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University  
of Glasgow

**The role of inflammation and cytokines in the  
pathogenesis of tendinopathy**

by

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

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## Abstract

Tendon disorders - tendinopathies - are the primary reason for musculoskeletal consultation in primary care in the UK and the US. The molecular pathophysiology of tendinopathy remains difficult to interpret while inflammation and its role in tendinopathy have been historically ignored due to its absence in human surgical specimens. This thesis explores the role of inflammation in human tendinopathy and dissects potential molecular pathways involved in its initiation and perpetuation.

Firstly I characterize inflammatory cell subtypes within a model of human tendinopathy highlighting a distinct inflammatory infiltrate particularly of mast cells and macrophages. Hypoxia and hypoxic cell death have been a long considered aetiology of tendon degeneration. In this thesis I demonstrate that hypoxia related proteins are present in early tendinopathy biopsies and thereafter in mechanistic studies demonstrate that hypoxia regulates inflammatory and apoptotic mediators in tendon cells associated with a significant shift in collagen matrix synthesis.

The cytokines, interleukins 17 and 33 are emerging inflammatory mediators known to play key roles in fibroblast biology. I explored the cellular sources of IL-17A in human tendinopathy with experiments revealing the majority of IL17A co-localised to mast cells. Moreover IL-17A induced proinflammatory cytokines and apoptosis in vitro and again resulted in a significant switch in collagen extracellular matrix production. IL 33 is a new member of the IL-1 superfamily that signals through the ST2 receptor. Herein I demonstrate that IL-33 expression is up regulated in human tendinopathic biopsies whilst rhIL-33 promotes proinflammatory cytokine release and significantly shifts matrix production toward a collagen III phenotype. WT mice undergoing a tendon injury model showed significant up regulation of IL-33 and ST2 while ST2<sup>-/-</sup> mice exhibit a reduced collagen response and biomechanical tendon strength at early time points post injury Addition of rh-IL33 increased type III collagen production and reduced the biomechanical strength of WT tendons. Furthermore mechanistic investigations has highlighted a key role for the microRNA 29 family in the modulation of collagen regulation in tendinopathy but also in controlling IL-33 induced changes as a direct target of sST2. Based on these experiments I propose IL-33 as an important and influential alarmin in early tendon injury and tendinopathy, which may be influential in the balance between reparation and degeneration in tendon disease.

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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.

.....

Neal L Millar

## Abbreviations

ACPA	Anti-Citrullinate Protein Antibodies
ACR	American College of Rheumatology
AS	Ankylosing Spondylitis
CBM	Chromatin binding motif
CIA	Collagen-Induced Arthritis
CRP	C-Reactive Protein
CTLA-4	Cytotoxic T Lymphocyte Associated Antigen 4
CVD	Cardiovascular Disease
DAMP	Danger-associated molecular pattern
DMARD	Disease modifying anti rheumatic drug
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic Reticulum
ETA	Etanercept
FCS	Foetal calf serum
FLS	fibroblast like synoviocytes
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HeLa	stromal tumor cell line derived from the patient Henrietta Largs
HLA	Human leukocyte antigen
HTH	helix turn helix
IgG	Immunglobulin G
IL	Interleukin
LPS	Lipopolysaccharide
MCSF	Macrophage colony stimulating factor
MHA	1-mercaptoundec-11-yl)hexa(ethylene glycol)
MMP	Matrix Metalloproteinase

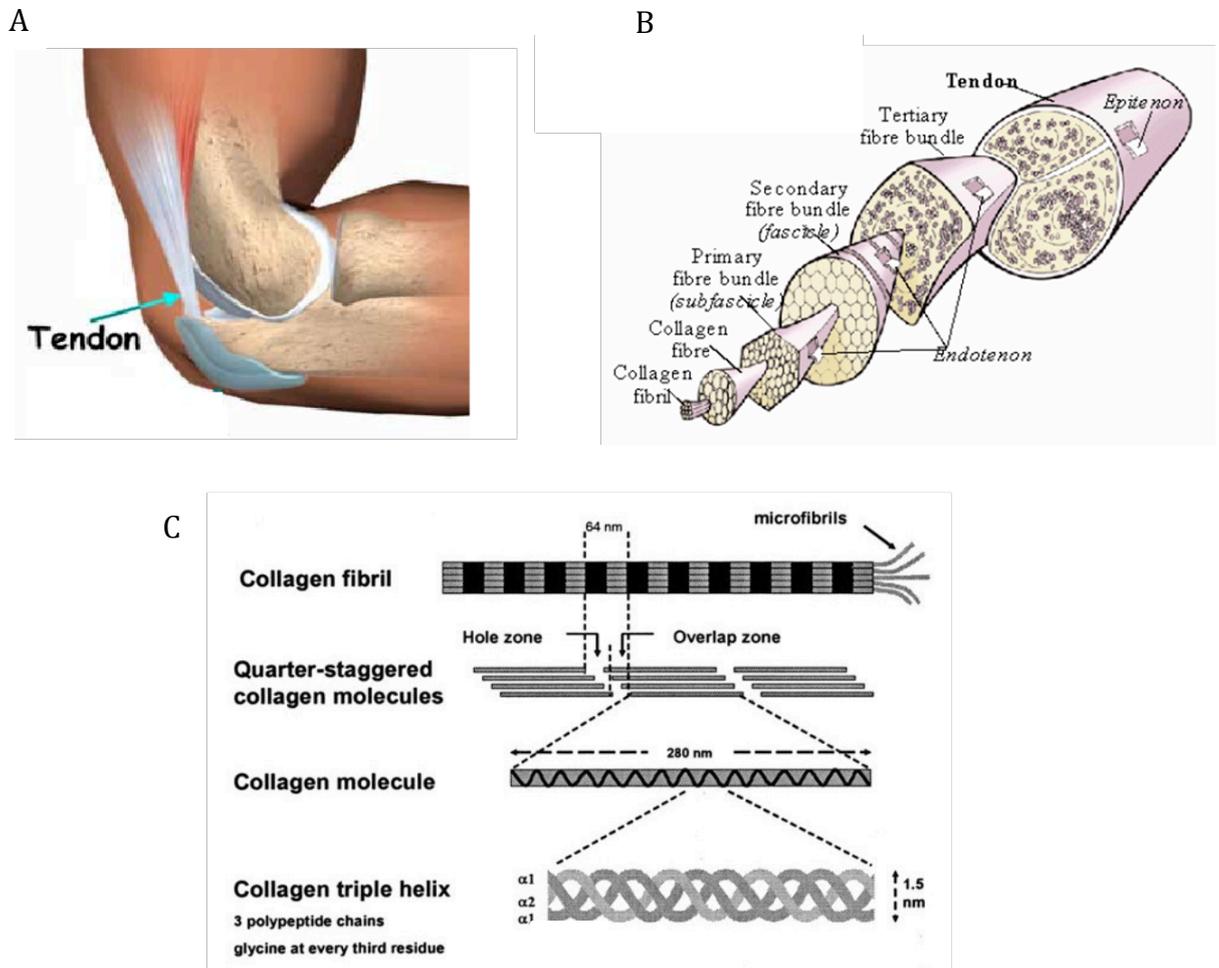
MNP	Magnetic nanoparticles
MRI	Magnetic resonance imaging
MTX	methotrexate
NIR	Near infrared
NLS	nucleus localisation site
NP	Nanoparticle
NT	Nanotag
OA	Osteoarthritis
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral Blood Mononuclear Cells
PET	Positron emission tomography
PsA	Psoriatic Arthritis
RA	Rheumatoid Arthritis
RANK	Receptor Activator of NF- $\kappa$ B
RANKL	Receptor Activator of NF- $\kappa$ B Ligand
RF	Rheumatoid Factor
SEM	Scanning electron microscope OR standard error mean
SERRS	Surface enhanced resonance Raman scattering
SERS	Surface enhanced Raman spectroscopy
SNP	Single Nucleotide Polymorphism
TcKs	Cytokine Activated T cells
TLR4	Toll Like Receptor 4
TNF $\alpha$	Tumour Necrosis Factor
TNFi	Tumour Necrosis Factor inhibitors
TMB	3,3',5,5'-tetramethylbenzidine
TPA	Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate

## Chapter 1 Introduction

## 1.1) Tendon Biology

Tendons are the connective tissue attaching muscle to bone; they allow the transduction of force from a contracting muscle to be exerted upon the attached skeletal structure at a distance from the muscle itself [1]. Force transduction in this way allows movement of the limbs, thorax, head and other structures during normal activity. Tendons also prevent joint displacement beyond anatomical barriers, thereby preventing injury and maintaining normal anatomical structure and function [2]. They consist primarily of water and type I collagen, with smaller amounts of other collagens and matrix materials, as well as various types of cells, most notably fibroblasts[3].

Tendons are a complex, systematically organised tissue and comprise several distinct layers. At their core is collagen, organised (in increasing complexity) into fibrils, fibres, fibre bundles, and fascicles (Figure 1-1), which are surrounded by a layer of loose, collagenous and lipid-rich connective tissue matrix known as the endotenon [4]. At the musculo-tendinous junction, the endotenon is continuous with the perimysium of the proximal muscle and the periosteum of the distal bone. It contains blood vessels which supplies adjacent tissue, as well as local nerves and lymphatics. The epitenon encloses the entire tendon and is continuous with the endotenon. Depending on the localisation of the tendon in question, the surrounding layers can vary. Some tendons are encased in a synovial tendon sheath which allows smooth gliding, particularly in areas of abrupt directional change or flexion (such as joints), in which synovial fluid between tendon layers acts as lubrication. Gliding is facilitated in tendons in which a synovial sheath is not present by an elastic, loosely arranged tissue layer known as the paratenon.



**Figure 1-1 The hierarchical structure of tendon.**

(A) Representation of the macromolecular organisation of the extracellular matrix (adapted from Lin, et al. 2004[5]). (b) Collagen fibril organisation (adapted from Riley, (2005)[6]). The collagen molecule takes a triple-helical formation which form up in staggered multimers to form microfibrils. Microfibrils organise together to form collagen fibrils, which pack together in fibres, fibre bundles, and fascicles.

The insertion of tendon into the attached bone has been described as occurring by one of two modes. The first is by the uniform transition of the tendon to fibrocartilage, which becomes steadily more ossified until it merges with the bone [7]. The second is somewhat more complex and involves the periosteum as an intermediate structure and superficial fibrils becoming fixed into the periosteum and then deeper, directly into the bone known as the enthesis. The archetypal enthesis organ [8] is that of the Achilles tendon where intermittent contact between tendon and bone immediately proximal to the enthesis leads to the formation of fibrocartilages on the deep surface of the tendon and on the opposing calcaneal tuberosity, but similar functional modifications are widespread throughout the skeleton. Many entheses have bursae and fat near the insertion site and both of these serve to promote frictionless movement. Collectively, the fibrocartilages, bursa, fat pad and the enthesis itself constitute the enthesis organ. Stress concentration at the enthesis itself is dissipated at many sites by fibrous connections between one tendon or ligament and another, close to the insertion site [9]. At a microscopic level, enthesis fibrocartilage is of paramount importance in ensuring that fibre bending of the tendon or ligament is not focused at the hard tissue interface. Normal enthesis organs are avascular in their fibrocartilaginous regions, but tissue microdamage to entheses is common and appears to be associated with tissue repair responses and vessel ingrowth.

### **1.1.1) Tendon ultrastructure**

Tendon is a roughly uniaxial composite composed mainly of type I collagen in an extracellular matrix (ECM) which comprises the majority of tendon volume providing strength and flexibility inherent in the tissue and essential for function. The main components of ECM include collagen, ground substance and neurovascular and connective tissue [10]. Tendons consist of 30% collagen and 2% elastin embedded in an extracellular matrix. Type I collagen has a large diameter (40-60nm) and links together to form tight fibre bundles. Type III collagen is smaller in diameter (10-20nm) and forms looser more reticular bundles. Collagen comprises 30% of wet tendon and 80% of dry tendon and confers stiffness, rigidity and strength when loaded and flexibility when bent, compressed or twisted [10].

## ***Collagen***

The structural unit of collagen is tropocollagen, a long, thin protein 280nm long and 1.5nm wide consisting mainly of Type I collagen (Figure 1-2).

Tropocollagen is formed in the rough endoplasmic reticulum of tenocytes as procollagen and subsequently in the golgi apparatus procollagen forms a helix and is transported to and secreted from the cell membrane in vacuoles via the cellular skeletal system of microtubules and microfilaments[11]. Procollagen is converted to insoluble tropocollagen in the extra cellular compartment where it is contained within the ECM [11]. The 100 amino acids join to form an alpha chain. There are 3 alpha chains which are surrounded by a thin layer of proteoglycans and glycosaminoglycans. Two of the alpha chains are identical and one differs slightly. The three polypeptide chains each form a left handed helix. The chains are connected by hydrogen bonds and wind together forming a rope like, right handed super helix giving collagen a rod like shape. Approximately two-thirds of the collagen molecule consists of 3 amino acids : glycine (33%), proline(15%) and hydroxyproline (15%) [12]. Collagen fibres acquire all their crosslink's shortly after synthesis. The key enzyme lysyl-oxidase is the rate limiting step forming the most prevalent hydroxylysin crosslinks which in turn provide tensile strength. Reduction of crosslinks results in an extremely weak, friable collagen fibre - crosslinking of collagen is one of the best biomarkers of aging.



### ***Elastin***

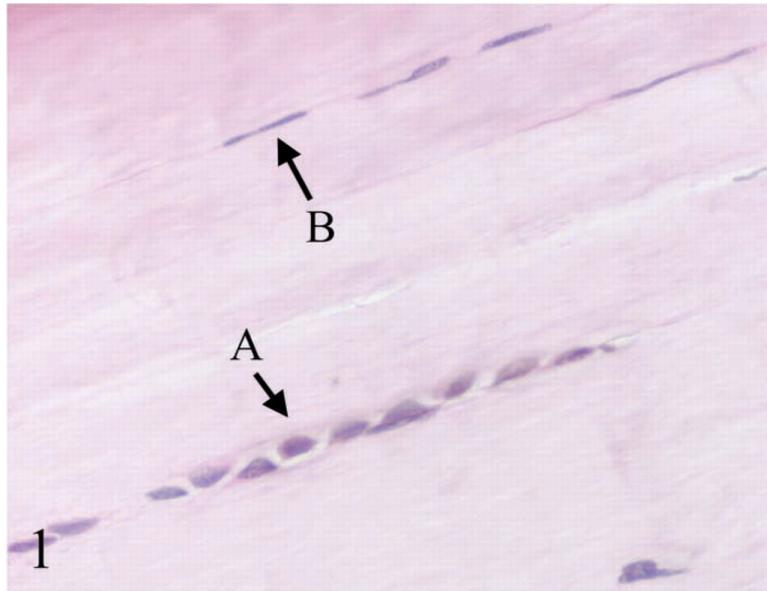
Elastin contributes to the flexibility of tendon. It is rich in glycine and proline but does not contain much hydroxyproline or lysine [13]. It has a large content of valine and contains desmosine and isodesmosine which form crosslinks between the polypeptides, but no hydroxylysine. Elastin does not form helices and is hydrophobic.

### ***Tenocytes***

Fibroblast-like cells are the major cell type in tendons, and have been histologically classified as elongated tenocytes or ovoid tenoblasts [14] (Figure 1-3). These cells are central for the maintenance of healthy tendon as they can proliferate, produce collagen and critically maintain the extracellular matrix [15]. Tenocytes are flat, tapered cells, spindle shaped longitudinally and stellate in cross section and are detected sparingly in rows between collagen fibres. They have elaborate cell processes forming a three dimensional network extending through the extracellular matrix and communicate via cell processes and may be motile. Tenoblasts are spindle shaped or stellate cells with long, tapering, eosinophilic flat nuclei and are motile and highly proliferative. They have well developed rough endoplasmic reticulum on which precursor polypeptides of collagen, elastin, proteoglycans and glycopeptides are synthesised.

During embryonic development, tenocytes originate from mesodermal compartments, as do skeletal myoblasts, chondrocytes and osteoblasts[16]. Some of the multipotent mesenchymal progenitor cells that arise from these compartments express the basic helix-loop-helix transcription factor scleraxis. However, once they are committed to become cells making up a specific tissue, only tendon progenitor cells and tenocytes retain the ability to express scleraxis. Therefore, scleraxis is a marker of tenogenic cells and mature differentiated tenocytes[17, 18]. The scleraxis gene is thus the first master gene found to be essential for establishing the tendon lineage during development. Tenomodulin is a type II transmembrane glycoprotein. Its robust expression is induced in mouse tendons in a late (embryonic day [E] 17.5) developmental phase and is also observed in adult tendons showing that tenomodulin is a marker of mature (differentiated) tenocytes[19]. In vitro experimental evidence shows that the genes composed of tendon-specific ECM are tightly regulated in tenocytes by mechanical forces. Very recently, tendon stem/progenitor cells have been discovered in

human and mouse tendon, and the proteoglycans biglycan and fibromodulin have been identified as essential elements in a microenvironment to keep phenotypes and differentiation of stem/progenitor cells[20]. However, the roles of these stem/progenitor cells in adult tendon homeostasis and/or wound healing remains unknown.



**Figure 1-3 :Tenocytes and tenoblasts**

Round tendon cells (A) and elongated tendon cells (B) were identified in healthy human patellar tendon by H and E staining. (modified from Chuen FS et al 2004)

### ***Ground substance***

Ground substance is a complex mixture of proteoglycans and glycoproteins surrounding collagen fibres. It has high viscosity thus providing structural support, lubrication and spacing of the fibres that is essential for gliding and cross-tissue interactions.

The proteoglycans and glycoproteins consist of two components, glycosaminoglycans and structural glycoproteins [21]. The main proteoglycans that are associated with glycosaminoglycans are dermatan sulfate, hyaluronic sulfates, chondroitin 4 sulfates, and chondroitin 6 sulfates. Other proteoglycans found in tendon include biglycan, decorin and aggrecan (Table 1-1).

Fibronectins are high-molecular weight, noncollagenous extracellular glycoproteins [22]. They play a role in cellular adhesion (cell-to-cell and cell-to-substrate) and in cell migration. Fibronectin may be essential for organisation of collagen I and III fibrils into bundles and may act as a template for collagen fibre formation during the remodelling phase of healing.

The synthesis of proteoglycans begins in the rough endoplasmic reticulum, where the protein portion is synthesized. Glycosylation starts in the rough endoplasmic reticulum and is completed in the Golgi complex, where sulfation occurs. Proteoglycans in the ground substance seem to regulate fibril formation as the content of proteoglycans decreases in tendons when the tropocollagen has reached its ultimate size. An adequate amount of ground substance is necessary for the aggregation of collagenous proteins into the shape of fibrils.

<b>Proteoglycan</b>	<b>Core Protein Size (kDa)</b>	<b>Properties and Role in Tendon</b>
<b>Decorin</b>	36	Binds to fibrillar collagen, inhibits collagen fibrillogenesis, binds TGF $\beta$ , and EGF
<b>Biglycan</b>	38	Binds to fibrillar collagen
<b>Fibromodulin</b>	42	Binds to type I collagen, facilitates formation of mature large collagen fibrils, modulation of tendon strength
<b>Lumican</b>	38	Binds to type I collagen, inhibits size of collagen fibrils, modulation of tendon strength
<b>Aggrecan</b>	220	Linked to hyaluronan, provides resiliency, low levels in tensional parts of tendon, high levels in compressed regions, particularly in fibrocartilage
<b>Versican</b>	265-370	Linked to hyaluronan, low levels in tensional parts of tendon, somewhat higher levels in compressed regions, increases viscoelasticity, maintains cell shape

**Table 1-1 Properties of the most abundant tendon proteoglycans**

### **1.1.2) Nerve supply**

Tendons are supplied by sensory nerve fibres from the overlying superficial nerves or from nearby deep nerves and are largely, but not exclusively, afferent [23]. The afferent receptors are found near the musculotendinous junction and the nerves tend to form a longitudinal plexus and enter via the septa of the endotenon or the mesotendon if there is a synovial sheath.

There are 4 types of receptors. Type I (Ruffini corpuscles) are pressure receptors that are extremely sensitive to stretch and adapt slowly. Type II (Vater-Pacini corpuscles) are activated by movement, Type III (Golgi Tendon organ) are mechanoreceptors and type IV are free nerve endings that act as pain receptors [24].

### **1.1.3) Blood supply**

The blood supply of tendons is very variable, and is usually described on the basis of three distinct anatomical regions: 1) the musculotendinous junction; 2) the length of the tendon; and 3) the tendon-bone junction. The blood vessels originate from the perimysium, periosteum or enter via the paratenon and mesotendon [25].

The blood supply to the musculotendinous junction is from the superficial vessels in the surrounding tissues. The main blood supply to the middle portion of the tendon is via the paratenon. The small blood vessels in the paratenon run transversely towards the tendon, branching several times before running parallel to the long axis of the tendon [26]. Vessels supplying the bone-tendon junction supply the lower one third of the tendon. There is no direct communication between the vessels because of the fibrocartilaginous layer between the tendon and bone; but there is some indirect anastomosis between the vessels. Blood supply is compromised at sites of friction, torsion or compression. This is found particularly in the tibialis posterior, supraspinatus and Achilles tendon.

#### 1.1.4) Biomechanics of tendon function

Tendons exhibit high mechanical strength, good flexibility and an optimal level of elasticity allowing them to perform their unique role. They are viscoelastic tissues that display stress relaxation and creep [27]. The mechanical behaviour of collagen depends on the number of intramolecular and intermolecular bonds. At rest, collagen fibres/fibrils display a crimped configuration (Figure 1-4) The initial concave portion of the stress strain curve where the tendon is strained up to 2% represents flattening of the crimp pattern. Beyond this point tendons deform in a linear fashion due to intramolecular sliding of collagen triple helices with the fibres becoming more parallel [27]. Microscopic failure occurs when the strain calculated by the mean deformation of the tendon exceeds 4%. Beyond 8-10%, macroscopic failure occurs from intrafibril damage[27]. Microscopic failure occurs when the strain exceeds 4%. Beyond 8-10%, macroscopic failure occurs from intrafibril damage by molecular slippage. As stress increases the gap between molecules increases leading eventually to slippage of adjoining molecules. Consequently, complete failure occurs rapidly and the fibres recoil at the ruptured end.

The ultimate tensile strength of tendons is related to thickness and collagen content (a tendon with an area of  $1\text{cm}^2$  is capable of bearing 500-1000kg) [2]. During strenuous exercise very high loads are placed on tendons and the rate of loading plays an important role in tendon rupture.

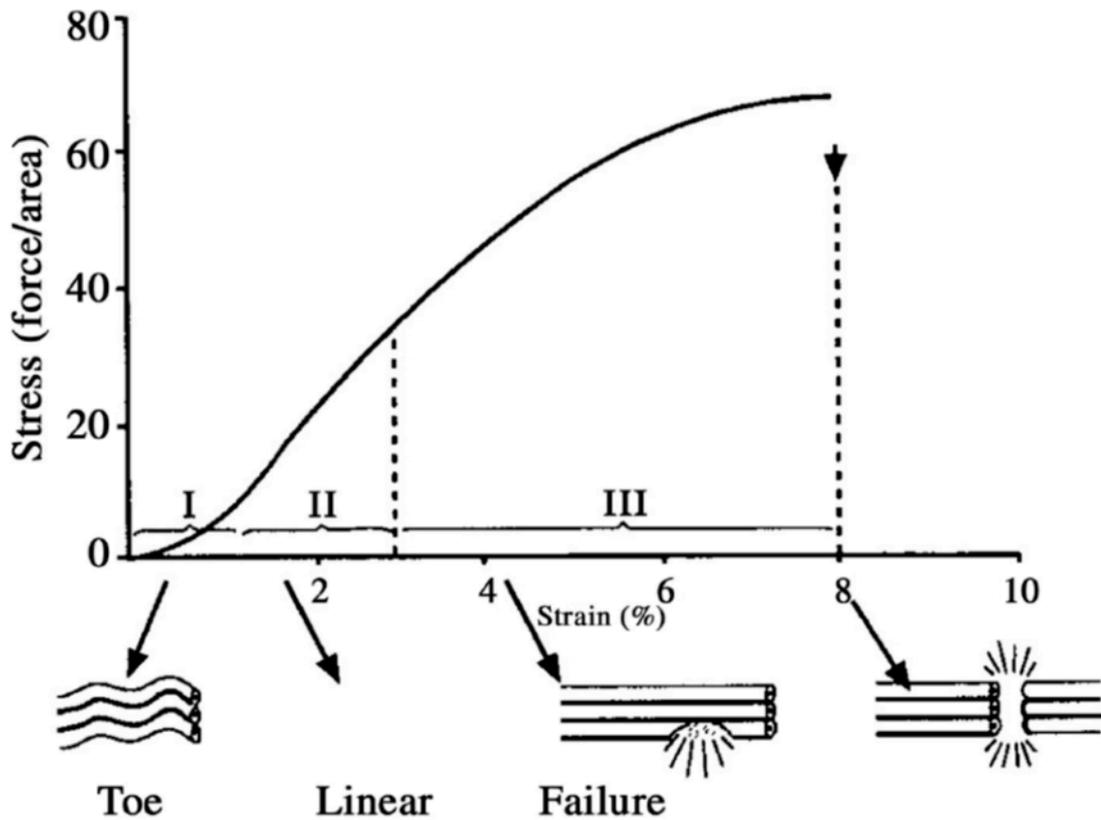


Figure 1-4: Graph showing the stress–strain curve for tendon.

Region I—the tendon has a wavy configuration at rest. The wavy configuration is lost when it is stretched more than 2%. Region II—collagen fibres exhibit a linear response to increasing strain as collagen fibres deform. Region III—one or two broken lines indicate that the collagen fibres are starting to slide past one another as the intermolecular cross-links fail. At strain levels greater than 8%, macroscopic rupture is produced by tensile failure. ( Reproduced from Sharma et al 2005 [28], © The Journal of Bone and Joint Surgery, Inc. with permission )

### 1.1.5) Injury and Healing of Tendon Tissue

The process of tendon healing represents an interesting paradigm. Though most tendons have the ability to heal spontaneously after injury, the scar tissue that is formed is almost always mechanically inferior and therefore much less able to perform the functions of normal tendon; it is also more susceptible to further damage lending itself to perpetual damage repair cycles with predilection for long term dysfunction in some patients as a result [29]. The formulation of effective treatments for tendon injuries has been based on traditional tissue-level reparative procedures, such as surgical reattachment. The lack of effective treatments has focused research toward understanding the mechanisms of tendon healing at the molecular level. This has ultimately been in an effort to develop therapies to facilitate tendon healing through the use of individual molecules or groups of molecules known to have beneficial roles in the process.

The process of tendon healing follows a pattern similar to that of other healing tissues [29] (Table 1-2), and can be roughly divided into three broad phases; that of inflammation, proliferation and remodelling.

#### The Inflammatory Phase

Upon tissue damage, blood vessels rupture and damage associated molecules and other extrinsic signalling moieties released by resident cells, together with the products of altered extracellular matrix trigger a coagulation cascade that will coordinate the formation of a clot around the injured area. The clot contains cells such as platelets that immediately begin to release a variety of molecules, most notably growth factors (such as PDGF, TGF $\beta$ , and IGF-I and -II), causing acute local inflammation and, within minutes to several hours, the influx of polymorphonuclear leukocytes and lymphocytes. Extrinsic cells are also attracted to the wound area by degradation products of extracellular matrix (ECM) components [30, 31]. The arrival of these cells initiates autocrine and paracrine signalling and begins to expand the inflammatory response, resulting in the recruitment of other types of cells to the wound, including a diverse immune cell population including mast cells, basophils and T cells. [32]. Cells recruited in this way adhere to the walls of vessels before passing through the basement membrane to invade the underlying tissue. Invasion by extrinsic cells such as neutrophils and macrophages will begin to 'clean up' necrotic debris by

phagocytosis, and as a result macrophages become the predominant cell type for several days. They also instigate granulation tissue formation and neovascularisation of the wound area through the proliferation of capillary endothelial buds by the secretion of various angiogenic growth factors. By the third day post-injury, the wound contains platelets, macrophages, polymorphonuclear leukocytes, lymphocytes, and multipotential mesenchymal cells, all of which secrete chemotactic and pro-proliferative cytokines [33], [34]. Together with intrinsic cells (such as endotenon and epitenon cells), these inflammatory cells will produce a second battery of cytokines to initiate the proliferative phase. At this stage **type III collagen** is the main matrix protein in production[28].

### **The Proliferation Phase**

After several days the inflammatory phase is superceded by a period of vigorous cell growth and division of both intrinsic and extrinsic cells if competent to do so. The proliferative phase sees collagen deposition and further granulation tissue formation and neovascularisation, as well as extrinsic fibroblast migration and intrinsic fibroblast proliferation. The fibroblasts are thought to be recruited from surrounding tissue and the systemic circulation [35], and are responsible for synthesizing new extracellular matrix. This new matrix consists largely of collagens and glycosaminoglycan, though at this stage it generally remains poorly organised. Any type III collagen that was formed by intrinsic fibroblasts during the inflammatory period starts to be replaced by type I collagen, and the mechanical strength of the injured tissue begins to increase as this new matrix is deposited. Total collagen content is greater at this time than in normal tendon, though collagen concentration is lower. Throughout the healing process this extracellular matrix, particularly type I collagen, plays a variety of vital roles. It acts as not only a physical substrate on which cells adhere and migrate, but also has the ability to directly induce intracellular responses through integrin-mediated signaling, both in inflammatory cells [36] and in fibroblasts [37]. For example, monocytes can be induced to release tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6), and oxygen free radicals simply by allowing them to come in contact with type I collagen [38]. These responses to extracellular matrix components are imperative for the progression of normal healing.

### **The Remodelling Phase**

Finally a remodelling phase begins, which sees decreases in the cellular and vascular content of the callus tissue, and further increases in collagen type I content and density. Eventually the collagen will become denser and more organised, and is longitudinally orientated and cross-linked with the healthy matrix outside the injury area. After 12-14 weeks, cellularity, vascularity, and collagen makeup will return to something approximating that of the normal tendon, though healing is not complete in the human for up to 1 year. Even after this time the diameters and covalent cross-linking of the collagen fibrils often remain inferior to normal tendon [39], and tensile strength remains diminished. This mechanically inferior repair tissue is more susceptible to tendon creep than uninjured tendon, and is therefore at higher risk of further damage [40]. It is not currently known why the tissue is unable to fully recover following acute injury, but is thought to be due to altered collagen ratios, proteoglycan levels or other unknown factors inherent to the healing process [41]. Throughout this phase although small amounts of collagen III is produced the main structural component of the matrix production is collagen I [28].

Time (days)	Phase	Process
0	Immediately post-injury	Clot formation around the wound
0-1	Inflammatory	First cassette of growth factors and inflammatory molecules produced by cells within the blood clot.
1-2	Inflammatory	Invasion by extrinsic cells, phagocytosis
2-4	Proliferation	Further invasion by extrinsic cells, followed by a second cassette of growth factors that stimulate fibroblast proliferation
4-7	Reparative	Collagen deposition; granulation tissue formation; revascularisation
7-14	Reparative	Injury site becomes more organised; extracellular matrix is produced in large amounts
14-21	Remodelling	Decreases in cellular and vascular content; increases in collagen type I
21+	Remodelling	Collagen continues to become more organised and cross-linked with healthy matrix outside the injury area. Collagen ratios, water content and cellularity begin to approach normal levels.

**Table 1-2: Summary of healing process in tendons and ligament**

## 1.2) Tendinopathy

Tendon injuries are common and account for up to 40% of musculoskeletal GP consultations in the UK [42]. Tendinopathy (often called tendinitis or tendinosis) is the most common tendon disorder. It is characterized by activity related pain, focal tendon tenderness and decreased strength and movement in the affected area. Tendinopathy can occur in almost any tendon. Common examples include plantar fasciitis, Achilles tendinitis, patellar tendinitis, tennis elbow (lateral epicondylitis), golfer's elbow (medial epicondylitis) and supraspinatus tendinitis.

### 1.2.1) Aetiology of tendinopathy

The current cause of tendinopathy remains unclear and many theories have been suggested. They are multifactorial in origin and interaction between intrinsic and extrinsic factors comprise a common factor in many.

#### ***Mechanical load***

Excessive loading of tendons is regarded as the main pathological stimulus for degeneration [43]. Tendons respond to repetitive overload beyond the physiological threshold with either inflammation of their sheath or degeneration of their body, or a combination of both. Traditionally pathology is reported to be caused by repeated strain that represents a sub rupture force [3]. The strain is hypothesized to disrupt collagen cross-links with subsequent vessel disruption resulting in impaired metabolic and oxygen delivery [5]. Physiologically mechanical load has been shown to deform tendon cells with the cells responding with an increase in nitric oxide levels and cytosolic calcium. The presence of load is then communicated to adjacent cells via gap junctions. Mechanical stretching of human tendon fibroblasts also increased production of PGE2 and LTB4[44] and is thought that they may be involved in the development of tendinopathy [45]. The modulation of MMP-1 gene expression was also studied in rat tail tendons in culture [46]. Increasing static tensile stresses as much as 2.6 MPa gradually inhibited the MMP-1 mRNA expression, whereas stress-deprivation for 24 hours resulted in a significant up-regulation of MMP-1 expression. Arnoczky et al[46] also reported that a 1% strain decreased MMP-1 mRNA expression, whereas 3% and 6% strain completely inhibited it. This strain effect on MMP-1 mRNA

expression was dependent on stretching frequency [47]. This combined with load-induced growth factor changes (state factors) are thought to be critical in the tendons response to excessive load. Ultimately the amount of load (volume, intensity and frequency) that induces pathological tendon changes remains unclear; moreover load seems to be modulated by complex interactions between intrinsic factors such as genes, age, circulating and local cytokine production, gender, biomechanics and body composition.

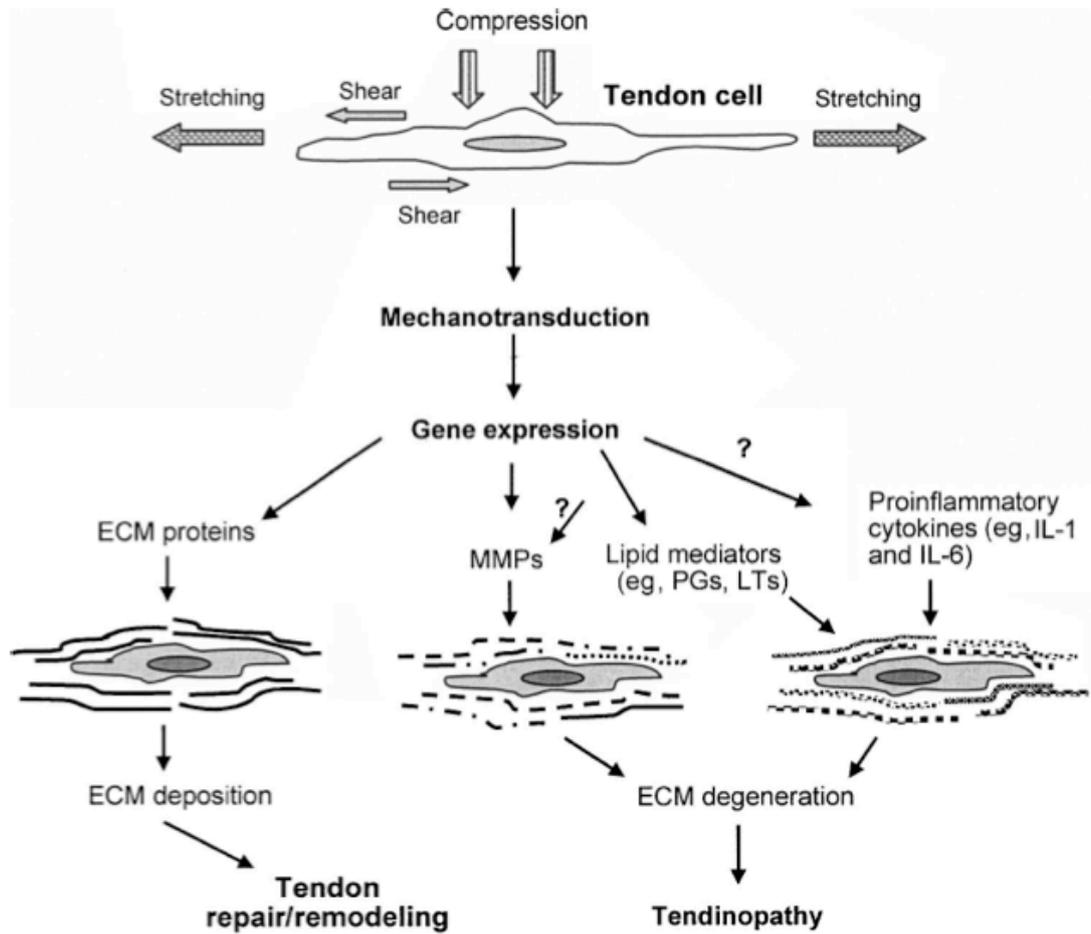


Figure 1-5 The biological responses of tendon fibroblasts to mechanical loading. Reproduced with permission from Lippincott Williams & Wilkins© , Wang et al 2006[48].

### ***Tendon impingement***

Impingement is a form of mechanical load adding compressive and or shearing load to interact with a tendons' normal tensile strength. This combination of loads may cause a tendon reaction to impingement resulting in cartilaginous and bony pathology [49]. Impingements in the Achilles tendon, rotator cuff, and the patellar tendon have been reported to cause tendinopathy. These tendon reactions may initiate or ultimately accelerate tendon damage alone or more likely in conjunction with other tendon responses.

### ***Thermal***

The storage and then release of elastic energy within tendons results in the discharge of intratendinous heat - equine studies suggest a resultant increase in tendon temperature and subsequent cell death[50]. The production of reactive oxygen species from hyperthermia has also been postulated to play a role in modulating tendon cell death[51].

### ***Oxygen free radicals and Drugs***

During tendon exercise and movement, bouts of ischaemia and reperfusion stimulate the formation of oxygen free radicals [52]. Tendon apoptosis has been shown to be induced with exposure to hydrogen peroxide [53] while peroxiredoxin, which eliminates hydrogen peroxide, has been shown to be up regulated in tenocytes in torn supraspinatus tendons [54]. The same group have highlighted the role of the short-lived oxygen free radical, nitric oxide in tendon disease [55]. Nitric oxide synthase peaks at day 7 and returns to baseline after rat Achilles tenotomise [56] in the same study inhibition of nitric oxide synthase reduced healing, reduced load failure and cross-sectional area. In an exercise overuse model of tendon degeneration in the rat, they found that iNOS, eNOS and bNOS mRNAs were overexpressed in the supraspinatus tendon of rats subjected to treadmill running at 14 days [57]. These results suggest that NOS activity is induced as a result of tendon injury in this model, and/or that expression of NOS is a part of supraspinatus tendinopathy.

Many studies indicate a strong association between fluoroquinolones and tendon pathology. Ciprofloxacin induces IL-1 $\beta$  mediated MMP-3 release [58] and reduces cell proliferation and collagen and matrix synthesis [59, 60] and thus may be a mechanism to induce tendinopathy.

### ***Inflammation***

Historically inflammation has not been demonstrated in tendon pathology in humans probably due to the end stage of disease that human biopsies represent [4, 61, 62]. End stage diseases in humans has shown no physiologic increase in PGE<sub>2</sub> in tendinopathic tissues at several different sites compared to normal tendon [63]. However it is becoming increasingly clear that the absence of inflammatory mediators at the end stage disease does not preclude its involvement in early stage disease. Animal and human studies in tendinopathy and tendon rupture have increasingly shown the presence of inflammatory mediators – this has been particularly evident with the use of microarray technology. The core paradox however resides in the essential nature of inflammation in the early phase of any injury and thereby the likelihood that inflammatory pathways will be evident during injury –what has been difficult has been to estimate their overall functional importance. This area is discussed in more depth in Section 1.4.

### ***Hypoxia and vascular changes***

Hypoxic injury has long been suggested as a cause of tendinopathy [64] although the exact mechanism remains to be defined. Hypoxic changes have been reported in tendon cells [65] and in the edge of tendon samples taken at the time of rotator cuff surgery [66]. However the hypoxic changes may have occurred anywhere during the sequence of pathology, rupture or post rupture and as such are difficult to define in terms of their functional and hierarchical import. The presence of hypoxia pre-rupture is supported by reports of increased lactate levels in Achilles tendinopathy compared to normal tendons [67] while recent in vivo work has shown decreased oxygen tension at the edge of torn rotator cuff sampled prior to surgical correction [68] and decreased microcirculation and vessels numbers in areas of degeneration or rupture [69]. Increased vascularity has been demonstrated histopathologically [70] on Doppler Ultrasound [71] and laser flowmetry [72]. However the connection between hypoxia and changes in vascularity remains unclear with the contradicting element that while hypoxia is a powerful stimulant to angiogenesis, impaired vascularity itself may lead ultimately to hypoxia.

### ***Neural***

As mentioned earlier the actual body of a tendon is poorly innervated however neurogenic inflammation and inflammation initiated by neuropeptides may also be important in tendon pathology. Neuropeptides substance P which induces mast

cell activation and calcitonin gene related peptide synthesis by a variety of cells which affects angiogenesis and vascular permeability have also been implicated in tendinopathy [73]. Microarray analysis of healing tendon with subsequent protein and mRNA analysis has also suggested a role for the neuropeptide glutamine, in the process of tendinopathy and tendon healing [74].

### ***Apoptosis***

Evidence for apoptosis and a potential role in pathology of tendinopathy was originally described in 2002 where the torn edge of ruptured supraspinatus tendons were examined and found to exhibit increased expression of tenocyte apoptosis compared to controls [75]. Lian et al [76] reported increased apoptosis in patellar tendinopathy in athletes and also showed tenocyte apoptosis could be induced by high-strain mechanical loading in a rat tibialis anterior model [77]. Activation of c-Jun N-terminal kinase (JNK) [78] and increase in cytochrome c-related activation of caspase 3 [53] may be two further potential pathways involved in apoptosis induction in tendinopathy. Cyclical strain applied to canine flexor tendon cells induces stress activated protein kinases that in turn induced apoptosis [79].

### ***Matrix metalloproteinases***

Matrix metalloproteinases (MMPs) are important regulators of ECM remodelling. Analysis of gene expression in human Achilles tendon showed that 23 MMP and 19 ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin Motifs) were detectable in normal tendon with widely varied expression. In a rat flexor tendon laceration model, the expression of MMP9 and MMP13 (collagenase 3) peaked between days 7 and 14 while MMP2 (gelatinase) and MMP3 (stromelysin) increased after surgery and remained high until day 28 [80]. There are changes in the expression and activity in various MMPs, and changes in the levels of tissue inhibitors of metalloproteinases (TIMPs) that are consistent with increased proteolytic activity in degenerate tendon [81]. Pathologic rotator cuff tendons showed greatly increased MMP1 activity and reduced MMP2 and MMP3 activity compared with normal tendons while ruptured tendons showed increased levels of MMP1, MMP9, MMP19, MMP25 and TIMP1 and decreased levels of MP3, MMP7, TIMP2, and TIMP3 [6]. More research is required to determine the mechanism of action and regulation of MMPs in tendinopathy to promote the development of specific therapeutic interventions.

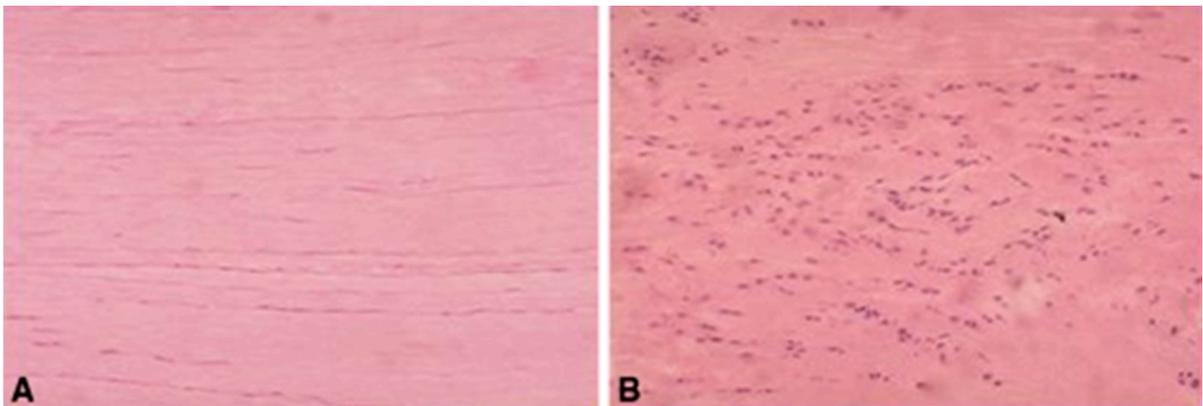
### **1.2.2) Pathology of tendinopathy**

The histopathological changes in tendinopathy are well documented (Table 1-3). Normal tendon is brilliant white in colour and has a firm fibroelastic consistency while tendinopathic tendon is grey or brown and is soft, thin and fragile. Microscopically collagen bundles are disorganised, there is increased ground substance and the nuclei are darkly stained, round and numerous in contrast to the highly organised parallel collagen bundles and spindle shaped tenocyte nuclei arrangement in normal tendon [82] (Figure 1-6). Electron microscopic evaluation reveals angulated collagen fibres which vary in diameter and orientation.

Changes consistent with hypoxia including lipid vacuoles, enlarged lysosomes and degranulated endoplasmic reticulum are also observed [82] while tendinopathic tendon often exhibits vascular infiltration and upregulation of VEGF. Others have demonstrated in growth of small nerves with an increase of substance P (SP)-positive nerve fibres in painful tendinopathic samples compared to normal tendon samples while increased adrenergic receptors and catecholamines have also been reported [73].

Findings	Macroscopic	Light microscopy	Ultrasound findings
Normal Tendon	<ul style="list-style-type: none"> <li>• Brilliant white</li> <li>• Firm Fibroelastic texture</li> </ul>	<ul style="list-style-type: none"> <li>• Organised parallel collagen bundles</li> <li>• Spindle shaped tenocyte nuclei</li> <li>• Parallel nuclei alignment</li> </ul>	<ul style="list-style-type: none"> <li>• Regular uniform fibre structure</li> <li>• Parallel hyperechoic features</li> </ul>
Tendinopathy	<ul style="list-style-type: none"> <li>• Grey or brown</li> <li>• Thin tissue, fragile and disorganised</li> <li>• Loose texture</li> </ul>	<ul style="list-style-type: none"> <li>• Disorganised collagen bundles</li> <li>• Round dark stained tenocyte nuclei</li> <li>• Increased number of nuclei with loss of parallel arrangement</li> <li>• Mucoïd degeneration and vacuoles</li> <li>• Increase of vascular and nerve ingrowth</li> <li>• Increased ground substance and GAG</li> </ul>	<ul style="list-style-type: none"> <li>• Local hypoechoic areas</li> <li>• Irregular fibre structure</li> <li>• Neovascularisation on power doppler</li> <li>• Widening of tendon</li> </ul>

**Table 1-3.: Summary of pathology of normal versus tendinopathic tendon tissue**



**Figure 1-6: Histology of normal tendon and tendinopathy.**

(A) Normal tendon has parallel, longitudinal architecture with scattered elongated tenocytes. (B) Tendinopathy tendon shows disorganized collagen architecture, high cellularity of rounded tenocytes. (This figure was published in Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE. Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg.* 2000;9:79–84, © Elsevier 2000, with permission.)

### **1.2.3) Strategies for tendon healing**

As the investigation into the basic science underpinning tendinopathy has expanded over the past 20 years so too have the treatment modalities. A summary of current therapies are shown in Table 1-4. Whilst many of these modalities are in routine clinical use, only a few randomised controlled trials exist and much of the evidence is either 'expert or eminence based, or remains at a pre-clinical stage. Many therapeutics are controversial – I interpret this as a lack of clear driving evidence to inform opinion, and this in part informs the biology exploration underpinning this thesis.

Treatment	Possible mode of action
Non-operative	Remove precipitating/aggravating factors
Rest/activity modification	Remove precipitating/aggravating factors
Eccentric exercising	Restoration of normal tissue architecture
Orthotics	Remove precipitating/aggravating factors
Cryotherapy	↓ inflammation
Heat treatment	↑ blood flow and cellular activity
Extracorporeal shock wave therapy	↓ neovascularization and ↓ nociception
Ultrasound	↑ blood flow and cellular activity
Laser Treatment	Analgesic effects
Drugs	
NSAID's	↓ inflammation through PGE <sub>2</sub>
Corticosteroid injection	↓ inflammation
Nitrate Patches	↓ inflammation
Sclerosant injection	↓ neovascularization and ↓ nociception
Platelet Rich Plasma Injection	↑ growth factors and matrix synthesis
Glycosaminoglycan polysulfate	↓ inflammation and MMP activity
Operative	
Surgery	Excision of degenerative tissue
Radiofrequency coblation (Topaz®)	↑ inflammation and cellular activity

**Table 1-4: Current treatment options for tendinopathy**

### ***Novel treatment areas***

As a result of the deficiencies of current treatment there is great interest in investigating the potential role for cellular based therapies.

Increased levels of growth factors, including insulin growth factor-1, transforming growth factor-beta-1, and platelet-derived growth factor, occur after tendon injury in animal models [83, 84]. In addition, several preliminary studies suggest adding exogenous growth factors to an injured tendon can enhance healing and repair [85, 86]. For instance, the addition of cartilage-derived morphogenic protein-2 to an animal tendon repair model generated an increase in the strength and organization of the repaired tendon [87]. Although the application of growth factors to augment tendon repairs seems feasible, it is unclear whether there is a role for growth factors in the treatment of tendinopathy at a pragmatic clinical level. Increased levels of transforming growth factor-beta1 and insulin growth factor-1 have been demonstrated in areas of tendinopathy, but this does not appear sufficient to heal the tendon injury [88]. One possible method of introducing an assortment of growth factors to an area of tendinopathy is through the injection of platelet rich plasma or autologous blood however recently a randomised controlled human trial has failed to show any improvement in tendon pain or function [89].

Stem cell technology in the treatment of degenerative conditions of the musculoskeletal system such as tendinopathy is appealing. In theory, pluripotent stem cells can be isolated and then delivered to an area of need such as an arthritic joint or degenerative tendon. Once the stem cells are in the desired location, either local signaling or the addition of exogenous factors can drive the pluripotent cells to differentiate into the needed cell line. Stem cell technology is currently being applied to the creation of tendon and ligament grafts and in enhancing graft incorporation [79, 88, 161]. Chong et al. applied bone marrow-derived stem cells to a rabbit Achilles tendon repair model [34]. They reported the addition of the stem cells in a fibrin carrier resulted in an increased modulus and improved collagen organization compared with control tendons at 3 weeks. Interestingly, no major differences were noted at later time points. This early work suggests stem cell technology may have a role in tendon grafting and repair, but

whether this technology will successfully be applied to the treatment of tendinopathy remains to be seen.

There are a number of studies, in both the human and the veterinary literature, suggesting that injection of glycosaminoglycan polysulphate (GAGPS) may lead to an improvement in disease of the human Achilles and equine superficial digital flexor tendon, respectively. In the human study, local injection of GAGPS was compared with oral indomethacin and at 1-yr follow-up two-thirds of the GAGPS group had a good response compared with only one-third of the indomethacin-treated group [181]. However, this study was confined to peritendinous lesions rather than lesions of the body of the Achilles and more data are needed in this area.

### **1.3) Models of tendon healing and tendinopathy**

Experimental in vitro studies allow the study of the effect of a single isolated factor. In vitro experiments are inexpensive and often serve as precursors for more complicated and expensive animal models, which may be simulated in vitro using different cell culture environments. In the case of tendon injury and repair, some examples of in vitro models have cultured flexor tendon cells [90, 91] and tested the strength of flexor tendon repair [92]. Additionally many in vitro studies have focused on the effect of applying cyclic loads to tendons [44, 93, 94].

The majority of experimental research on tendon injury and repair has been performed in animal models, which possess advantages such as fewer ethical issues, less variability, and greater availability of numbers when compared to clinical trials. However, animal models also have their shortcomings as well and researchers must be cautious in applying their results to humans, just as care must be taken when extrapolating in vitro results for in vivo settings. Some disadvantages of animal models include inherent biologic variability, metabolic and hormonal differences from humans, anatomical differences, and potential difficulty in working with animals, such as noncompliance. The models of naturally occurring tendinopathy in horses and dogs are of some theoretical interest but in practice are not valuable for invasive or explorative research such as that planned here due to their large size and cost [95].

#### **1.3.1 Current animal models of tendon healing and tendinopathy**

Numerous animal models have been generated and assessed for their utility to investigate tendon healing and tendinopathy. Whilst human studies provide the clinical characteristics of tendinopathy, the aetiology of degeneration and injury remains uncertain. One reason is that human tissue can normally only be examined during the end stage of what is by definition a chronic pathology. It remains difficult to obtain early pathologic tendon tissue because these injuries are often initially asymptomatic or treated non operatively. By providing tissue throughout all time points of tendinopathy, animal models provide an excellent investigative approach to tendon injury and tendinopathy.

### ***Tendinopathy in animal models***

Two general categories of animal tendinopathy models exist based on the mode of injury; mechanically induced versus chemically induced.

The most frequently used mechanical model is repetitive treadmill running. In particular this method has been extensively used to evaluate injury to the supraspinatus tendon of the rotator cuff. Rats are subjected to a repetitive exercise programme consisting of treadmill running at 17m/min on a 10° decline for 1 hour/day, 5 days/week [96]. Following several weeks the supraspinatus tendons in exercised animals demonstrate significant changes consistent with tendinopathy. Despite large numbers of studies using this model the biological response to the overuse stimulus is still not well understood. Importantly this model has been unable to be translated to smaller rodents such as mice due to lack of compliance with the exercise regime and such as reduced the ability to use 'knock-out' models to study gene specific targets. Other mechanically induced models include a novel rat model of patellar tendinosis [97] where the patella and tibia are gripped and distracted to apply repetitive sub-failure loads to the patellar tendon and a rabbit model of FDP tendinosis [98] which applies electrical stimulation to the FDP muscle to recreate tendinosis.

### ***Tendon healing in animal models***

In order to study tendon healing in an animal model, a distinct and reproducible injury must be created. The two most common types of tendon transection models have been complete and partial transections. Complete transections are a better simulation of what occurs clinically, but the effect of sutures, which are necessary to reduce gap formation, must be taken into account. Partial transections can be made in the middle half or along one side of the tissue. Although they are unlikely to occur clinically, injuries only to the middle half leave the marginal fibres intact, circumventing the need for suture repair and allows for immediate postoperative tendon mobilization [99, 100].

Complete transections have been shown to be reproducible in location, pattern, and extent of damage that they induce [101]. As a result of a complete transection, there exists a gap between two free ends. Sutures are utilised to achieve tendon apposition in order to minimize scar tissue formation, hasten collagenisation, and minimise the presence of adjacent non-tendinous tissue between the two ends. Animal model studies have confirmed the clinical finding

that the highest suture strand technique from each study provided the highest tensile strength [102, 103]. When using sutures, other factors to consider include suture knot location, suture material, and suture size [104]. One disadvantage with using sutures is that they may disrupt the intrinsic blood supply and reduce overall tensile strength of the repair site [105].

Animal models have also been used to study partial transections, which involve injuring a percentage of the tendon. Some examples include window defects, removal of the central third of the tendon, and transection of the medial half of the tendon. Although a partial transection injury is not as clinically relevant as a complete transection injury, it still allows the tendon healing response to be studied. In addition, partial injuries have the advantage of not requiring sutures to reduce gap formation since the tendon is still partially attached. The most commonly used model was developed by Lin et al [5] where a partial (0.75mm) transection is made with a punch biopsy and then animals resume normal cage activity and are then culled as per the protocol necessary to explore a given pathway. The advantage of this model has been its reproducibility in mice and thus has allowed the use of gene targeted or transgenic strains to study specific targets.

## 1.4) Cytokines in health and disease

Cytokines are mediators that transmit signals between cells in an autocrine or paracrine manner acting either in soluble or membrane bound form. Initially, cytokine families were named for their origin—for example, lymphokines, monokines, and interleukins. However, as many of these molecules also act on and/or are produced by non-lymphoid cells, the term *cytokine* is more appropriate. Cytokines are divided into families reflecting either their core functional domains and/or their shared structural homology, including, for example, hematopoietins, chemokines, interferons, TNF superfamily, IL-6 superfamily, IL-10 superfamily, and the IL-12 superfamily. Cytokines may exist as monomers, homo- or heterodimers, trimers, or tetramers. Receptors mainly comprise heterodimers; cytokine receptor families often utilize common receptor subunits (e.g. common  $\gamma$  chain receptor and IL-1RAcP). Receptors, like cytokines, can exist as membrane-signalling molecules, or may be released as soluble entities as a result of enzymatic cleavage from the cell membrane, or through the generation of alternatively spliced mRNA species. Soluble cytokine receptors can act as inhibitors of their ligands or act to facilitate signalling. Cytokine-receptor complexes may thereafter be internalized by endocytosis, fused with a lysosome where the cytokine will be destroyed in the proteasome. The receptor may either shuttle back to the cell surface or itself undergo degradation.

### Inflammation in tendinopathy

Historically the term tendinitis was used to describe chronic pain from tendons, thus implicating inflammation as a central pathological process. However, traditional treatment modalities aimed at modulating inflammation have limited success [106] and as previously mentioned histological lesions from surgical specimens have consistently shown the absence of inflammatory cells [107]. Insights into the role of inflammation in tendon disorders are however available from tendon healing models where inflammation is vital for the return of normal tendon function. Whilst not a perfect model for the study of tendinopathy they do provide us with some key steps in tendon repair biology. I described in section 1.1.5 that there is a huge influx of inflammatory cells particularly neutrophils at the site of tendon injury with the subsequent release of growth

factors and cytokines. These factors chemotactically attract leukocytes (neutrophils, lymphocytes and macrophages) to immigrate into the injured tissue [108] and to produce cytokines and growth factors (e.g. TGF $\beta$ 1, insulin-like growth factor [IGF]-I, basic fibroblast growth factor [bFGF], platelet-derived growth factor [PDGF], growth and differentiation factor [GDF]). In addition, enhanced IL-1 $\beta$  expression was evident in ruptured tendon [109] and TNF $\alpha$  also seems to be involved in healing processes at this time, as analysed in a rat Achilles tendon healing model [110].

Endogenous expression of various cytokines such as TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, VEGF and TGF $\beta$  has been also demonstrated in tenocytes [111-114]. Heat stress in tenocytes induced TNF $\alpha$  but not IL-1 $\beta$  expression in equine tendon fibroblasts [115]. Increased amounts of IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$  was demonstrated in inflamed native equine tendon [116]. Mechanical factors also influence tendon cytokine profile whereby cyclic strain has been shown to induce VEGF expression in tenocytes [117] while stress deprivation lead to an over-expression of IL-1 $\beta$  and TNF $\alpha$  and other cytokines such as TGF $\beta$  in the patellar tendon with subsequent mechanical deterioration of the tendon [118].

#### **TNF $\alpha$ , IL-1 $\beta$ , and IL-6 in tendinopathy**

TNF is a homotrimeric cytokine that is typically produced by monocytes, fibroblasts, and endothelial cells but has been shown to be produced in a variety of key immune cells including macrophages, T-cells and B-lymphocytes, granulocytes and eosinophils [119]. Its core biology is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, sepsis (through IL-1 & IL-6 production), cachexia, inflammation and to inhibit tumorigenesis and viral replication [120]. Dysregulation of TNF production has been implicated in a variety of human diseases, including Alzheimer's disease, cancer, major depression, and inflammatory bowel disease (IBD) [121]. TNF $\alpha$  was found to be up-regulated in inflamed equine tendon and also expressed in scar-formed tendon [122] while TNFR1 and -R2 co-localized on the same tenocyte and could be up-regulated by TNF $\alpha$  in equine tenocytes suggesting auto-amplification of the TNF response. TNFR-associated factor (TRAF)2 was also detected in tendon [123] while TNFR1 and -R2 co-localized on the same tenocyte were up-regulated by TNF $\alpha$  in equine tenocytes. Tenocytes can be highly activated by TNF $\alpha$  to produce further pro- and anti-inflammatory cytokines such as

IL-1 $\beta$ , TNF $\alpha$ , IL-6 and IL-10 and matrix degradative enzymes such as MMP1 [114] and these cytokines suppressed ECM synthesis such as that of type I collagen [124]. However, the expression of other ECM components such as elastin was up-regulated by TNF $\alpha$  [114]. TNF $\alpha$  expression was lower in loaded compared with unloaded tendon repair callus during healing, which underlines the important influence of mechanobiology on healing [110]. Cytoskeletal alterations toward a more stellate shape and loss of F-actin fibers were also visible in tenocytes treated with TNF $\alpha$  [114]. Thus TNF $\alpha$  is considered detrimental to matrix remodelling and the ultimate strength of injured tendons.

IL-1 exists within a family of structurally related trefoil molecules including IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra, IL-18 and IL-33. IL-1 typically is produced by activated macrophages, as well as neutrophils, epithelial cells, and endothelial cells possessing many metabolic, physiological, haematopoietic activities, and plays one of the central roles in the regulation of the immune responses [125]. IL-1R comprises two distinct forms. The type I receptor is primarily responsible for transmitting the inflammatory effects of IL-1 while the type II receptors may act as a suppressor of IL-1 activity by competing for IL-1 binding. The IL-1 receptor accessory protein (IL-1RaCP) is a transmembrane protein that interacts with IL-1R and is required for IL-1 signal transduction [126]. In human tenocyte cultures, IL-1 $\beta$  induced inflammatory and catabolic mediators such as cyclo-oxygenase (COX)-2, prostaglandin PGE2 and various MMPs while IL-1 $\beta$  regulated tenocytes cytoskeletal polymerization, and hence, cell stiffness, which was an important precondition for the cell adaptiveness to mechanical loading in tendon [124]. A disruption of cytoskeletal actin filaments leading to a more stellate cell shape and down-regulation of actin in IL-1 $\beta$ -treated tenocytes was observed whereby cytoskeletal tubulin was up-regulated [124]. Again IL-1 $\beta$  acts in a proinflammatory manner with an ultimate decrease in biomechanical strength.

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis [127]. The main sources in vivo are stimulated monocytes, fibroblasts, and endothelial cells. On target cells, IL-6 first binds to the IL-6 receptor (IL-6R). The complex of IL-6 and IL-6R associates with the signal-transducing membrane protein gp130, thereby promoting its dimerization and the subsequent initiation of intracellular signaling [128]. Gp130 is expressed by most, if not all, cells in the body, whereas IL-6R is mainly expressed by hepatocytes, neutrophils, monocytes/macrophages, and

some lymphocytes [129]. A naturally occurring, soluble form of the IL-6R (sIL-6R), which has been found in various body fluids, is generated by two independent mechanisms: limited proteolysis of the membrane protein and translation from an alternatively spliced mRNA [130].

Increased IL-6 production and activator of transcription (STAT3) phosphorylation was found in ruptured rotator cuff tendon [131]. IL-6 is a multifunctional cytokine, which exhibits immunoregulatory functions in tissues and is involved in matrix regulation. Mechanical properties of healing tendons in IL-6 knock-out mice were inferior compared with normal controls while tendon healing was also impaired underlining an essential role of IL-6 in tendon healing [132]. IL-6 was also up-regulated in tendon and peritendinous tissue during tendon exercise, while cyclic mechanical stretching enhanced the secretion of IL-6 in human tendon fibroblasts [133]. Despite this, IL-6 did not induce its own expression or that of TNF $\alpha$  or IL-1 $\beta$ , however, it had a marginal but significant stimulatory effect on IL-10 gene expression [114] which has been shown to modulate type I collagen and matrix metalloprotease gene expression in cultured human skin fibroblasts [134]. IL-6 was highly up-regulated by TNF $\alpha$  and IL-1 $\beta$  in tenocytes [112] and as reported in other cell types, produced then promoted the activation of the STAT3 signalling pathway, which is implicated in cell proliferation and survival [135].

These facts underline the putative functions of pro-inflammatory cytokines in injured and healthy tendon. It is probable that pro-inflammatory cytokines are essential regulators of tendon healing simultaneously acting in a positive and negative manner acting as a regulatory link between several catabolic and anabolic systems. The application of cytokine inhibitors and anabolic cytokines e.g. by using gene therapeutic strategies has been proposed to support tendon healing. In this context, the involvement of multiple cytokines in overlapping phases of tendon healing and tendinopathy, has to be further considered.

#### **1.4.1) Candidate cytokines to be explored**

The main thrust of this thesis, namely the role of cytokines in tendinopathy was born out of preliminary studies carried out in animal and human models of tendinopathy during a period of research in a collaborating laboratory in Sydney, Australia. There the running rodent model of tendinopathy was utilised and microarray analyses carried out in an attempt to identify genes and genetic

pathways that may contribute to the progression of degeneration and tendon damage [136]. Microarray analysis revealed several immune and stress response genes to be altered. Up regulation of cytokine genes including IL-18, and IL-17R were noted. Other key regulated genes included the apoptotic molecules, caspase 3 and 7, and heat shock proteins 27 & 70 all of which are known to play key roles in programmed cell death. Several signalling pathways including the mitogen activated kinases (MAPK), and other regulatory machinery including hypoxic inducible factor 1 were also noted to be significantly altered. While microarray analysis can indicate novel transcripts, further validation is required to evaluate quantitative message and protein change. Therefore these experiments were extended to samples of tendon from patients undergoing rotator cuff repair which confirmed overproduction of key cytokines and apoptotic pathway related molecules in torn supraspinatus and intact matched subscapularis tendon samples [137]. Thus the premise for key inflammatory molecules involvement in tendinopathy was set.

We know that fibroblasts are a diverse population of cells. Substantial evidence supports the concept that fibroblasts from different anatomic regions exhibit distinct phenotypes in culture. Characteristic expression patterns of receptors [138], gangliosides [139] [140], glycosaminoglycans [139, 141-143], plasminogen activator inhibitor type 1 [144-146], and PG endoperoxide H synthase-2 [147] have recently been documented in different populations of fibroblasts. Moreover, fibroblasts are capable of responding to their microenvironment in a complex manner. In some tissues, such as the orbit and lung, fibroblasts can be subtyped on the basis of the surface expression of the glycoprotein Thy-1 [148]. Despite this heterogeneity, many phenotypic attributes are common to fibroblasts regardless of the tissue of origin. Thus, they are capable of expressing multiple regulatory and communicative molecules, including cytokines, prostanoids, growth factors and adhesion molecules [149-151]. When activated, they can express chemokines such as IL-8 [150], RANTES [152], monocyte chemoattractant protein-1 and -2 [151]. Fibroblasts are also responsible for matrix homeostasis, continuously synthesizing and degrading a diverse group of extracellular molecules and their receptors. Rates of turnover of matrix molecules and the proteases that degrade them are normally under the control of diverse chemical and mechanical cues, with cytokines, growth factors, proteases, lipid mediators and mechanical forces playing roles. The maintenance of this

homoeostasis is vital to the preservation of normal tissue function and is clearly lost in chronic diseases of the joints and tendon disorders where destruction and excessive deposition is seen. A key example in tendinopathy remains the 'switch' in collagen matrix synthesis from the biomechanically superior type I collagen to type III which is inferior and produced in a haphazard fashion. The mechanisms underlying how fibroblast-like tenocytes manages this change remain incompletely understood. Thus, the fibroblast can function as a key orchestrator of diverse aspects of tissue reorganization. The opportunity to further investigate the role of cytokines within tendon fibroblasts should allow further expansion of the diverse role of the fibroblast as a central regulator of inflammation and matrix regulation.

Several IL-1 family members were detected as differentially expressed in the microarray and subsequent quantitative analysis. As introduced above, cytokines of the interleukin-1 (IL-1) family, such as IL-1 $\alpha$ / $\beta$  and IL-18, have important functions in host defence, immune regulation, and inflammation [153]. Investigation of their biological functions has led to novel therapeutic approaches to treat human inflammatory diseases [154]. Interleukin (IL)-33 is a novel member of the IL-1 family that exhibits close structural homology to IL-18. IL-33 is expressed mainly in endothelial cells and fibroblasts and as such is likely rapidly implicated in response to tissue insult, particularly its role as a novel alarmin. Furthermore its role in musculoskeletal pathogenetic processes such as rheumatoid arthritis and fibrosis [155] suggested that this cytokine may also be involved in the pathogenesis of tendinopathy. This thesis will directly address its role as a possible novel alarmin in tendon injury/damage.

The interleukin-17 (IL-17) family of cytokines is emerging as a key player in immune responses and increasing evidence shows that IL-17 family members play an active role in autoimmune and inflammatory diseases [156]. In humans, IL-17 is a potent inducer of other proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 from monocytes, macrophages or fibroblasts [157, 158]. IL-17 has been detected in synovial fluids and tissues of RA [159], inflammatory bowel disease [160] and psoriasis [161] patients. These findings suggest that IL-17 is an important cytokine located upstream of two pathways that are TNF- $\alpha$ -dependent and IL-6-dependent respectively [161]. It is notable that the organ systems that protect the host from the environment, specifically the lungs, intestinal mucosa and skin, seem to contain mostly IL-17 producing innate cells [162]. We chose to

investigate IL-17 as the initial rodent microarray showed a modest up regulation of IL-17A receptor (1.2 fold up regulation, unpublished data Millar et al) and human microarray data has highlighted a role for IL-17F in chronic tendinopathy. This combined with parallel laboratory studies investigating the mast cell/IL-17 axis in rheumatoid arthritis patients

In the following sections, these novel cytokines will be introduced in the context of their cytokine family, their structure, expression and biology as well as their potential role in disease thus far elucidated.

***Interleukin 17***

IL-17 (IL-17A) was discovered in 1993 originally as a rodent T cell cDNA transcript cytotoxic T lymphocyte associated antigen 8 (CTLA8). Human IL-17 was subsequently discovered and to date five additional members of the IL-17 family has been identified and termed IL-17 B,C,D,E and F. While historic data suggest IL-17 is produced mainly by T-cells, its receptor is expressed ubiquitously on various cell types, including myeloid cells, epithelial cells and fibroblasts. Thus, IL-17 exerts various biological functions in vivo, which might be involved in the pathogenesis of a wide range of infectious conditions and inflammatory disorders (Figure 1-7)

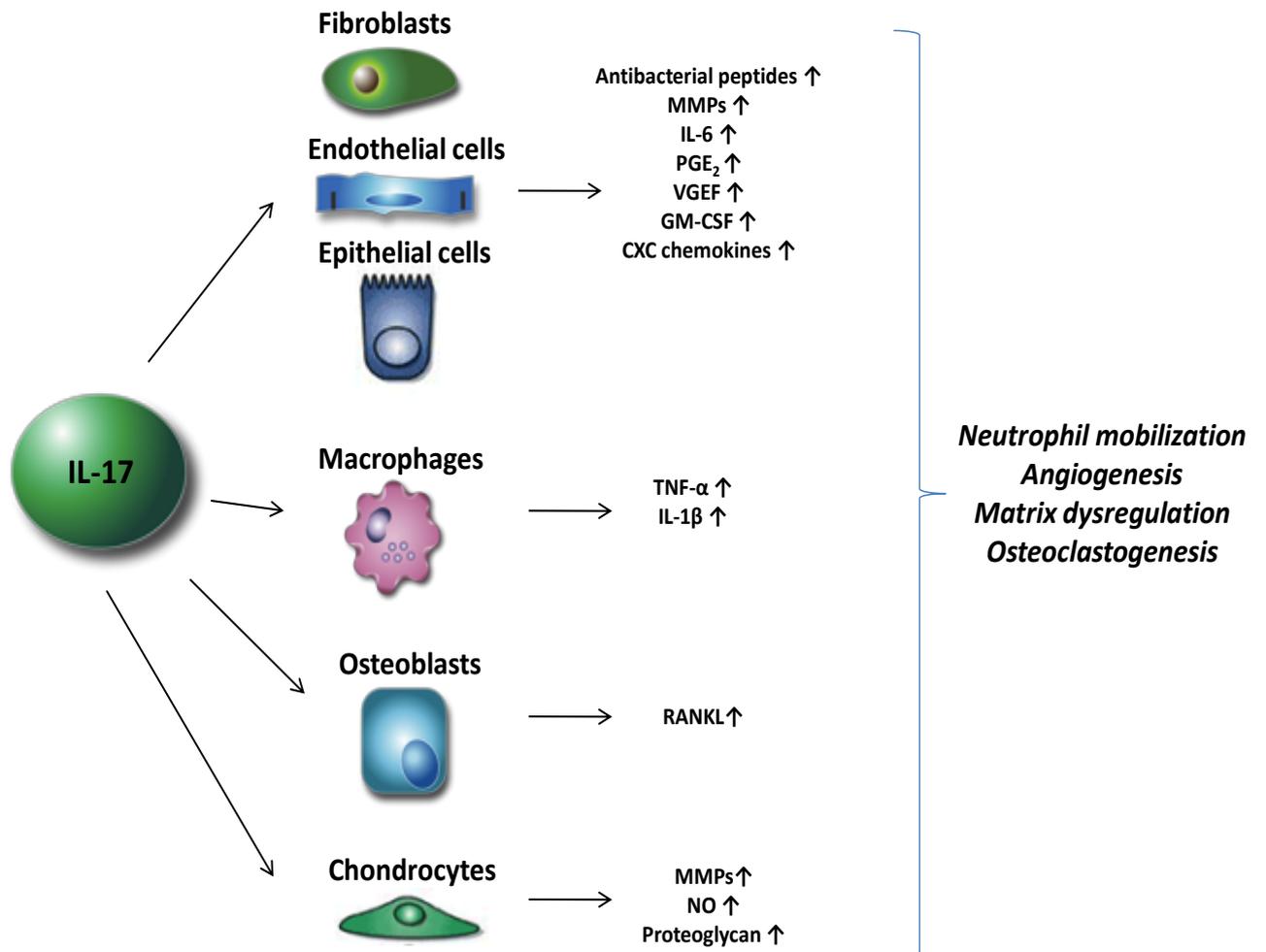
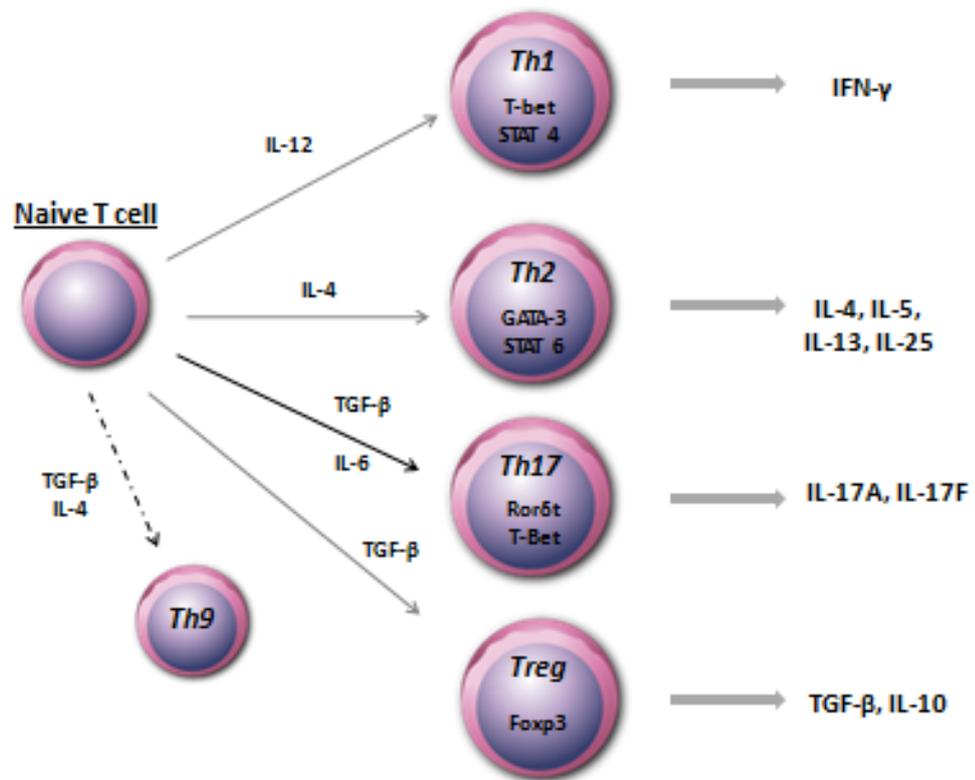


Figure 1-7: Effects of IL-17 on host defence, inflammation and tissue destruction

***Th 17 cells***

The differentiation of naive CD4<sup>+</sup> T cells into effector T helper cells is initiated by engagement of their T cell receptor (TCR) (signal 1) and co stimulatory molecules (signal 2) in the presence of specific cytokines produced by the innate immune system upon recognition of particular pathogens. IL-12 initiates differentiation of Th1 cells that produce high levels of IFN- $\gamma$  and are responsible for clearing intracellular pathogens. IL-4 triggers the differentiation of Th2 cells which organise host defence against extracellular pathogens and in help B cells to produce antibodies. The initial source of the differentiation factors for Th1 and Th2 cells are cells of the innate immune system responding to microbial products, including bacterial, viral, fungal or parasitic antigens, or allergens; however the cytokines produced by Th1/2 cells are capable of feedback mechanisms to further enhance differentiation of the respective T cell subset. This model first proposed by Mosmann and Coffman helped explain many phenomena in adaptive immunity. Recently this model has been expanded following the discovery of further subsets called Th9 and Th17 cells, in addition to distinct regulatory T cell subsets (Treg) (Figure 1-8)



**Figure 1-8: T helper cell differentiation (overview).** Differentiation driven by various cytokines to either proinflammatory cells such as Th1 and Th17, but also regulatory cells (Tregs).

Th17 cells were identified as an independent subset of T helper cells by the identification of differentiation factors and transcription factors that are unique to Th17 cells. In mice, a series of studies have shown that IL-6 and/or IL-21 and TGF- $\beta$  are key cytokines regulating the development of Th17 cells [163-165]. The Th17 lineage defining transcription factor was found to be ROR $\gamma$ t [166]. Interestingly, key cytokines of Th1 and Th2 cells, namely IFN- $\gamma$  and IL-4, block Th17 cell differentiation [167] commensurate with the existence of complex cross regulatory feedback loops in the effector T cell system. The cytokine IL-23 (comprising the subunits IL-12p40 and IL-23p19) is essential for the maintenance and expansion of murine Th17 cells [168].

Similar to their mouse counterparts, human Th17 cells express RORC2, the human orthologs of ROR $\gamma$ t [169] however development of human Th17 cells include more variables. Firstly, Th17 cell development depends on the T cell type, differing between naïve T cells and memory T cells. Naïve CD45RA<sup>+</sup>CD161<sup>+</sup> T cells derived from umbilical cord blood differentiate to Th17 cells under the influence of IL-1 $\beta$  and IL-23 [170]. This process is TGF- $\beta$  independent; however, it is thought that TGF- $\beta$  supports Th17 differentiation by inhibiting T-bet and hence Th1 development [171].

In peripheral blood, differentiation of Th17 cells from naïve T cells is not fully elucidated as yet. Specifically, the need for TGF- $\beta$  is controversial as serum containing media might be contaminated with platelets and TGF- $\beta$ . Serum free approaches demonstrated an essential requirement for low dose TGF- $\beta$  in conjunction with IL-1 $\beta$  and IL-23. However, the different approaches used in many of these studies, such as various purification techniques and culture conditions, make direct comparison of results difficult (reviewed in [172]). Interestingly, in human blood serum concentrations are usually higher than the concentrations which were used for culturing T cell *in vitro* in any of the described studies, and therefore most likely do not represent the *in vivo* situation. However, TGF- $\beta$  is not the only cytokine involved in the differentiation and maintenance of Th17 cells. Other cytokines such as IL-6, IL-21 and TNF- $\alpha$  have also been shown to support Th17 polarisation from naïve Th cells.

More clear is the development of Th17 cells from the memory pool. These effector cells can be identified by the combined expression of CCR4 and CCR6 [173] - another group described a CD4<sup>+</sup>CD45RO<sup>+</sup>CCR7<sup>-</sup>CCR6<sup>+</sup> population which

produce IL-17 [174]. CCR6 is thought to be involved in the recruitment of Th17 cells to sites of inflammation via the ligand CCL20. IL-1 $\beta$ , IL-23 and IL-6 promoted production of IL-17; however, TGF- $\beta$  inhibited IL-17 [174, 175] from this particular cell population. To summarize, due to a combination of ex vivo approaches including purification techniques and different cell culture conditions human Th17 biology is still elusive.

### ***Biology of IL-17***

Besides being produced by Th17 cells, both IL-17A and IL-17F are also produced by a variety of cell types, including  $\gamma\delta$  T cells, NKT cells, NK cells, neutrophils, eosinophils and mast cells. Thus IL-17 and IL-17F are effector cytokines produced by cells of both innate and adaptive immune systems. Indeed the IL-17 mediated immune pathway is induced within hours following epithelial cell injury or activation of pattern recognition receptors (PRRs) [176], which is not enough time for the development of TH17 cells. It is important to remember that the earliest reported functions of IL17A were its effects on innate immune responses [177].

One of the most important functions of IL-17 is the mobilization of neutrophils, which is mediated by the production of CXC chemokines, including IL-8 (CXCL8) and growth regulated oncogene-alpha (GRO $\alpha$ , CXCL1), and growth factors, including granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF), from epithelial cells, smooth muscle cells and fibroblasts. Murine models of infection have identified IL-17 in neutrophil mediated host defence against extracellular bacteria and fungi, such as *Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans*. IL-17 also participates in the elimination of pathogens by inducing antimicrobial peptides, such as  $\beta$ -defensins, especially in co-operation with IL-22. All of the aforementioned chemokines and growth factors have been identified in rodent models of tendinopathy or tendon healing.

### ***IL-17 in inflammatory disease***

IL-17 mediates various biological activities, which potentially cause tissue destruction and degeneration during chronic inflammation. IL-17 stimulates

macrophages to produce various cytokines such as IL-1 $\beta$  and TNF $\alpha$ . Furthermore, IL-17 acts synergistically with TNF- $\alpha$  in IL-6 and GM-CSF production from fibroblasts. In rheumatoid arthritis IL-17 induces cartilage destruction via induction of metalloproteinases and inhibition of proteoglycan synthesis while also inducing expression of RANKL on osteoblasts leading to bone destruction. IL-17 stimulates MMP, IL-6 and IL-8 production from cultured colonic subepithelial myofibroblasts in intestinal inflammation. Increased expression of IL-17 mRNA has been detected in biopsies from lesional psoriatic skin while IL-17 up regulates production of GM-CSF, IL-6 and GRO- $\alpha$  from keratinocytes. A summary of current inflammatory diseases with IL-17 implicated pathology is shown in Table 1-5

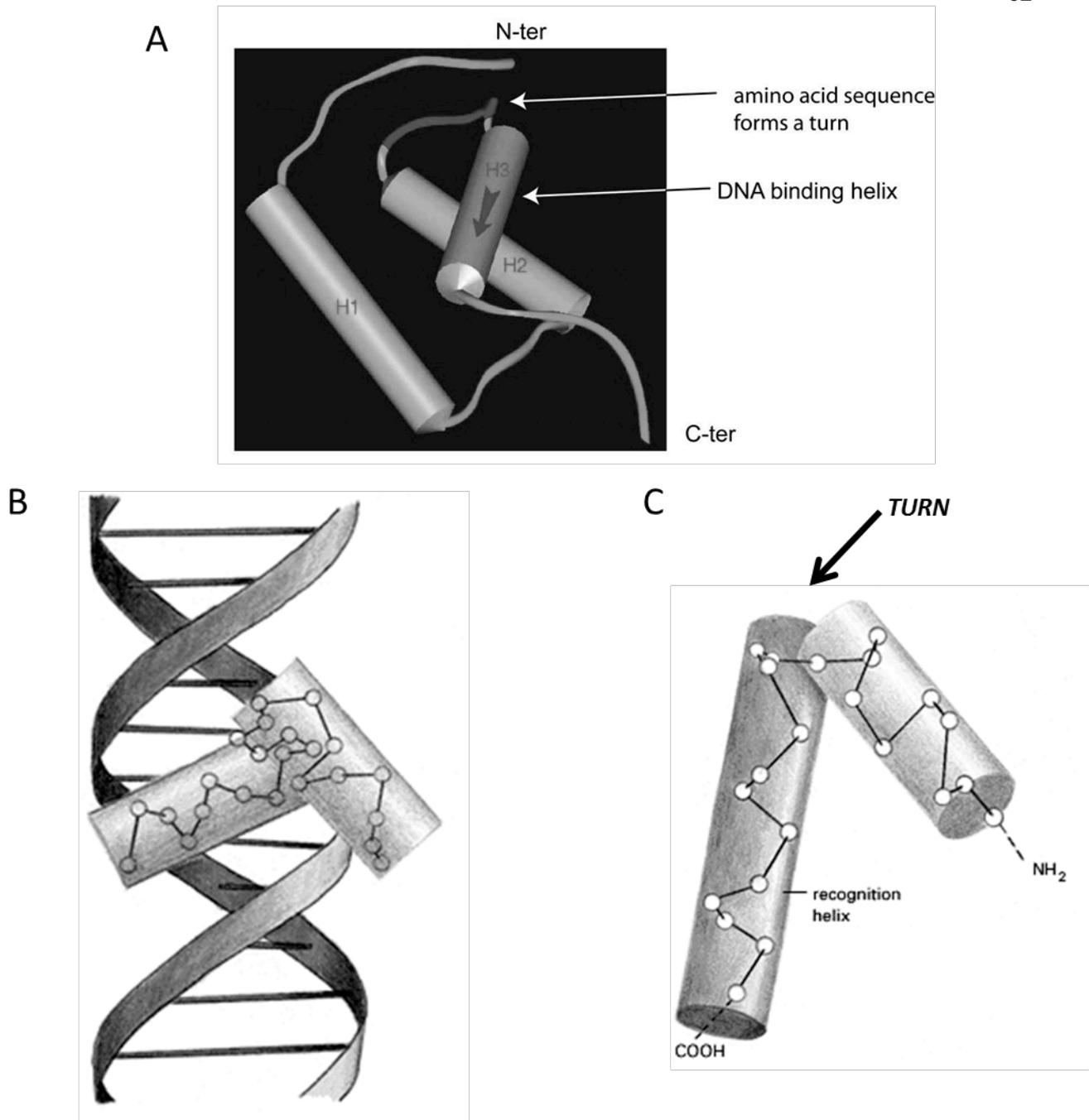
Disease	Role of IL-17	References
EAE(Experimental autoimmune encephalomyelitis)/Multiple sclerosis	<p>IL-17A KO mice have milder EAE</p> <p>Anti IL-17A antibody treatment attenuates EAE but does not abrogate disease</p> <p>IL-17 highly expressed in chronic MS lesions</p> <p>IL-17 found in CD4+ and CD8+ astrocytes of MS lesions</p>	[178-181]
Inflammatory skin disease/Psoriasis	<p>Increased Th17 cells in psoriatic lesions</p> <p>Anti TNF therapy in psoriasis works by decreasing Th17 cells</p>	[182, 183]
Inflammatory bowel disease	<p>IL-17 produced in healthy gut</p> <p>Anti IL-17A monoclonal antibody treatments aggravates DSS induced colitis</p> <p>Increased Th-17 related molecules in human IBD lesions</p>	[181, 184, 185]
Experimental arthritis/ rheumatoid arthritis	<p>IL-17 KO mice have reduced arthritis</p> <p>Vaccination against IL-17A reduces arthritis</p> <p>Expansion of Th17 cells in RA</p>	[186-188]

**Table 1-5 Functions of Th-17 / IL-17 in disease processes**

### ***Interleukin 33***

IL-33 (IL-1F11) is the latest IL-1 family member to be discovered. Initially described as nuclear factor of high endothelial venules (NF-HEV) by Girard and colleagues in 2003, Schmitz et al defined its function and role in the IL-1 family in 2005 [189, 190]. This group described the *IL33* gene after searching a computational derived database of the IL-1 family members where they identified a ligand for the then orphan receptor, ST2.

The sequence of IL-33 has been mapped to human chromosome 9 (9p24.1) and mouse chromosome 10 (19qC1). The cDNA encodes for 270 and 266 amino acids for human and mouse, respectively, and are approximately 55% identical at the amino acid level [189]. Its molecular weight is ~ 30 kDa. Using sequence and secondary structure alignment, Girard and colleagues demonstrated that IL-33 consists of a homeodomain like helix turn helix motif (HTH) on the amino terminal side [190]. This is followed by a nuclear localisation signal and further 12 predicted  $\beta$  strands comprising an IL-1 like cytokine domain with a typical  $\beta$ -trefoil structure (Figure 1-9). The 1-65 amino acid HTH motif of IL-33 has homology with drosophila transcription factors showing the best match with *Engrailed* and these HTH motifs are known to be repressors of transcription. *Engrailed* is a Drosophila homeodomain protein required for proper segmentation and maintenance of the posterior compartment identity. The repressor activity of *Engrailed* lies in the sequence containing amino acids 1–298 which has been shown to confer transcriptional repression when fused to heterologous DNA binding domains. Use of dominant-negative transgenes has been successful in analysing certain pathways and using this technique to convert transcription factors into dominant repressors can be achieved by fusion to repressor domains such as *Engrailed* [191]. Accordingly, translational fusions with the *Engrailed* repressor domain have efficiently converted plant transcription factors, b-catenin and c-myc, into dominant-negative proteins. Also, *in vivo*, an airway-targeted GATA6-*Engrailed* dominant-negative fusion construct was able to alter epithelial differentiation [192].

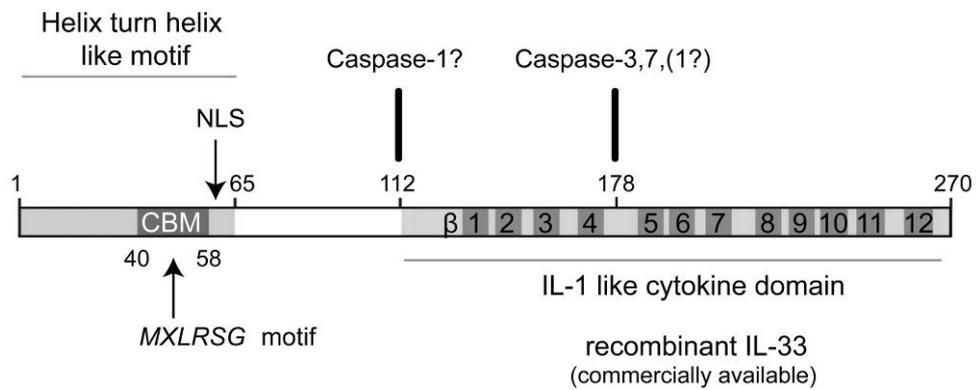


**Figure 1-9 Structure of IL-33 HTH like motif**

Example of HTH like motif in comparison to other HTH genes. Figure A demonstrates the structure of IL-33 HTH like part with three helices where the helix number 3 (arrow) is able to bind DNA; this figure has been altered from [190]. B demonstrates a schematic picture of a HTH in which one  $\alpha$ -helix binds DNA in the major groove. Black arrow indicates the turn.

Carriere et al [193] demonstrated that IL-33, particularly the HTH like part is co-located to heterochromatin. By truncation of IL-33 HTH-like part this heterochromatin association was abolished and IL-33 expression became unrelated to the heterochromatin. Thus, similar to *Engrailed*, IL-33 is thought to be a suppressor of transcription. More interestingly, by fusing IL-33 to the Gal4-DNA-binding domain in gene reporter assays with a GAL4-responsive luciferase reporter, transcriptional activity was reduced illustrated by decreased luciferase activity. This could be re-established by mutating the DNA binding parts of IL-33 [194].

Next to the chromatin binding domain in the HTH like motif, IL-33 possesses a nuclear localisation signal (NLS), near the N-terminal domain. Truncation of the NLS (e.g. a construct containing only IL-33<sub>112-270</sub>) leads to retention of IL-33 in the cytoplasm [193, 195]. The HTH like motif and NLS (important for nuclear localisation and DNA binding) are linked to the IL-1 like cytokine domain consisting of 12  $\beta$  strands. The latter part has mainly been used to study biological effects of IL-33, as it has been assumed that IL-33 is cleaved by caspase-1 leading to “mature IL-33” (the IL-1 like cytokine domain) which is thought to be released in a similar way to IL-1 or IL-18 [189]. Interestingly, recent studies revealed that IL-33 is not cleaved by caspase-1, but instead cleaved by apoptosis associated caspases 3 and 7 as well as calpain and as a result is therefore inactivated rather than activated [196-198]. Although, caspase 3 treated IL-33 breakdown products are not able to bind ST2 (the IL-33 receptor), the N terminal part still translocates to the nucleus [196]. Moreover, different groups showed that full length IL-33 is bioactive and can be released by necrosis classifying IL-33 as an alarmin [197-200].



**Figure 1-10: IL-33 structural components and cleavage sites.**

N terminal domain shows HTH like motif including chromatin binding domain (CBM) with the specific amino acid motif and the nucleus localisation site (NLS).  $\beta$ 1-12 demonstrates the IL-1 like cytokine domain with 12  $\beta$  strands. At localisation amino acid 112 the initial described caspase-1 cleavage site from Schmitz et al [189] is shown. Further after the residue Asp178 Cayrol et al showed a truncation by caspase 1 and 3 which deactivated IL-33 signalling through ST2 [199]. One month later, Luthi et al published truncation at Asp178 by caspase 3 and 7, but did not confirm caspase 1 [200]. It should be emphasised that the structure in biological assay using recombinant IL-33 is a product reflecting the IL-1 like cytokine domain truncated at amino acid 112.

### ***IL-33 receptor complex***

IL-33 signals through a receptor complex comprising ST2L, a subunit that is specific for IL-33 [189] and IL-1R accessory protein (IL-1RAcP) that is widely shared between other IL-1 receptor family members [201, 202]. ST2 (also named T1, IL1RL1, DER4, and FIT-1) was first described in 1989 [203]. Differential splicing leads to formation of 3 isoforms: transmembrane ST2, a soluble form (soluble ST2 or sST2) and a variant ST2. ST2 is a member of the IL-1 receptor family and has 38% amino acid homology to the IL-1 receptor [204]. Transmembrane ST2 is membrane-bound with 3 extracellular immunoglobulin-G domains, a single transmembrane domain, and an intracellular domain homologous to toll-like receptors and other IL-1 receptors. Soluble ST2 lacks the transmembrane domain as well as the intracellular domains and is thought to act as a decoy receptor for IL-33 signaling. ST2 deficient mice are healthy and display no overt phenotypic abnormalities [205]. The initial manuscript describing ST2 deficiency demonstrated that mice challenged with a pulmonary parasite infection, exhibited significantly impaired levels of Th2 cytokine production.

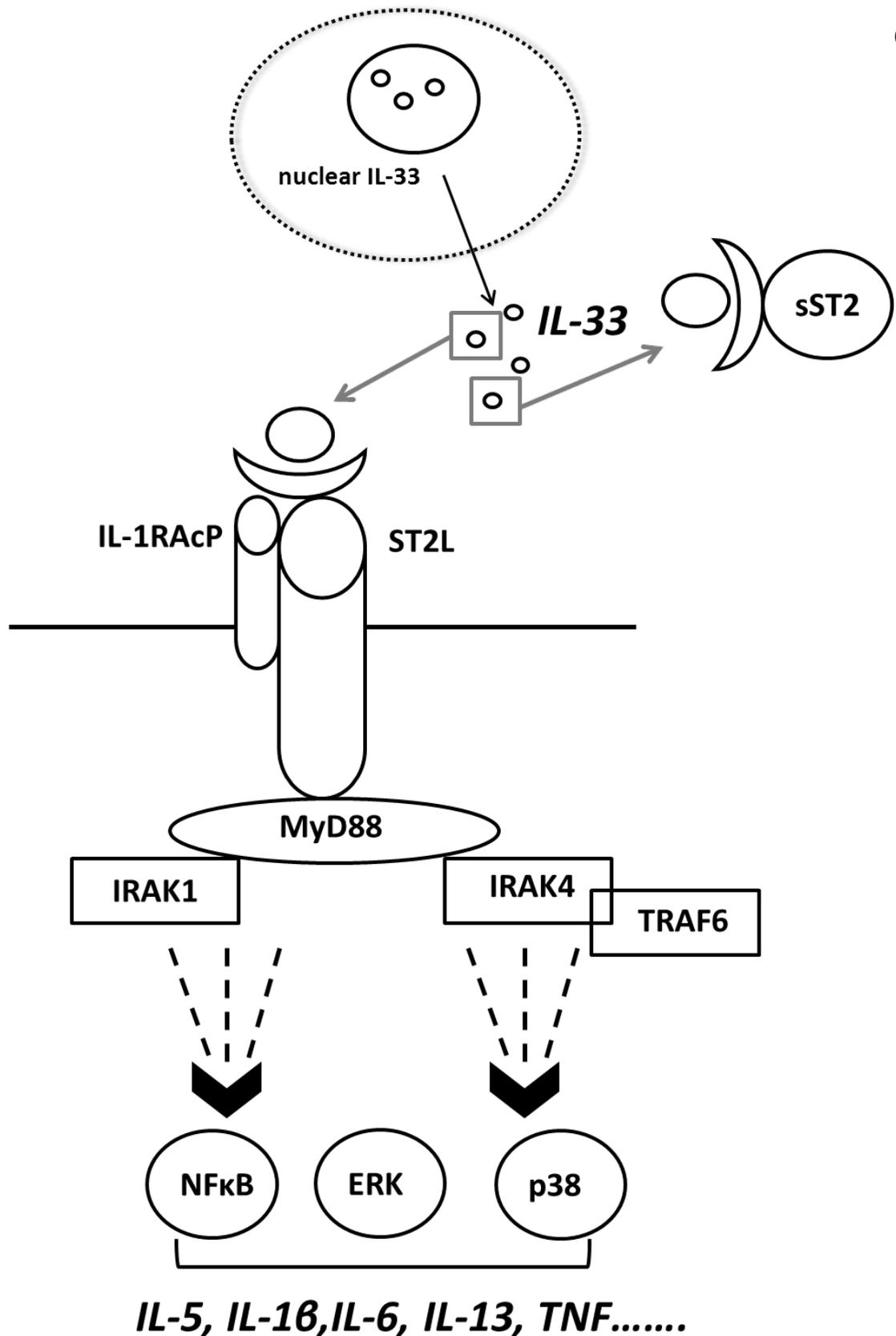
ST2 is mainly expressed on mast cells and Th2 cells but not on Th1 or Th17 cells [205-207]. Further expression has been detected on many cell types including fibroblasts endothelial cells and other stromal cells but also on dendritic cells, macrophages [208-210] and basophils [211-213]. Both ST2L and IL-1RAcP seem essential for IL-33 action as mice deficient either for ST2 or IL-1RAcP are unresponsive to IL-33 administration, whereas wild type animals exhibit a marked inflammatory response [202]. Interestingly, soluble IL-1RAcP enhances the ability of sST2 to inhibit IL-33 signalling [214]. Signal transduction in ST2 and IL-1RAcP is mediated via the Toll-IL-1R (TIR) domain which is shared among the entire TLR/IL-1R receptor superfamily. Upon binding of IL-33 the TIR domain of IL-1RAcP directly interacts with the TIR domain of ST2/IL-33 contributing to the IL-33 mediated signal [202]. Downstream of the IL-33/ST2L/IL-1RAcP complex, the IL-33 signal is transmitted via the recruitment of the myeloid differentiation primary response protein 88 (MyD88), and TNF receptor-associated factor (TRAF-6) to the TIR domain in the cytoplasmic portion of ST2L (and IL-1RAcP) where these molecules are activated [189]. This in turn leads to recruitment of IL-1R-associated kinase (IRAK) 1 and IRAK4 and TNFR-associated factor 6 (TRAF6) [189, 215]. MyD88 is essential for IL-33 signalling as MyD88 deficient mice do not respond to

IL-33 administration [202]. IL-33 stimulation also leads to phosphorylation of inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ ) as well as the kinases Erk1/2, p38, and JNK [189]. Further key signalling events downstream of MyD88 are summarised as follows [216]:

Further key signalling events downstream of MyD88 include [216]:

- MyD88 recruits IRAK1 and IRAK4; IRAK4 phosphorylates IRAK1
- IRAK1 phosphorylates Pellino-1 (E3-ligase), this leads to IRAK1 polyubiquitination
- further recruitment of NEMO/IKK1/IKK2 complexes
- polyubiquitination of TRAF6 with TAK1 recruitment to the NEMO/IKK1/IKK2 complex
- activation of IKK2 by TAK1
- TAK1 also couples to the upstream kinases for p38 and JUN N-terminal kinase-1 (JNK).

However this intracellular activation cascade by IL-33 (or IL-1) is likely to be more complex [216]. Furthermore in mast cells, IL-33 triggers Ca<sup>2+</sup> influx by the activation of phospholipase D and sphingosine kinase [217]. Although ST2 receptor signalling is understood in the broad context, it is not clear why signalling cascades through MyD88 by different receptors leads to different cellular responses.



**Figure 1-11 IL-33 binding to ST2 and IL1RAcP signals via MyD88**  
 Schematic overview for binding and signalling of IL-33: nuclear IL-33 is released by necrosis and binds to ST2 which leads to recruitment of IL-1RAcP, MyD88, IRAK1, IRAK4. TRAF6 is necessary for recruitment of IRAK. This signalling complex leads through further events to activation of NF-κB and p38 and JNK pathways. These pathways induce gene expression leading to cytokine and chemokine synthesis

### ***Tissue localisation and cellular expression of IL-33***

To date most reports visualising IL-33 expression demonstrate that IL-33 is almost exclusively expressed in the nucleus. Using antibodies against the C terminal region (e.g. Nesy-1) most analyses demonstrate (in nearly every tissue) nuclear expression of IL-33 although a few notable exceptions exist. IL-33 is localised within the cytoplasm of airway smooth muscle cell bundles from asthmatic patients. These cells also secrete small but significant amounts of IL-33 [218]. Cytoplasmic IL-33 staining is also evident in intestinal epithelial cells taken from patients suffering from ulcerative colitis and Crohn's disease with correspondingly high levels in serum from the same patients [219]. The nuclear expression pattern mainly occurs in stromal cells such as endothelial cells and epithelial cells. IL-33 was initially described as a "nuclear factor of high endothelial venules" consistent with this reported expression pattern. In addition, Schmitz et al reported IL-33 expression in cDNA libraries. They showed that IL-33 is broadly expressed across different tissues; however, on the cellular level differences emerge. Mouse organs expressing high levels of IL-33 cDNA include stomach, lung, spinal cord, brain, and skin. In humans, IL-33 cDNA was detected in adherent stromal cells like epithelial/endothelial cells, smooth muscle cells, fibroblasts, and keratinocytes where the latter two upregulated IL-33 after TNF- $\alpha$  and IL-1 stimulation [189].

Kuchler et al demonstrated that IL-33 is globally expressed in nuclei of vascular endothelium in normal human tissues staining vessels in human skin, small intestine, umbilical cord and lung [220] and further show *in vitro* that vascular IL-33 is downregulated by proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and VEGF. More surprisingly, IL-33 is strongly down-regulated in endothelium undergoing tumor or wound healing angiogenesis. These findings are distinct and contradict fibroblast derived data, where IL-1 and TNF- $\alpha$  upregulated IL-33 [210]. However, cell type and stromal origin could explain this difference, as could culture conditions as Kuchler et al used superconfluent cultures of human umbilical vein endothelial cells (HUVECs) and Xu et al used synovial fibroblasts with ~80% confluency. In contrast to IL-33 expression in endothelial cells, Choi et al studied the response of HUVECs to IL-33. They reported that IL-33 has a significant effect on angiogenesis and vascular permeability by rapidly increased endothelial nitric

oxide (NO) production [215]. Overall, a more careful dissection of the role of IL-33 in endothelial cells is required.

### ***IL-33 release and nuclear function***

Initial studies hypothesized that IL-33 is released in a caspase-1 dependent manner [189]. NLRP3 (NLR family, pyrin domain-containing 3) inflammasome activation by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) leads to oligomerisation of NLRP3 and clustering of pro-caspase-1. This results in caspase-1 auto-activation and caspase-1-dependent processing of cytoplasmic targets, including the pro-inflammatory cytokines IL-1 $\beta$  and IL-18, which require this step to be biologically active and mediate repair/inflammation responses such as angiogenesis and neutrophil influx to remove cellular debris or fight pathogens [221]. Full length IL-33 with ~30 kDa was reported initially to be cleaved to a 20-22 kDa product in a similar way to IL-1 and IL-18 [189]. However IL-33 orthologs from various other species lack conserved caspase I sites which cast doubt on the necessity of caspase I cleavage for bioactivity. Subsequent studies have shown that IL-33 like IL-1 $\alpha$  is biologically active in its full length form and more importantly cleavage by caspase I resulted in a truncated form of IL-33 that was unable to signal [199]. IL-33 is also cleaved by the apoptosis associated proteases, caspase 3 and 7 which attenuated IL-33's ability to signal [200].

IL-33 expression and active secretion have been observed in only a limited number of studies. It was shown that inflammatory cells including monocytes, macrophages, astrocytes and synovial fibroblasts, in response to stimulation with a range of pro-inflammatory signals including LPS, IL-1 $\beta$  and TNF $\alpha$  [198, 210, 222] release IL-33. Increasing numbers of different groups established the fact that either chemical (H<sub>2</sub>O<sub>2</sub>, NaN<sub>3</sub>, Daunorubicin, TritonX100) or mechanical (scratching, freeze/thaw) necrosis of IL-33 expressing cells release full length IL-33 into the media [199, 200]. Interestingly some investigators report increased extra-cellular IL-33 expression in diseases with high number of necrotic cells such as RA, SLE [223, 224] but the mechanism/pathway as to how IL-33 is released in such disease states remains ill-defined. This has led to the suggestion that IL-33 acts as a DAMP or alarmin whereby its passive release from damaged or dying cells sends a signal to cells of the innate immune system alerting them to the presence of potentially harmful pathogens. In this regard IL-33 is thought to act in

a similar manner to the archetypal alarmin, High mobility group box 1 (HMGB1) as both are released from dying cells and both are nuclear factors with HMGB1 having well characterized roles in gene regulation, chromatin remoulding, and DNA replication and repair [225]. Like HMGB1 IL-33 has also been shown to interact with heterochromatin and mitotic chromatin and is released from necrotic cells whilst being actively retained complexed within chromatin of apoptotic cells.

In a similar fashion to IL-1 $\beta$ , IL-18 and HMGB1, IL-33 lacks a signal peptide and thus cannot be mobilized via the classical ER-Golgi exocytotic pathway. Some reports suggest that IL-33 secretion may involve similar processes to that for IL-1 $\beta$  and IL-18. The release of IL-1 $\beta$  and IL-18 requires two signals, an initial signal in the form of PAMP (e.g. LPS) which stimulates gene expression with subsequent exposure to ATP resulting in an influx of K<sup>+</sup> via the nucleotide gated ion channel P2X7 which activates the inflammasome resulting in activation of caspase 1 with subsequent bioactivity and release [226]. Whilst as generally accepted it is unlikely IL-33 is a substrate of caspase 1 it may still be required for secretion as is the case for HMGB1 [227] with a recent study showing significant release of IL-33 from astrocytes treated with LPS and ATP [228]. This study showed that LPS induces expression with localization to the nucleus followed by mobilization of IL-33 from the nucleus to the cytoplasm with ATP incubation. However this does not hold true for all inflammatory cell types as while IL-33 expression could be induced by PAMP stimulation in primary human monocytes and a monocyte cell line [222], no secretion of IL-33 was detected upon incubation with ATP, conditions which elicited the secretion of both IL-1 $\beta$  and IL-18.

One of the most interesting aspects surrounding the biology of IL-33 remains its nuclear expression profile. Its presence in the nucleus represents a significant barrier to its secretion as unlike its IL-1 family members, IL-1 $\beta$  and IL-18 it must negotiate passage through both nuclear and cell membranes. HMGB1 undertakes a similar passage by encoding a nuclear export sequence (NES) and a variety of post-translational modifications all of which serve to redirect HMGB1 out of the nucleus. IL-33 does not contain a NES and presently no post-translational modifications have been described. Although the nuclear functions of IL-33 remain to be fully investigated, the association of IL-33 with heterochromatin has been suggested to be functionally linked to transcriptional repression and chromatin compaction [193]. Recently nuclear IL-33 expression was reported in pancreatic stellate cells, where inhibition of IL-33 expression, mediated by small interfering

RNA, decreased cell proliferation [229]. Thus, like pro-IL-1 $\alpha$ , IL-33 may act as a dual-function cytokine that exerts unique biological activities in the cell nucleus independently of cell-surface receptor binding. One could further postulate complex intracellular interactions analogous to the internalization and subsequent signalling of some TLRs.

### ***Biological cellular function of IL-33***

Early studies showed selective expression of ST2L by T<sub>H</sub>2 but not T<sub>H</sub>1 cells [206, 230] and thus IL-33 was initially investigated in T cells. T<sub>H</sub>2 cells treated with IL-33 enhanced IL-5 and IL-13 production but not IL-4 [189]. Further it acts as chemoattractant for human Th2 cells [231]. IL-33 also stimulates NK and NKT cells to produce IFN- $\gamma$  [232]. DCs express low levels of ST2; however, upon stimulation with IL-33, DC can induce Th2 cytokines in CD4 T cells [209].

IL-33 is a potent inducer of cytokines and chemokines in mast cells (TNF, IL-1, IL-6, IL-13, CCL2 and CCL3) [233]. In particular it induces the degranulation of IgE primed mast cells and enhances mast cell maturation and survival [234]. Three reports describe basophils and their response to IL-33 [211-213]. Schneider et al studied murine basophils and found that cells express ST2 and respond in an unprimed state with the production of IL-4 and IL-6 [212]. *In vivo* indirect increase of GM-CSF and IL-5 promoted expansion of basophils. In humans, Smithgall et al and Suzukawa et al confirmed ST2 expression in basophils [211, 213]. Stimulated with IL-33, these cells produced IL-4, IL-5, IL-6 and IL-13 and increased their adhesive capability.

A role for IL-33 in the activation and maturation of other myeloid lineage cells is also emerging. IL-33 enhances the production of TNF by macrophages treated with lipopolysaccharide and lipoteichoic acid [235]. Importantly, IL-33 can amplify IL-13-mediated polarization of alternatively activated macrophages to produce increased levels of CCL17 and CCL24, which contribute to airway inflammation [236]. Dendritic cells (DCs) express ST2 at low levels but respond to IL-33 by upregulation of MHC class II molecules and the co-stimulatory molecule CD86 [209]. IL-33 can also activate DCs to produce IL-5, IL-6 and IL-13 by CD4<sup>+</sup> T cells [209], suggesting an additional indirect role of IL-33 in the induction of adaptive immune responses. The ability of IL-33 to target various immune cell types highlights its potential in influencing the outcome of a wide range of diseases.

### ***IL-33 in experimental models***

Mice treated with IL-33 exhibit splenomegaly, blood eosinophilia and increased levels of IgA and IgE. This relates to a Th2 phenotype with increased IL-4, IL-5 and IL-13 [189]. Also anatomical changes of stomach and mucus- and bile-filled duodenum as well as lung were observed with recruitment of myeloid cells and epithelial reaction with either hyperplasia (esophagus) or massive mucus production (lung). In contrast, IL-33 administration was beneficial in a model of atherosclerosis [237]. In ApoE deficient mice fed on a high fat diet, injection of IL-33 significantly reduced atherosclerotic lesions in terms of size and leukocyte infiltration. Also serum levels were increased for IL-4, IL-5 (especially) and IL-13 with decrease of IFN- $\gamma$  suggesting a switch from a Th1 to Th2 phenotype. This atheroprotective effect could be blocked with anti-IL-5 suggesting rather indirect effect whereby IL-33 operated via modulated IL-5 production. IL-33 also induced oxidized low-density lipoprotein (ox-LDL) antibodies which are thought to be atheroprotective. Interestingly, it has also been shown that IL-33/ST2 signalling is a crucial biomechanically activated system that controlled cardiomyocyte hypertrophy and cardiac fibrosis after pressure overload [238]. Soluble ST2 levels also negatively correlate with survival and directly with cardiac injury and microvascular injury post myocardial infarction [239].

In a mouse model of RA, collagen induced arthritis was diminished in ST2 deficient mice [210]. In addition, CIA mice treated with recombinant IL-33 led to exacerbation of disease. This had no effect in ST2 deficient mice. Furthermore, reconstituting ST2 deficient mice with mast cells (expressing normal levels of ST2) again led to exacerbation of arthritis [210]. This supports the important role of mast cells in proinflammatory cytokine production. It is interesting, that despite the Th2 profile of mast cells, secretion of proinflammatory cytokines like IL-1, IL-6 and IL-17 can bias the balance to a Th1/Th17 autoimmune phenotype. It is noteworthy, that Raza et al and Kokkonen et al detected increased Th2 cytokine patterns immediately after the onset of RA disease [240, 241]. The potency by which IL-33 increases severity of CIA was confirmed by Palmer et al using anti-ST2-antibodies [242]. Furthermore they showed a decrease in IFN- $\gamma$  as well as IL-17 production in the draining lymph nodes. Therefore IL-33 clearly drives Th1/Th17 responses in experimental arthritis. This is confirmed by higher levels of IFN- $\gamma$  and IL-17 in the IL-33 treated CIA mice [210]. Focussing on autoantibody induced arthritis (AIA),

IL-33 exacerbated arthritis, where AIA in ST2 deficient mice developed attenuated disease [243].

Administration of IL-33 exacerbates experimental asthma and induces features of asthma in naïve animals. Furthermore blocking ST2 or IL-33 attenuates disease in some models [233]. More evidence is emerging that allergic IL-33/ST2 pathology is antigen mediated with induction of antigen specific IL-5 expressing T cells [244]. Even more strikingly is the fact, that a SNP in a region flanking *IL33* is associated as risk factor for asthma in a large-scale, consortium-based genome wide association study [245].

IL-33 deficiency did not influence the pathogenesis of experimental autoimmune encephalitis [246]. The severity of dextran sodium sulfate-induced colitis was attenuated in IL-33-deficient mice in the early stages of disease, but recovery was delayed in these mice [246]. Subcutaneous administration of IL-33 to wild-type mice led to the accumulation of inflammatory cells and the development of skin fibrosis, which was dependent on IL-13 and the presence of eosinophils [247]. Of note, IL-33 levels have been shown to correlate with collagen type III expression in this particular study which as previously mentioned is a key feature of collagen change in tendinopathy.

### ***IL-33 in human disease***

Serum levels of IL-33 are significantly higher in patients with RA than in those with OA or in healthy individuals [223]. IL-33 levels correlated with RA disease activity [223], and with the presence and titre of rheumatoid factor and antibodies against cyclic citrullinated peptides [248]. In general, IL-33 is present primarily in the nuclei of endothelial cells and in some cases also in synovial fibroblasts, without any specific difference in IL-33 tissue expression pattern between RA, osteoarthritis and psoriatic arthritis [249]. In addition, the expression of ST2 on neutrophils is decreased in patients who respond to therapy with the TNF inhibitor infliximab [250], suggesting that both production of and cellular responses to IL-33 are modulated by proinflammatory cytokines. In early systemic sclerosis, nuclear expression of IL-33 in dermal endothelial cells and keratinocytes was down regulated; by contrast, IL-33 expression was enhanced in dermal and pulmonary fibroblasts [208]. Serum levels of IL-33 were comparable in patients with systemic lupus erythematosus (SLE) and healthy subjects in one study,

whereas other investigators have detected elevated levels in patients with SLE [251, 252].

Serum concentrations of sST2 are elevated in patients suffering from disorders associated with an abnormal TH2-like immune response, including atopic dermatitis and asthma [253]. Indeed in endobronchial biopsies obtained from adults with varying severities of asthma, higher levels of IL-33 transcripts were detected in biopsies compared with control subjects [218]. In the same study airway smooth muscle cells (ASMCs) showed IL-33 expression at both protein and mRNA levels and TNF- $\alpha$  up-regulated IL-33 expression by cultured ASMCs in a time- and dose-dependent manner with the authors proposing IL-33 as a novel inflammatory marker of severe and refractory asthma.

IL-33 has also been linked with inflammatory bowel disease. IL-33 production is increased in active lesions of ulcerative colitis but not in Crohn's disease with subepithelial myofibroblasts identified as the major cellular sources of IL-33 in ulcerative colitis lesions [254, 255]. The TLR3 ligand polyinosinic–polycytidylic acid, administered alone or in combination with transforming growth factor  $\beta$ , strongly induced IL-33 expression in myofibroblasts [254]. It is noteworthy that serum levels of IL-33 decreased following the administration of infliximab in patients with ulcerative colitis [219].

Overall these findings demonstrate clearly that IL-33 production is enhanced in several inflammatory diseases, and that resident cells, including epithelial cells, endothelial cells and fibroblasts, are major sources of IL-33.

## 1.5) Novel regulation of cytokine production

Intercellular signalling by cytokines is a vital feature of the innate and adaptive immune system. Cells respond to extracellular stress or stimuli by driving intracellular signalling cascades that coordinate cellular gene expression through complex networks of kinase activation, protein phosphorylation, and activation of DNA-binding proteins that translate signals at the cell surface to actions of transcriptional regulation of cellular genes. Cytokines modulate communication between cells of the immune system and between immune cells and differentiated somatic cells. Upon binding to their cognate receptor on the cell surface, cytokines trigger transcriptional changes and balance cellular activities ranging from growth to differentiation and cell survival. Cytokine-directed transcriptional induction of yet other cytokines may further enhance the innate immune response in an increasingly entangled network of signals.

Cytokines are heavily regulated at the transcriptional level, but emerging evidence suggests that regulatory mechanisms that operate after transcription play a key role in balancing the production of cytokines. Thus I wished to explore two more recently identified mechanisms, namely hypoxia and microRNA, promoting cytokine regulation which have been shown in similar fibroblast driven inflammatory disorders to have key functional consequences on cytokine production. The next section introduces these two regulatory pathways and their possible relevance to tissue damage in tendon disease.

### **Hypoxia and cytokine regulation**

Normal oxygen delivery is essential for survival of organisms and evolutionarily conserved mechanisms have been developed to maintain oxygen homeostasis. Changes in tissue oxygen levels in pathologic conditions are caused by an imbalance between cellular oxygen demand and tissue oxygen delivery. Thus, tissue hypoxia is a feature of a number of pathologic conditions ranging from cancer to inflammatory diseases. Oxygen homeostasis is largely maintained through a family of hypoxia-inducible transcription factors (HIFs), composed of a constitutively expressed HIF-1 $\beta$  subunit and a HIF- $\alpha$  subunit (HIF-1 $\alpha$  and HIF-2 $\alpha$ ) that is tightly regulated by tissue oxygen levels [256]. Accumulation of the HIF- $\alpha$  subunit is triggered by a decrease in oxygen levels, with subsequent transcriptional activation of a number of genes whose products are involved in

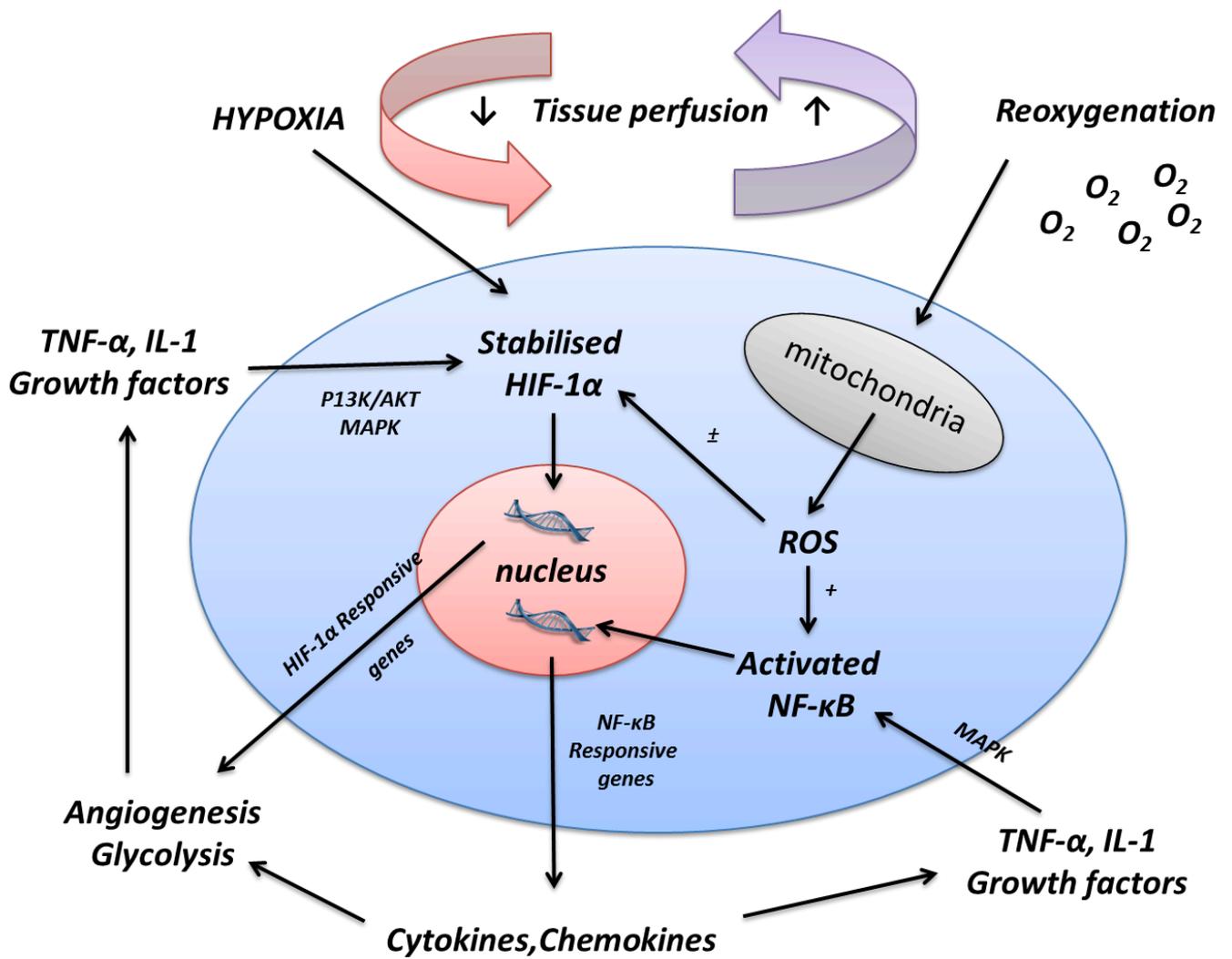
pathways aimed at restoring oxygen delivery and maintaining energy requirements, including erythropoiesis, angiogenesis, and glycolytic metabolism.

Increasing evidence supports the concept that hypoxia can act as a regulator of innate and adaptive immunity. Intuitively this seems obvious a priori given the fundamental import of tissue oxygenation and the obvious importance of protecting tissue in the event of ischaemia. The extent however to which such regulatory interactions exist and can if modulated lead to immune perturbation is illuminating. Mice with HIF-1 $\alpha$  deficient lymphocytes have elevated levels of anti-double stranded DNA antibodies and rheumatoid factor in serum as well as proteinuria and depots of IgG and IgM in the kidney [257]. Hypoxia induced signalling pathways stimulate the differentiation and proliferation of regulatory T cells accompanied with and increase extracellular level of adenosine thus protecting the tissues by restricting effector functions of T cells [258]. Myeloid cells have HIF-dependent ways functioning in the hypoxic microenvironment. HIF-1 $\alpha$  null phagocytes cannot efficiently eliminate bacterial loads instead forming persistent ulcerative lesions [259]. HIF-1 $\alpha$  also prolongs the lifespan of neutrophils in hypoxia by inhibiting apoptosis [260]. Cross-talk between hypoxic and non-hypoxic signalling pathways may further amplify inflammatory responses by activating HIF as well as other oxygen-sensitive transcription factors, for example nuclear factor- $\kappa$ B (NF- $\kappa$ B). Indeed, recent evidence indicates that NF- $\kappa$ B and HIF-1 $\alpha$  are linked in a regulatory loop that substantiates the involvement of hypoxia in innate immunity and inflammation [261]. Members of the NF- $\kappa$ B family interact with members of the prolyl hydroxylases (PHDs) - HIF pathway with mouse studies of inflammatory bowel disease showing that PHDs regulate anti apoptotic effects of NF- $\kappa$ B in intestinal inflammation [262]. Hypoxia also amplifies the NF- $\kappa$ B pathway by increasing the expression and signalling of TLRs, which subsequently enhance the production of antimicrobial factors stimulating phagocytosis and leukocyte recruitment [263].

Accumulating evidence suggests that pro-inflammatory cytokines can act as oxygen-sensitive mediators, indicating the potential to integrate oxygen-linked pathways mediated by cytokines via ROS-dependent mechanisms [264, 265]. ROS, for instance, can induce pro-inflammatory cytokine biosynthesis and this response can be abrogated by selective antioxidants, suggesting an integral role of endogenous ROS [266]. As such, cytokines could form a pivotal link in ROS-dependent pathways leading to the activation of redox-sensitive transcription

factors, such as HIF-1 $\alpha$ , whose up regulation determines the specificity of cellular responses to oxidative stress. TNF- $\alpha$  was reported to have a stimulatory effect on HIF-1 DNA binding activity in a manner similar to that of IL-1 [267]. In addition, the induction of adhesion molecules by hypoxia was reported to have been induced by IL-1 and TNF- $\alpha$  that was caused with anoxia/reperfusion [268]. This was corroborated with the observation that the upregulation of redox factor-1 (Ref-1), a transcription factor, promoted endothelial cell survival in response to hypoxia and TNF through NF- $\kappa$ B-independent and NF- $\kappa$ B-dependent signalling cascades, respectively, indicating that Ref-1 may act as a critical cofactor mediating the TNF-induced NF- $\kappa$ B response in the vascular endothelium [269]. The expression of HIF-1 during acute inflammation has also been investigated in experimental wounds. HIF-1 $\alpha$  induction in primary inflammatory cells was reported to be TNF- $\alpha$ -dependent, the expression of which in early wounds may contribute to the regulation of iNOS and VEGF, two HIF-1-responsive genes intimately related to the process of repair [270]. Hypoxia and IL-1 $\beta$  were reported to stimulate VEGF production in human proximal tubular cells, ostensibly due to increased DNA binding of HIF-1 to hypoxia-responsive elements in the VEGF gene promoter, thereby contributing to microvascular leakage and monocyte extravasation [271]. The upregulation of IL-8 by hypoxia in human macrophages of the lung revealed a potential role in the pathogenesis of acute respiratory distress syndrome (ARDS).

Several signal transduction pathways have been proposed to act downstream of putative oxygen sensors and lead to the activation of MAPK [272]. HIF, IL-1 $\beta$  and TNF- $\alpha$ , initiate a cascade of receptor-mediated mechanisms, which diverge at the level of G-coupled receptors (Ras), proximal kinases involved with the phosphorylation of MAPKp38/MAPKJNK, and phosphatidylinositol-3 kinase pathway [272]. Subsequent phosphorylation/activation mechanisms up regulate the biosynthesis of the  $\alpha$  subunit of HIF, which couples with the constitutively expressed  $\beta$  subunit, thereby allowing nuclear translocation and transactivation of this transcription factor. The activation of HIF-1 $\alpha$  requires phosphorylation. This scenario is likely provided with the normoxic induction by cytokines, which stabilize the  $\alpha$  subunit, ostensibly via downregulation of VHL and/or up regulation of upstream kinases such as MAPKs.



**Figure 1-12 Regulation of the hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB) pathways by reactive oxygen species (ROS) and cytokine stimulation.** The complex and interrelated activation of these two critical transcription factors is central to most of the processes that sustain inflammation in tissues, such as endothelial activation, leukocyte recruitment, angiogenesis, and enhanced cell survival. IL, interleukin; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; TNF, tumour necrosis factor.

One of the best characterized hypoxia-responsive genes is the angiogenic stimulus vascular endothelial growth factor (VEGF), expression of which is dramatically up regulated by hypoxia in many cells types. This has been particularly well explored in RA in which angiogenic e.g. VEGF, FGF, expression and intercurrent hypoxia have been closely compared. This has led to an apparent paradox, whereby there is abundant synovial vasculature (particularly of neovessels expressing  $\alpha v\beta 3$  integrins) and angiogenic expression (which might be expected to restore oxygen levels to normal) occurring together with regions of substantial synovial hypoxia. It has been shown in a number of studies that vascular endothelial growth factor blockade is effective in animal models of arthritis; these findings suggest that hypoxia may activate the angiogenic cascade, thereby contributing to RA chronicity. Recent data also suggest that, as well as activating angiogenesis, hypoxia may regulate many other features that are important in RA, such as cell trafficking, matrix degradation and bone erosion [273].

Thus hypoxia and inflammation are intertwined at the molecular and cellular levels. The prominent transcription factor HIF-1 is a key regulator of oxygen dependent gene expression, particularly in immune cells with subsequent cytokine dysregulation. Its prominence in wound healing and arthritis warrants its investigation in tendon disease as a possible mediator of tissue damage.

### ***Hypoxia and tendinopathy***

Biopsy and *in vivo* model studies suggest that tendon hypoperfusion [274] and subsequent hypoxia [275] are central elements of tendon failure but few data have addressed this at a mechanistic level in humans. Hypoxia was first described in 1982 when Jozsa and colleagues [65] examined 34 tendons under the electron microscope, excised within 48 h after rupture of the tendons and showed marked hypoxic alterations in the tenocytes. This remained the foundation of the concept of hypoxic induced tendon damage for around 20 years until Sivakumara et al examined *in vivo* oxygen tension measurements of rheumatoid tendons at the time of hand surgery and also analysed tissue for the expression of HIF and cultured cells to assess cytokine production. Here profound hypoxia in the synovium of RA tendons and joints *in vivo* despite immunohistochemical evidence of markedly increased vascularity was noted as was markedly increased production of TNF in hypoxic cell cultures. In rodent models, cyclic strain regulates the expression of

the HIF-1 $\alpha$  in rat tendon fibroblasts [117] and recently Benson et al [68] described hypoxic damage throughout the spectrum of rotator cuff disease. Experiments on primary cell cultures of normal human dermal fibroblasts in hypoxic conditions reveal up regulation of VEGF with early increases in Type I collagen that regresses at 48 hours suggesting a functional role for hypoxia in matrix adaptation [276]. Microarray approaches exploring hypoxic versus normoxic synovial fibroblasts from healthy controls and RA patients show the number of differentially expressed genes to be significantly increased by hypoxia [277]. The main biological pathways modified by hypoxia include carbohydrate and lipid metabolic pathways both of which are established HIF targets. In particular this elegant study found the AMP-activated protein kinase pathway (AMPK) to be one of the most important canonical pathways induced by hypoxia in synovial fibroblasts. AMPK is HIF independent factor critical for maintaining energy homeostasis in cells exposed to stress or cytokines [278] and AMPK and its agonists can suppress proinflammatory signaling in models of inflammation [279, 280].

To date no studies have explored the functional role of hypoxia in modulating apoptosis, inflammation and matrix regulation in tendinopathy. I was intrigued by this and as such this thesis has as one of its aims an endeavour to investigate the role of hypoxia and underlying molecular mechanisms of hypoxic induced tendon damage.

### **MicroRNA**

MicroRNA (miRNA) are a group of short non-coding RNA functioning as post transcriptional regulators of gene expression. It is estimated that there are more than 1200 human miRNAs that are believed to affect a large number of biological processes including cell differentiation and development. Typically, miRNAs are 19–24 nucleotide long, single-stranded molecules that suppress the expression of protein-coding genes at the posttranscriptional level by directing translational repression, mRNA destabilization, or a combination of the two [281]. miRNAs mediate their regulatory action through imperfect binding to the 3' untranslated region (3' UTR) of target mRNAs carrying complementary sites. The interaction of miRNAs and their target mRNAs is mediated primarily through nucleotides 2–7 in the 5' region of the miRNA, although other nucleotides of the miRNAs probably also have a modifying effect. Moreover, each mRNA can be regulated by dozens of miRNAs (depending on the length of the 3' UTR) that may

interact with each other by synergism or competition. It is estimated that expression of ~8000 genes (30% of the human genome) are likely to be regulated by miRNAs. Thus, the gene regulatory effect of miRNAs is potentially enormous.

### ***MicroRNA expression and effector function***

Most miRNAs are transcribed by RNA polymerase II enzyme as long, mRNA-like polyadenylated primary transcripts (pri-miRNA), which can be several thousand bases long [282]. In the first processing step, the RNase III enzyme, Drosha, together with other proteins in the cell nucleus cleaves the long pri-miRNA to a shorter, 70 nucleotide stem-loop-structure known as the precursor miRNA (pre-miRNA) [283]. This is then exported from the nucleus by exportin 5 to the cytoplasm, where the second processing step is carried out. Another RNase III enzyme, Dicer, together with other proteins cleaves the pre-miRNA into a short (19–24 nt) double-stranded miRNA duplex that is processed further with one of the strands being incorporated into the RNA-induced silencing complex (RISC) [283, 284], that directs miRNA to its target site on mRNA and leads to post-transcriptional repression (Figure 1-13). An alternative pathway of miRNA biogenesis has been discovered involving splicing from introns. These 'mitrons' mimic the structural features of pre-miRNAs and are spliced without Drosha processing.

Similar to protein coding genes, miRNA may also be subject to post-transcriptional regulation. For example, SMAD proteins, regulators which are induced by TGF family members, have been shown to enhance pre-miR-21 processing from its primary transcript through SMAD association with Drosha and the p68 RNA helicase. It has also been shown that primary miRNA transcript processing can be inhibited whereby the developmentally regulated RNA binding protein, Lin28, binds pri-let-7 transcripts thus preventing processing in ES cells [285].

Transcription factors (TFs) regulate gene expression responsible for various cell phenotypes and the response of cells to environmental stimuli. Increasing evidence supports the role of miRNAs in combination with TFs controlling the expression of thousands of mammalian genes in a so called 'fine tuning' of the transcriptional network. An example is c-Myc activated E2f1, a transcription factor, and miR-17-5-p and miR-20 [286]. E2F1 binds directly to the promoter of MiR-17-

92 cluster and thus regulates its ultimate transcription [287]. The presence of multiple miRNA target site in immune genes and studies of miRNA expression in brain and haematopoietic cells may suggest that like TFs, codes of miRNAs may exist [288]. However TFs can act as either activators or repressors and due to their size and structure can simultaneously interact with distinct tissue specific proteins. miRNAs are primarily repressors, although recent data has suggested that miRNAs may activate certain genes in growth arrested cells [289]. Additionally others have shown that miRNAs can bind sites in promoters to activate transcription and that sites in 5'UTR may be capable of enhancing translation [290].

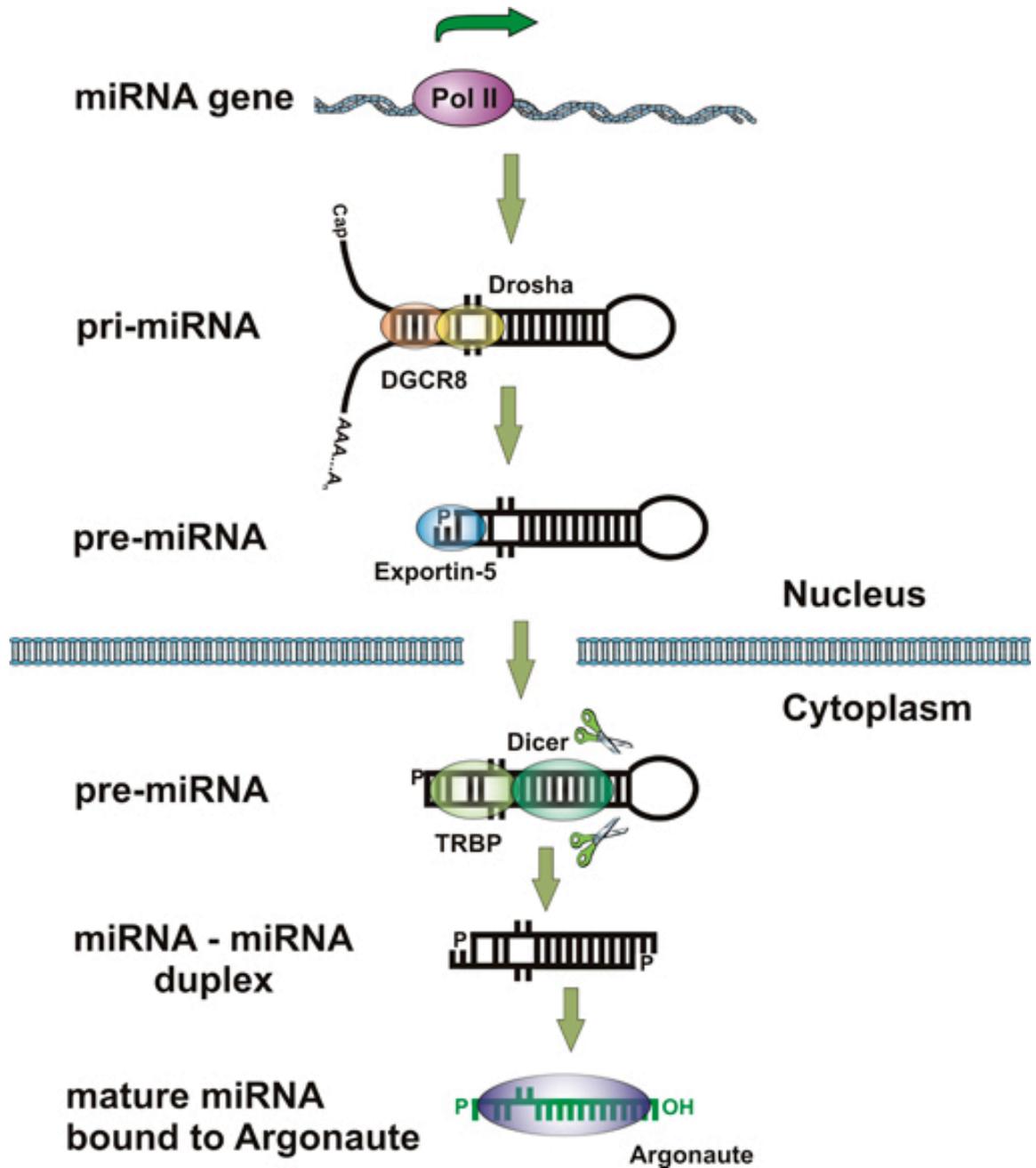


Figure 1-13 A schematic representation of miRNA processing and activity. MicroRNAs are transcribed by RNA polymerase enzymes as part of primary miRNA molecules. Two dsRNA-specific ribonuclease, Drosha and Dicer sequentially cleave the long primary miRNA into the precursor hairpin miRNA and the mature miRNA. Adapted from University Of New South Wales Embryology Image Bank © Dr Mark Hill.

### ***miRNA mediated regulation of the immune system***

The first studies implicating miRNAs in immunological processes originated from expression profiling of haematopoietic cells during their development. Systematic investigation of miRNA levels in hematopoietic cell lineages, using microarray profiling, cloning, northern blotting, and quantitative real-time polymerase chain reaction (PCR), has identified miRNAs that are now considered as “markers” of these lineages [291, 292]. These studies also showed that miRNAs are dynamically regulated during the development of T cells, B cells, and granulocytes, and are involved in the regulation of these processes. Recent investigations identified critical roles for miR-150 in B cell differentiation, by inhibiting the transition to the pro-B to the pre-B cell stage [293, 294]. Moreover, miR-181a was identified as a positive regulator of B lymphocyte differentiation [291]. miR-181a is also involved in thymic T cell differentiation, by defining the activation threshold of T cell receptors [295]. The miR-142s have also been demonstrated to be positive regulators of T cell differentiation [291]. miR-223 was identified as an essential modulator of granulocytic differentiation [296]. Collectively, these results demonstrate that miRNAs play critical roles in hematopoiesis.

Global downregulation of miRNAs was observed in antigen-induced T cell differentiation, suggesting that miRNAs are involved in the regulation of T cell responses [297]. Since activated T cells are metabolically active, it is thus plausible that downregulation of miRNAs is needed to allow the increased protein synthesis in these cells. These results also suggest that miRNAs may contribute to immune-mediated diseases known for inadequate T cell activation such as autoimmune conditions or chronic inflammation.

### ***miRNA and inflammatory diseases***

As detailed above miRNAs has so far been shown to play an essential role in the development, homeostasis and function of both innate and adaptive immunity. Consequently the dysregulation of these processes can induce and be critical to inflammatory and autoimmune pathology. Table 1.5 summarises the current microRNA implicated in inflammatory and immune pathology. In an attempt to illustrate how microRNAs can regulate key signal pathways in inflammatory disorders I have chosen to focus on the role of miR155 in rheumatoid arthritis as I became involved with this work in the host laboratory during my PhD studies and it

was this that ultimately led to a hypothesis driven question concerning the role of miRNA in tendinopathy.

<i>microRNA</i>	<i>Disease</i>	<i>Species</i>	<i>References</i>
<i>miR-155</i>	RA	Human	[298]
<i>miR-146a</i>	RA OA SLE Psoriasis	Human	[299] [300] [301] [302]
<i>miR-101</i>	SLE	Mouse	[303]
<i>miR-21</i>	Psoriasis Asthma	Human Mouse	[302] [304]
<i>miR-125b</i>	Psoriasis	Human	[302]
<i>miR-9</i>	OA	Human	[305]
<i>miR-133a</i>	Asthma	Human	[306]
<i>miR-126</i>	Cystic fibrosis	Human	[307]
<i>miR-203</i>	Peridontal Disease	Human	[308]

**Table 1-6 MicroRNAs involved in inflammatory and autoimmune diseases**

<i>microRNA</i>	<i>Immune Function</i>	<i>Transcriptional Target</i>
<b><i>miR-16</i></b>	Binds to UA rich elements in the 3' UTR and induces TNF $\alpha$ mRNA	TNF $\alpha$
<b><i>miR-17-5p</i></b>	In combination with miR-20a and miR-106a inhibits monocyte proliferation,	AML-1
<b><i>miR125b</i></b>	Expression downregulated by LPS and oscillations in expression after NF $\kappa$ B	TNF $\alpha$
<b><i>miR-146b</i></b>	LPS induced expression induced in macrophages IRAK1	TRAF6
<b><i>miR-155</i></b>	Increased expression following activation of the innate immune response.	AP-1
<b><i>miR-181a</i></b>	Positive regulator of B-cell development and CD4 <sup>+</sup> T-cell selection	
<b><i>miR-17-92</i></b>	Regulates pro- to pre- transition during B- and T-cell development	PTEN

**Table 1-7 MicroRNAs involved in immune cell regulation**

### ***miRNA 155 in rheumatoid arthritis***

miR-155 is the first miRNA whose function was investigated in transgenic mouse models and it is one of the best characterized microRNAs. To date, more than 130 articles have been published about miR-155. In vitro and in vivo studies implicated miR-155 in various immune functions involving innate as well as adaptive immune responses and the development of immune cells. Moreover, miR-155 has also been shown to be associated with several malignancies and it functions as an oncogene [309, 310]. However, miR-155 has all the characteristics that represent difficulties when trying to identify the function of a miRNA: it is expressed in many cell types and seems to have a different function and regulation in those, it has multiple targets that are regulated in a cell-type-specific manner, and it has relatively modest effects on the biological functions it has been associated with so far.

Two independent groups initially generated miR-155 knockout mice and showed the essential role of miR-155 in both B and T cell responses [291, 311]. Rodriguez et al. observed that miR-155-deficient mice had defects in dendritic cell–T cell interactions and that T cells from miR-155-null mice displayed an increased tendency to differentiate into Th2 type cells when activated in vitro, as indicated by Th2-type cytokine and chemokine production. Most importantly, vaccination of miR-155-null mice with live attenuated vaccine against *Salmonella typhimurium* failed to protect them against challenge with virulent *Salmonella* demonstrating that miR-155 is indispensable for normal adaptive immune responses [311]. Subsequently Thai et al. reported that miR-155 is required for germinal centre response. While miR-155-null mice developed fewer and smaller germinal centres in response to antigenic challenge in comparison to control mice [291], mice ectopically expressing miR-155 developed more and larger germinal centres with more class-switched antibody [291].

Given the aberrant expression of miR-155 in RA patients we sought to focus on the functional contribution of miR-155 in clinical and experimental arthritis models. In situ hybridization revealed that miR-155 was strongly expressed in RA but not OA synovial biopsies, mainly in the lining layer and to a lesser extent in the sublining layer. Double immunofluorescence staining showed that miR-155 was expressed in a majority of CD68+ macrophages in the RA membrane lining. The increased expression of miR-155 in SF CD14+ cells was associated with lower

expression of the miR-155 target, SHIP-1, an inhibitor of inflammation. Similarly, SHIP-1 expression was decreased in synovial lining layer CD68+ cells in RA patients, compared to OA patients. Over-expression of miR-155 in PB CD14+ cells led to down-regulation of SHIP-1 and an increase in the production of pro-inflammatory cytokines. Conversely, inhibition of miR-155 in RA synovial CD14+ cells reduced TNF- $\alpha$  production. Finally, miR-155-deficient mice are resistant to collagen-induced arthritis, with profound suppression of antigen-specific Th17 cell and autoantibody responses, and markedly reduced articular inflammation. The data produced from this study identified an unrecognized role of miR-155 in clinical and experimental arthritis.

### ***miRNA and tendinopathy***

The above work was an obvious catalyst for me to investigate a possible role for miRNA regulation of cytokine and matrix dysregulation in tendinopathy. Whilst to date no work exists on the role or presence of miRNA in tendon disease some recent work in other disease processes where collagen changes predominate are pertinent.

Dermal fibroblasts are critical components of the wound healing process, responsible for collagen and proteoglycan production in the remodelling phase. They regulate the activity of other key players in tissue repair by secreting growth factors promoting chemotaxis, cell proliferation, and angiogenesis [312]. Using expression profiling with miRNA micro assays, Gu and Lyer [313] analysed the alterations in miRNA expression in dermal fibroblasts during transition from quiescence to proliferation. A cluster of 33 miRNAs were identified, which were induced by both serum and fibroblast growth factor early in proliferation, suggesting the involvement of miRNAs in regulating the expression of genes important for the re-entry of fibroblasts in the cell cycle and for fibroblast proliferation. miRNA expression also correlated with the expression of genes encoding early transcription factors, including c-Myc, SRF, JUNB, EGR2 and EGR3.

Transforming growth factor (TGF)- $\beta$  isoforms are secreted by platelets, fibroblasts, and macrophages at the site of injury and act in various capacities, influencing keratinocyte, fibroblast and inflammatory cell action [314]. The effects of TGF $\beta$  are widespread in morphogenesis and adult tissue homeostasis, and specific miRNAs have emerged as key modulators of TGF $\beta$  signalling in several

cellular processes. miR-17-92, miR-24, and miR-224 have been implicated in TGF $\beta$ -mediated apoptosis [315], differentiation [316], and proliferation [317], respectively. Because of their effects on wound fibroblasts, TGF $\beta$  isoforms are key mediators of post-injury scar formation [318, 319], and emerging evidence supports miRNA involvement in TGF $\beta$ -mediated collagen synthesis and regulation of ECM [320-322].

Most relevant to this thesis however has been the very recent implication of miRNA in the regulation of collagen synthesis. Ogawa and colleagues [323] were the first to implicate mir29b as a suppressor of type I collagen at the mRNA and protein level via its direct binding to Col1A1 3'UTR. miR-29b also had an effect on SP1 expression suggested that miR-29b is involved in the regulation of type I collagen expression by interferon-alpha in hepatic stellate cells. Maurer and colleagues [324] then suggested mir 29 as a key regulator in collagen expression in systemic sclerosis. They found that miR-29a was strongly down-regulated in SSc fibroblasts and skin sections as compared with the healthy controls. Overexpression in SSc fibroblasts significantly decreased, and accordingly, knockdown in normal fibroblasts increased, the levels of messenger RNA and protein for type I and type III collagen. They suggest miR-29a as a posttranscriptional regulation of collagen and as such a therapeutic target in SSc.

Thus although we are still in the preliminary stages of this area of research, there is convincing evidence available so far that miRNA provide a means to understanding the physiologic and pathologic processes in dysregulation matrix production which may ultimately translate into novel therapeutic approaches.

## **1.6) Aims of this Work**

In this work I sought to define the role for inflammatory and regulatory cytokines in tendon disease with a focus on the novel entities IL-17 and IL-33. Further I sought to define their effector biology and to thereafter investigate the regulation of cytokine production in tendinopathy paying particular attention to hypoxia and post transcriptional microRNA biology.

Therefore, the ultimate aim of these studies was to generate new leads and novel ideas about several facets of tendon healing, with the intention of identifying new areas of research in the field with potential to be of interest in the future.

## Chapter 2 Material and Methods

## 2.1) General reagents & buffers

### Materials and reagents

**General chemicals:** All chemicals were purchased from Sigma (Dorset,UK) unless otherwise stated.

**Plastics:** All plastics used for cell culture were purchased from Corning and Gibco unless otherwise indicated.

### Buffers and culture media

**Complete media:** Basic media: RPMI1640, DMEM or IMEM plus 10% heat inactivated foetal bovine calf serum, Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-Glutamine (2mM) (all at final concentration from Invitrogen).

**PBS:** Phosphate buffered saline was purchased from Invitrogen.

**Tris-acetate-EDTA (TAE) buffer:** 50x stock: 242 g of Tris base in 750 ml dH<sub>2</sub>O. Mixed with 57.2 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0). Final volume was made up to 1000 ml with dH<sub>2</sub>O. Buffer was used at 1x concentration.

**TBS Tween (TBST):** 10x TBS buffer was made using: 876.6 g NaCl, 121.1 g Tris, 40 ml HCl and adjusted to pH 8.0. 10x solution was diluted 1:10 with dH<sub>2</sub>O and 0.05% Tween added.

## **2.2) Patients**

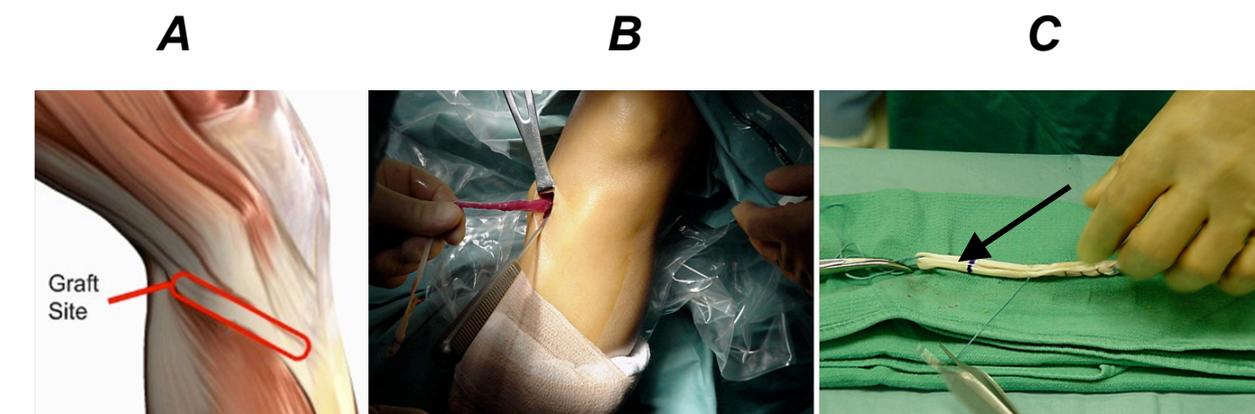
South East Sydney Area Research Ethics Committee granted approval for sampling of shoulder tendon biopsies for cytokine and inflammation analysis in June 2004. Patients with rotator cuff tears or shoulder instability, who were greater than 18 years of age and capable of providing informed consent, were invited to take part in the project. Such patients were identified from the Orthopaedics Clinics in St George Orthopaedic Research Institute, Sydney Australia under the guidance of our collaborator Professor George Murrell. Tonsils were used as control tissue and were obtained by Mr Simpson, Stobhill Hospital, Glasgow. Demographic data were collected and stored in a password-protected database and samples were blinded to the researchers.

### **Ethics Approval**

All procedures and protocols were approved by the South east Area Sydney Ethics Committee under ACEC No. 99/101 for collection of human supraspinatus and subscapularis tissue. 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.

### **2.3) Tendon explant biopsy**

Patients between 18 and 25 years of age undergoing arthroscopic anterior cruciate ligament reconstruction using an autograft hamstring tendon graft were identified in the Orthopaedic department (Mr William J Leach and Mr Brian P Rooney), Western Infirmary, Glasgow prior to surgery. Having identified a suitable patient informed consent was sought to use any residual tendon tissue not utilised at the time of surgery under the NHS Greater Glasgow Residual Tissue consent procedures. During arthroscopic knee surgery performed under general anaesthesia a standard hamstring graft was obtained. The hamstring tendon used for a reconstruction often comes from one of the medial hamstrings, the semitendinosus or semimembranosus. The semitendinosus is most commonly used, because of its easily accessible insertion at the medial tibia. The tendon is harvested from an anterior-medial incision, and the tissue is dissected down to the tendon / bone interface (Figure 2-1). A tendon stripper is used to remove the tendon. Once the tendon is removed, it is folded over itself twice, producing a graft that is 4 layers thick. Following placement in the knee the standard hamstring tendon graft is cut flush to the bone and residual tendon is normally disposed of (Figure 2-1). It is this residual tendon, which was used for explant culture to establish tenocyte cultures.



**Figure 2-1 Illustration of protocol for tendon explant tissue.**

**(A) Graft site highlighted illustrates the normal anatomical position of the semitendinosus or semimembranosus tendons used in hamstring ACL reconstruction. (B) Surgical incision on the anteromedial aspect of the knee joint is used to access the insertion site of the tendons. A tendon stripper is then used to retrieve these tendons for use as graft material. (C) The tendons are folded over making a 'four strand' section. Area highlighted with black arrow is excess surgical tissue and it is this section which is used for explant culture.**

## **2.4) Tissue preparation**

For cell harvest, explant tissue was transferred to complete medium and cultured at 37°C in 5%CO<sub>2</sub>/95% O<sub>2</sub> to generate fibroblast-like tenocytes (FLT) following prolonged incubation and passage of cells.

### **Paraffin embedded tissue**

Tissue sections were prepared and stored for future analysis of tissue protein e.g. cytokine expression by immunohistochemistry (IHC). For paraffin embedded sections, tissue was stored in formalin for 24 h and transferred to 70% ethanol to retard the fixation process. Tissues were paraffin embedded and subsequently cut to thickness of 5 µm. 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 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.

## 2.5) Immunohistochemistry (IHC) of paraffin embedded sections

### Single staining for light microscopy

Sections were prepared as previously mentioned (Section 2.4). 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<sub>2</sub>O<sub>2</sub>/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 h at RT 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 (see working concentrations and companies in Table 2-1, page 100). 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, Peterborough, UK) 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<sub>2</sub>O<sub>2</sub> for up to 5 min at RT until brown reaction product was apparent. Sections were then washed in water and counterstained using Harris's haematoxylin (BDH Ltd. Lutterworth, Leicester, UK). 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 mins. Further staining of the sections was carried out as described above.

### Double staining for light microscopy

Similar to single staining the 0.5% hydrogen peroxidase/methanol incubation and heat retrieval in 0.5M citrate buffer (pH 6) was followed by incubation in 2.5% species/2.5% human serum with AvidinD (4 drops/ml) (Vector Laboratories, Petersborough, U.K.). Surface expression of CD68 was detected by staining with a mouse anti-CD68 antibody in the presence of Biotin (4 drops/ml, Vector) in 2.5% horse serum TBST for 1 h at RT, 2 washes with TBST, followed by

a biotinylated secondary antibody for 30 min (1:200; Vector Laboratories). The reaction was developed using VIP. Following incubation for 1 h in 2.5% species/2.5% human serum, goat anti-IL-17 in TBST was added overnight at 4°C. On the following day sections were washed with TBST, and then incubated with a biotinylated antibody for 30 min and stain developed using either DAB or Nickel-DAB. The chosen counterstain for DAB was methyl green whereas Nickel-DAB sections were left unstained. Sections were washed dH<sub>2</sub>O, dehydrated in serial ethanol (from 70%, 90% to 100%), cleared in xylene and mounted in DPX.

### **Double staining for fluorescent microscopy**

Sections were prepared as described above. For double immunofluorescent staining ten paraffin embedded samples were incubated for 1 h with Abs (mouse anti-CD3 [1.25 µg/ml; Vector Laboratories], mouse anti-CD4 [7.56 µg/ml; Dako UK, Cambridgeshire, U.K.], mouse anti-mast cell tryptase [MCT] [0.43 µg/ml; Dako UK], or mouse anti-CD68 [1 µg/ml; Dako UK]) followed by 30 min incubation with biotinylated Abs (1:200; Vector Laboratories) with subsequent staining with streptavidin DyLight Fluor (1:500; Invitrogen, Paisley, U.K.) for 45 min. Goat anti-IL-17 (5 µg/ml; R&D Systems, Abingdon, U.K.) was added overnight at 4°C, then incubated with a biotinylated Ab for 30 min, and stained with Avidin FITC (1:500; Vector Laboratories) for 45 min. Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed on a fluorescent imaging microscope (BX50; Olympus, Essex, U.K.). Images were captured using Apple Open laboratory software.

<i>Antibody</i>	<i>Manufacturer and clone</i>	<i>Source</i>	<i>Working Concentration</i>
<b>CD3</b>	Vector	mouse monoclonal	1.25 µg/ml
<b>CD4</b>	Dako	mouse monoclonal	7.56 µg/ml
<b>MCT</b> ( <i>Mast cell tryptase</i> )	Dako	mouse monoclonal	0.43 µg/ml
<b>Anti Histamine</b> ( <i>mouse mast cells</i> )	Sigma	rabbit polyclonal	1 µg/ml
<b>CD34</b>	Vector	mouse monoclal	0.5 µg/ml
<b>CD68</b>	Dako clone PG-M1	mouse monoclonal	1 µg/ml
<b>CD206</b>	R&D	goat polyclonal	5 µg/ml
<b>Collagen I</b>	Abcam	mouse monoclonal	2 µg/ml
<b>Collagen III</b>	Abcam	mouse monoclonal	13 µg/ml
<b>F4/80</b>	Serotec	mouse monoclonal	1 µg/ml
<b>IL-17A</b>	R&D	goat polyclonal	5 µg/ml
<b>IL-1RAcP</b>	ProSci	Rabbit polyclonal	1 µg/ml
<b>IL-33</b>	Alexis (nessy-1)	mouse monoclonal	5 µg/ml
<b>ST2</b>	Sigma-Aldrich	rabbit polyclonal	1 µg/ml
<b>IL-33</b>	R&D	mouse monoclonal	10 µg/ml
<b>Clusterin</b>	R&D	Goat	10 µg/ml
<b>HIF</b>	R&D	rabbit polyclonal	2 µg/ml
<b>BNIP-3</b>	Abcam	rabbit polyclonal	2.5 µg/ml
<b>VEGF</b>	Abcam	mouse monoclonal	20 µg/ml
<b>Isotype</b>	various	various	various

**Table 2-1 Antibodies for IHC**

Shown are surface marker or cytokine antibodies using in IHC. Left column shows antibody target, 2<sup>nd</sup> column manufacturer, 3<sup>rd</sup> the source and right column the working concentration. Dilutions were not mentioned as stock concentration of antibodies can vary.

<b><i>Target molecule</i></b>	<b><i>Company</i></b>
<b><i>human sST2</i></b>	R&D systems
<b><i>human COL I</i></b>	Biosource
<b><i>human COL III</i></b>	Biosource
<b><i>mouse COL I</i></b>	Biosource
<b><i>mouse COL III</i></b>	Biosource

**Table 2-2 ELISAs used and respective companies**

**Quantification of fluorescent IHC**

Images were captured digitally and the total number of IL-17A+, ST2+ and IL33+ found within duplicate tissue areas (two representative 10x fields) of 0.52 mm<sup>2</sup> was calculated. Double staining allowed the calculation of the proportion of IL-17A, ST2 or IL33 expressing cells per cell surface marker. Four tissues were double stained for all markers (mouse and human CD68 and MCT) with studies performed on a further 6 tissues to confirm the co-expression with MCT and CD68.

## **2.6) ELISA and Luminex cytokine analysis**

Cytokine expression was tested in culture supernatant. ELISAs were carried out according to the manufacturer's protocol (summarized in Table 2-2) A 25-Plex human cytokine assay performed on supernatants from cultured human tenocytes was performed according to manufacturers' instructions.

## 2.7) Signaling analysis

Phosphorylation status of mitogen-activated protein kinases (MAPKs), extracellular signal regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38 isoforms were evaluated using the Human Phospho-MAPK Array (R & D Systems Europe, UK) as per the manufacturer's instructions. The ERK inhibitor (FR180204) and Akt inhibitor (1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate) were purchased from CalbioChem (Merck KGaA, Germany) and used at  $IC_{50} = 10\mu\text{M}$ , a concentration previously determined to offer optimal specific inhibition relative to off target effects which was used previously in our laboratory [244].

Phosphorylation of NF $\kappa$ B p65 was assessed using the InstantOne ELISA in cell lysates from treated and untreated tenocytes. NF kappa B is a family of transcription factors with five members that includes Rel (c-Rel), RelA (p65), RelB, NF $\kappa$ B1 (p50 and its precursor p105), and NF kappa B2 (p52 and its precursor p100). NF kappa B dimers containing p65 are activators of transcription. In a majority of unstimulated cells, NF kappa B remains in its inactive form and is retained in the cytoplasm by the bound inhibitory I $\kappa$ B proteins. Upon stimulation by inducers such as TNF $\alpha$ , I $\kappa$ B $\alpha$  is phosphorylated and degraded. This results in the release of the NF kappa B complex from the IKK complex and the p105 subunit is cleaved into its active p50 form. Subsequently the p50/p65 translocates to the nucleus where it activates transcription of many genes, including its own inhibitor I kappa B alpha, causing an auto-regulatory mechanism of NF kappa B. The absorbance was measured at 450 nm by microplate reader with positive and negative controls supplied by the manufacturer. The relative absorbance of stimulated versus unstimulated cells was used to assess the total or phosphorylated NF $\kappa$ B p65 in each sample.

## 2.8) RNA extraction and Quantitative PCR analysis

The cells isolated from the normoxic or hypoxic experiments were placed in Trizol prior to mRNA extraction. QIAgen mini columns (Qiagen Ltd, Crawley UK) were used for the RNA clean-up with an incorporated on column DNase step as per manufactures instructions. RNA purity and concentration was assessed using the Nanodrop 2000 machine. cDNA was prepared from RNA samples according to AffinityScript™ (Agilent Technologies, CA, USA) multiple temperature cDNA synthesis kit as per manufactures instructions. SYBR green Real time PCR was performed using SYBR green mastermix (Applied Biosystems, CA,USA). Prior to setting up the SYBR green the cDNA was diluted 1 in 5 using RNase-free water. Each sample was analysed in triplicate. Primers (Integrated DNA Technologies, Belgium) used are shown in. Conventional PCR was undertaken to test the respective primers with cDNA from diseased synovial fibroblasts that were known to express many inflammatory genes. After ensuring a band was present the primers were again tested on the quantitative machine were melt curves were performed on all primers to ensure specificity.

<b>Gene</b>	<b>Primers</b>
<b>Human IL-33</b>	GGAAGAACACAGCAAGCAAAGCCT (f) TAAGGCCAGAGCGGAGCTTCATAA (r)
<b>Mouse IL-33</b>	GGAAGAACACAGCAAGCAAAGCCT (f) TAAGGCCAGAGCGGAGCTTCATAA (r)
<b>Human ST2</b>	ACAACCTGGACAGCACCTCTTGAGT (f) ACCTGCGTCCTCAGTCATCACATT (r)
<b>Human sST2</b>	GAGACCTGCCACGATTACAC (f) TGT TAA ACC CTG AGT TCC CAC (r) CCC CAC ACC CCT ATC CTT TCT CCT (probe)
<b>Human ST2L</b>	ACAAAGTGCTCTACACGACTG (f) TGTTCTGGATTGAGGCCAC (r) CCCCATCTGTAAGTGGATTTGTAGTTCC G (probe)
<b>Mouse sST2</b>	TCTGCTATTCTGGATACTGCTTTC (f) TCTGTGGAGTACTTTTGT CAC C (r) AGA GAC CTG TTA CCTGGGCAAGAT G (probe)
<b>Mouse ST2L</b>	CCAATGTCCCTTGTAGTCGG (f) CTT GTT CTC CCC GCA GTC (r) TCC CCA TCT CCT CAC CTC CCT TAA T (probe)
<b>Heat shock protein 72</b>	TGCTGGACAAGTGTCAAGAGGTCA (f) TCCTCTTGTGCTCAAACCTCGTCCT (r)
<b>Heat shock protein 27</b>	AGACCTCAAACACCGCCTGCTAAA (f) TGCTCTGGACGTCTGCTCAGAAA (r)
<b>HIF-1<math>\alpha</math></b>	TTGGCAGCAACGACACAGAACTG (f) TTGAGTGCAGGGTCAGCACTACTT (r)
<b>Col 3A Human</b>	TATCGAACACGCAAGGCTGTGAGA (f) GGCCAACGTCCACACCAATTCTT (r)
<b>Col 3A Mouse</b>	GCTTTGTGCAAAGTGGAACCTGG (f) CAAGGTGGCTGCATCCCAATTCAT (r)
<b>COL 1A1 Human</b>	CCATGCTGCCCTTTCTGCTCCTTT (f) CACTTGGGTGTTTGTGAGCATTGCCT (r)
<b>COL 1A1 Mouse</b>	TTCTCCTGGCAAAGACGGACTCAA (f) GGAAGCTGAAGTCATAACCGCCA (r)
<b>Smac</b>	TGCTGGCCAAGTGACAAGACGTCA (f) CAAGGAGGCTGCCTCCCAATTCAG (r)

Table 2-3 Quantitative PCR primers

### 2.8.1) RNA isolation and quantitative real time PCR analysis of miRNA

Total RNA was isolated by miRNeasy kit (Qiagen). miScript Reverse Transcription Kit (Qiagen) was used for cDNA preparation. TaqMan mRNA assays (Applied Biosystems) or miScript primer assay (Qiagen) were used for semi-quantitative determination of the expression of human miR-29a( MS (MS00001701) 29b (MS00006566) and c (MS00009303) and mouse 29a (MS00003262), 29b(MS00005936) and c (MS00001379). The expressions of U6B small nuclear RNA or beta-actin were used as endogenous controls.

### 2.8.2) miRNA transfection

Cells were transfected with synthetic precursors miRNA (pre-miR) for miR 29 a&b or with negative controls (pre-miR mimic negative labelled control, Thermo Scientific Inc ) at a final concentration of 20nM with the use of Dharmacon® DharmaFECT® 3 siRNA transfection reagents (Thermo Scientific Inc). At 48 hours after transfection cellular lysates were collected to analyse the expression of genes of interest.

Transfection efficiency was assessed by flow cytometry using the labelled D547 mimic and confirmed by quantitative PCR of control scrambled mimic and the respective miR29 family mimic.

### 2.8.3) Luciferase reporter assay for targeting Collagen I & III and soluble ST2

The human 2 miRNA target site was generated by annealing the oligos: for COL I & III and soluble ST2 3'UTR's which were cloned in both sense and anti-sense orientations downstream of the luciferase gene in pMIR-REPORT luciferase vector (Ambion). These constructs were sequenced to confirm inserts and named pMIR-COL I/COL III/ sST2-miR29a/b/c and pMIR(A/S)- COL I/COL III/ sST2-miR29a/b/c, and used for transfection of HEK293 cells. HEK293 cells were cultured in 96-well plates and transfected with 0.1 µg of either pMIR-COL I/COL III/ sST2-miR29a/b/c, pMIR(A/S)- COL I/COL III/ sST2-miR29a/b/c or pMIR-REPORT, together with 0.01 µg of pRL-TK vector (Promega) containing *Renilla* luciferase and 40 nM of *miR-155* or scrambled miRNA (Thermo Scientific Dharmacon®). Transfections were done using Effectene (Qiagen) according manufacturer's instructions. Twenty-four hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega).

## 2.9) Matrix regulation analysis

Tenocytes were evaluated for immunocytochemical staining of Collagen I, and Collagen III (Abcam, Cambridge UK) to assess tenocyte matrix production. Total soluble collagen was measured from cell culture supernatants and from digested mouse tendon samples using the Sircol assay kit (Biocolor Ltd, Carrickfergus, Northern Ireland) according to the manufacturer's protocol. 1 ml of Sircol dye reagent was added to 100  $\mu$ l test sample and mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000  $\times$  g for 10 min; and then washed twice with 500  $\mu$ l of ethanol. The pellet was dissolved in 500  $\mu$ l of alkali reagent. The absorbance was measured at 540 nm by microplate reader. The calibration curve was set up on the basis of collagen standard provided by the manufacturer. Additionally the concentration of human and mouse Collagen I and III was assessed using ELISA with colour change measured at 450 nm by microplate reader along with standards supplied by the manufacturer (USCNK Life Science Inc).

## **2.10) Cell culture**

### **Culture of adherent cells**

Primary tenocytes, were cultured in tissue culture flasks with appropriate complete media (RPMI,) and split when cells reached 80% confluence. Detachment of cells was achieved by incubation with 0.5 – 1 ml 5% trypsin in PBS for 5 min. Trypsinization was stopped by adding media containing 10 % FCS, cells were washed, counted and reseeded to a final concentration of  $10^5$ /ml.

### **Culture in Hypoxic conditions**

The tenocyte cells were filtered through nylon mesh, and were plated at  $1 \times 10^6$ /ml into 75 cm<sup>2</sup> culture flasks (BD Falcon, Leuven, Belgium) under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions using an air-tight hypoxic incubator with inflow and outflow valves (Wolf Laboratories Limited, York, UK). Oxygen concentrations were continuously measured with a built-in oxygen sensor and the percentage of oxygen was adjusted by addition of nitrogen. To ensure these conditions were reflective of hypoxia cell lysates were then analysed at 24 and 48 hours by quantitative PCR to ensure upregulation of HIF-1 $\alpha$ .

## 2.11) FACS analysis

The expression of ST2 on cultured human tenocytes was analysed by FACS. To this end  $0.5 \times 10^6$  cells were washed and unspecific antibody binding to Fc-receptors was blocked by incubating cells with 50  $\mu$ l Fc-Block (Sigma) for 15 min. 5  $\mu$ l of anti-ST2 FITC were added and incubated for 15 min at RT in the dark. Cells were washed with PBS EDTA and analysed using a FACS calibur machine (BD). To test cell death of tenocytes due to both recombinant cytokine and hypoxic conditions cells were stained with Annexin-V and 7-AAD (Apoptosis kit, BD biosciences) according to manufacturer's instructions. FACS data were analysed using FlowJo software (TreeStar).

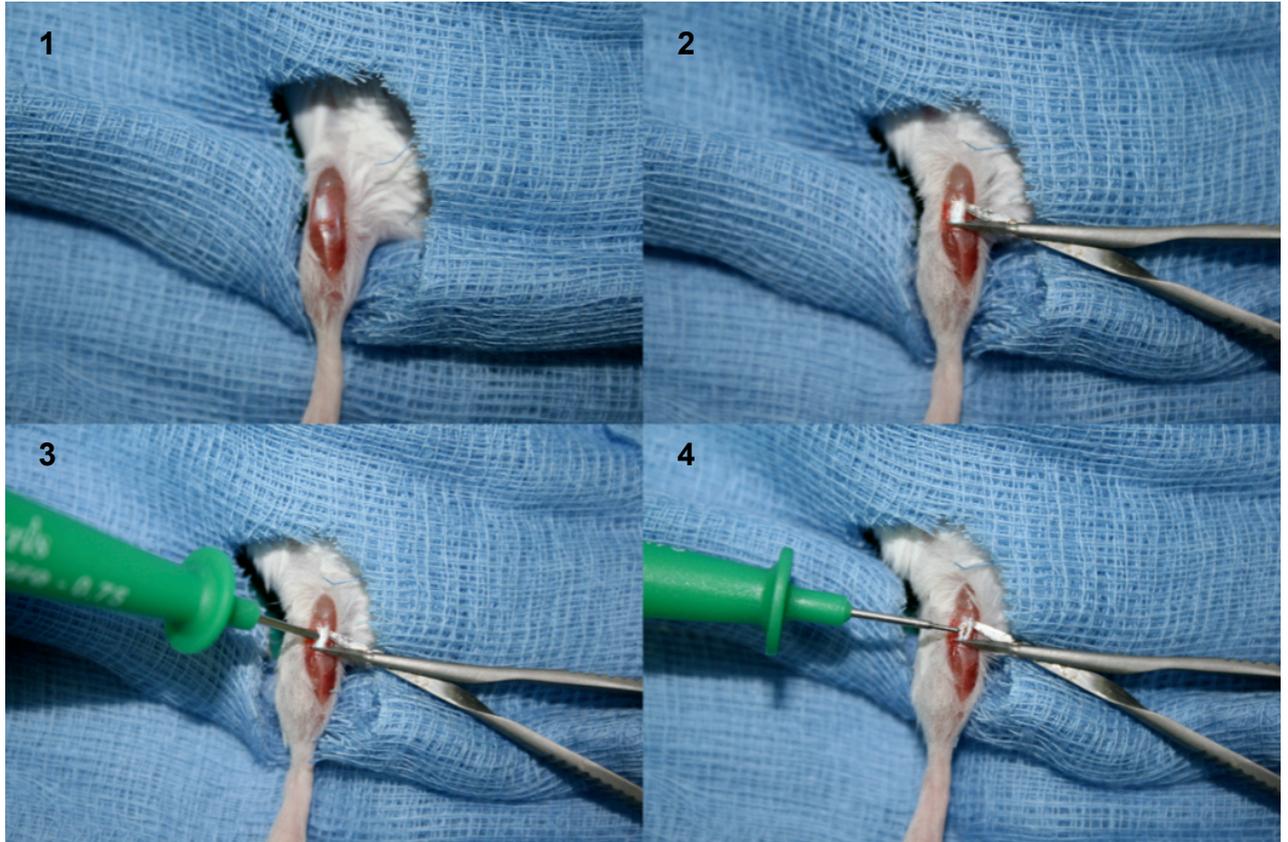
## 2.12) Animals

Mice were maintained at the Central Research Facility, University of Glasgow. All animal experimentation and husbandry was under the authority of a UK Government Home Office Project License. All protocols were approved by the Glasgow University local Ethical Review Panel. Normally, mice were used at the age of 10- 12 weeks and were age-matched for each independent experiment. Control mice(BALB/c) were bought from Charles River. ST2-/- mice were kindly provided by Prof McKenzie (Cambridge) and Prof Liew (Glasgow).

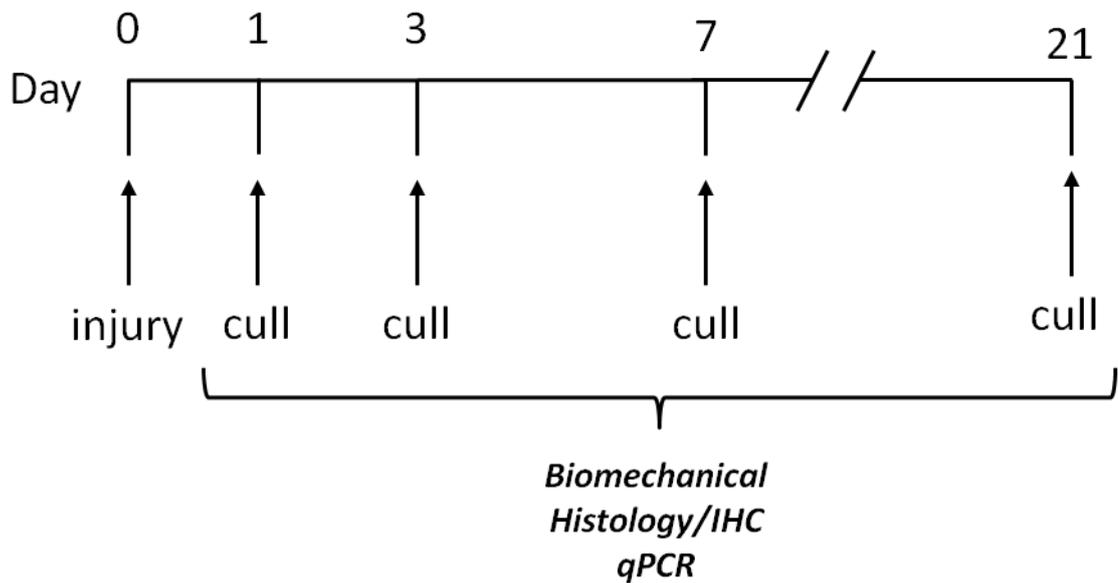
### Patellar tendon injury model

In preparation for the surgical procedure, mice were anaesthetised with a mixture of isoflurane (3%) and oxygen (1%) and both hind limbs were shaved. During the surgical procedure, anaesthesia was delivered via a nose cone with the level of isoflurane reduced to 1% with the oxygen. Following a skin incision, two cuts parallel to the tendon were made in the retinaculum on each side, a set of flat faced scissors were then placed underneath the patellar tendon. With the scissor blades serving as a support, a 0.75mm diameter biopsy punch (World Precision Instruments) was used to create a full thickness partial transection in the right patellar tendon. The left patellar tendon underwent a sham procedure, which consisted of only placing the plastic backing underneath the tendon without creating and injury. The skin wounds were closed with skin staples and the mice were sacrificed at 1 day, 3 days 7 and 21 days post-surgery. Mice were sacrificed by CO<sub>2</sub> inhalation and immediately weighed. Mice from two groups BALB/c control (CTL) and ST2-/- BALB/c were used. Each group contained 30 mice (n=30 ST2-/- BALB/c and 30 BALB/c). This set experiments was repeated on two separate occasions.

A



B



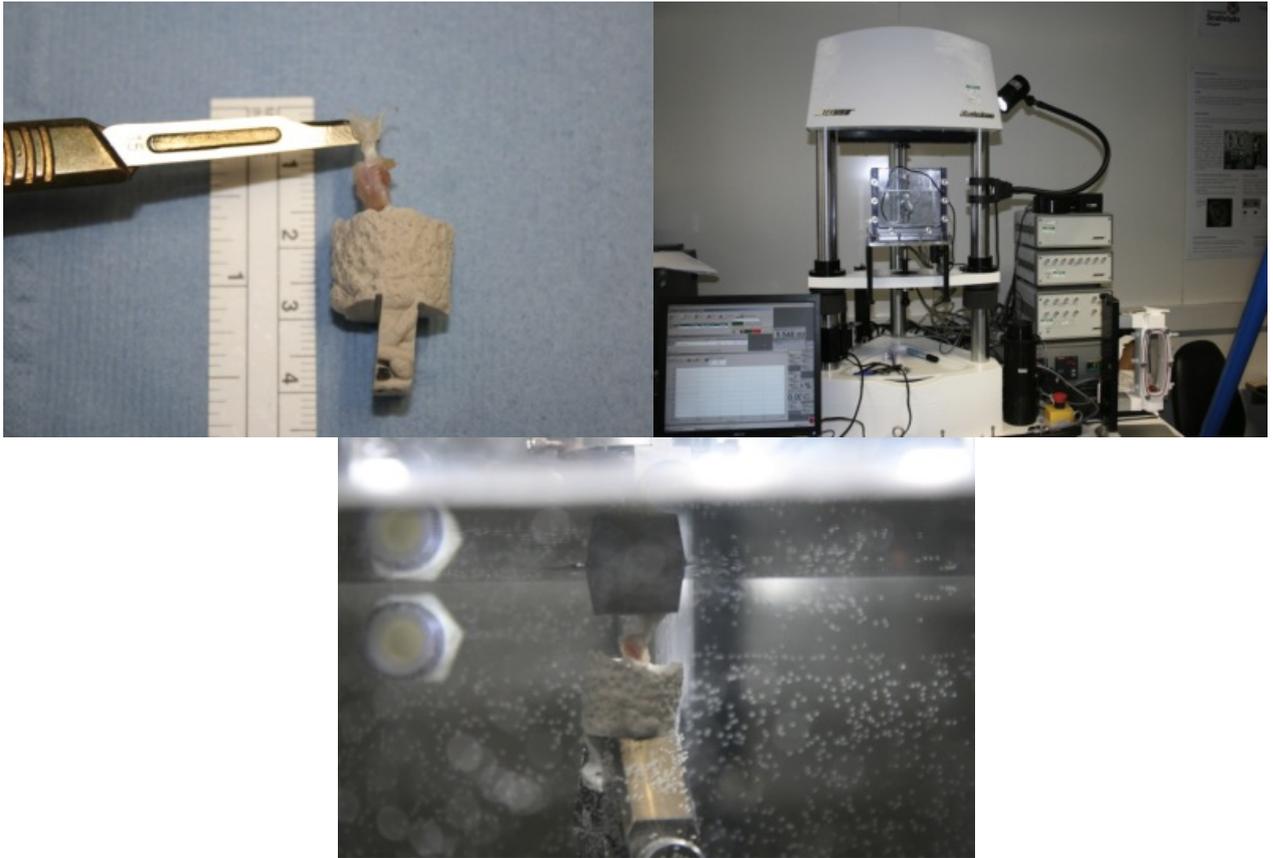
**Figure 2-2 Mouse patellar tendon model**

(A) Images of various steps in surgical protocol. (1) Exposure of patellar tendon, (2) Dissection of retinaculum and placement of dissecting scissors behind patellar tendon, (3) placement of 0.75mm biopsy punch over midportion of patellar tendon and (4) post injury with small defect left in midportion of tendon.

(B) Timeline for patellar tendon model. n=30 mice per group, 30 ST2<sup>-/-</sup> Balb/c and 30 Control Balb/c with 6 mice culled in each group on Day 1,3,7 and 21 post injury. On each cull day tendon was dissected and used in biomechanical (n=3), Histological/ Immunohistochemical (n=2) and quantitative PCR (n=2)

### **Biomechanical analysis**

For the biomechanical analysis, the patellar tendons of mice from each group were injured and three mice sacrificed at one of four time points for mechanical testing as described previously by Lin et al [132]. Briefly, the patellar tendons were dissected and cleaned, leaving only the patella, patellar tendon and tibia as one unit. Tendon width and thickness were then quantified and cross sectional area was calculated as the product of the two. The tibia was embedded in Isopon p38 (High Build Cellulose Filler) in a custom designed fixture and secured in place in a metal clamp. The patella was held in place by vice grips used with the BOSE ElectroForce® 3200 test instrument. Each tendon specimen underwent the following protocol immersed in a 37<sup>0</sup>C saline bath – reloaded to 0.02N, preconditioned for 10 cycles from 0.02 to 0.04 at a rate of 0.1%/s (0.003mm/s), and held for 10s. Immediately following, a stress relaxation experiment was performed by elongating the tendon to a strain of 5% (0.15mm) at a rate of 25% (0.75mm/s), followed by a relaxation for 600s. Finally a ramp to failure was applied at a rate of 0.1%/s (0.003mm/s). From these tests, maximum stress was determined and modulus was calculated using linear regression from the near linear region of the stress strain curve.

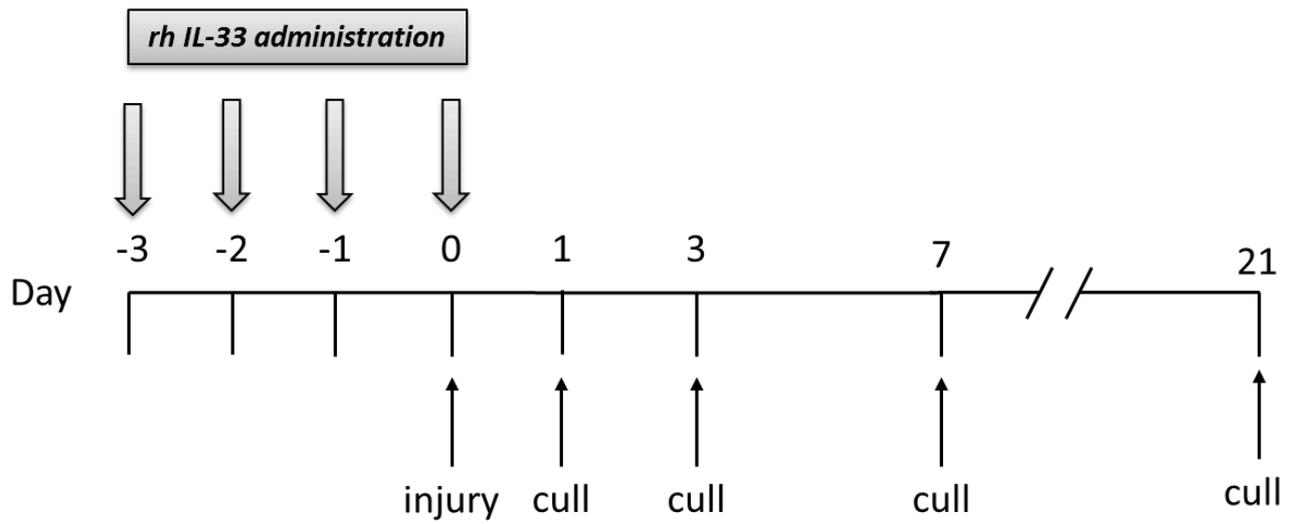


**Figure 2-3 Biomechanical testing apparatus.**

**(A) Image of patella, patellar tendon and tibia complex embedded in Isopon cellular filler prior to mechanical testing, (B) BOSE ElectroForce® 3200 test instruments feature with saline bath at 37°C and (C) image of mounted patellar tendon and tibia in saline bath prior to testing**

### **Cytokine injection model**

To test if IL-33 induces tendon matrix dysregulation a cytokine injection model was established. IL-33 was tested in a previously reported model initially described for the application of IL-23 or IL-22 [325, 326]. ST2<sup>-/-</sup> mice (n = 6/group/treatment/experiment) were injected i.p. daily with IL-33 (0.2 µg per mouse diluted in 100 µL PBS) on days-3, -2, -1 and the day of injury. 24 hours following the final injection mice the cull protocol was followed (Figure 2-4). Control mice similarly received an equal volume of PBS.



**Figure 2-4 Overview of the mouse injection model.**  
**(A) Injection of recombinant IL-33. n=16 mice per group, ST2<sup>-/-</sup> Balbc and Control Balbc. 6 mice culled in each group on days 1,3, and 7 post injury.**

## 2.13 Statistical analysis

The statistics used in each chapter are summarised below :-

### *Chapter 3*

Results are reported as mean values  $\pm$  SEM. Comparisons between groups were made with two-way paired Student's t tests (parametric), Mann Whitney U tests (non-parametric) and Kruskal-Wallis One Way Analysis of Variance on Ranks, using Graph Pad Prism 5 software. A power analysis was performed with the beta error set at 0.2 (power = 0.8) as previously described. Based on the results of the power analysis, it was determined that each group required 10 tissue samples to detect a difference of 20% between each of the groups with regard to inflammatory cell subset expression. Pearson's correlation coefficient was used to calculate the relationship between inflammatory infiltrate and rotator cuff tear size. In addition the inter rater reliability of our Modified Bonar score was calculated using intraclass correlation calculation (Graph pad Prism) with five independent observers between two international centres.

### *Chapter 4*

Results are reported as mean values  $\pm$  SEM/SD. Comparisons between groups were made with two-way paired Student's t tests, Mann Whitney U tests and Kruskal-Wallis One Way Analysis of Variance on Ranks, using Graph Pad Prism 5 software. Statistics reported on real time PCR data represent the mean of each experiment repeated in triplicate and then the pooled mean of n=3 or 5 depending on the experiment undertaken (see figure legends). Once again a similar power analysis to chapter 3 was performed requiring a minimum of 10 tissue samples to detect a 20% difference in hypoxic markers. Pearson's correlation coefficient was used to calculate the relationship between hypoxic cell markers, inflammatory infiltrate and rotator cuff tear size.

## *Chapter 5*

Results are reported as mean values  $\pm$  SEM/SD. Comparisons between groups were made with two-way paired Student's t tests, Mann-Whitney U tests and Kruskal-Wallis One Way Analysis of Variance on Ranks, using Graph Pad Prism 5 software. Analysis of IL-17 and immune cell staining was using the pooled mean of ten HPF per patient sample. Statistics reported on real time PCR data represent the mean of each experiment repeated in triplicate and then the pooled mean of n=3 or 5 depending on the experiment undertaken (see figure legends).

## *Chapter 6*

Once again results are reported as mean values  $\pm$  SEM/SD. Comparisons between groups were made with two-way paired Student's t tests, Mann Whitney U tests and Kruskal-Wallis One Way Analysis of Variance on Ranks, using Graph Pad Prism 5 software. Analysis for IL-33/ST2/IL-1RAcP immunostaining was using the pooled mean of each samples mean of ten high powered fields microscope examination. Statistics reported on real time PCR data represent the mean of each experiment repeated in triplicate and then the pooled mean of n=3 or 5 depending on the experiment undertaken (see figure legends). Analysis of mouse biomechanical data was based on the pooled mean of 3 or 4 mice depending on the experiment (See figure legend).

## **Chapter 3 Inflammation in human tendinopathy**

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

Millar NL, Hueber AJ, Reilly JH, Xu Y, Fazzi UG, Murrell GA, McInnes IB.  
Inflammation is present in early human tendinopathy.

Am J Sports Med. 2010 Oct;38(10):2085-91.

### 3.1) Aim and introduction

Overuse tendon injuries, namely tendinopathies, pose a significant problem in sports and exercise medicine [327]. The intrinsic pathogenetic mechanisms underlying the development of tendinopathies are largely unknown and debate remains as to whether inflammatory processes play a prominent role in the disease process [328]. Empirically, by dint of the 'danger hypothesis' [329], innate immune mechanisms should subservise, *prima fasciae*, core effector biology upon early damage mediated by biomechanical stress. Historically, 'tendinitis' was used to describe chronic pain from a symptomatic tendon [330] implying that inflammation was a central pathological process. However, traditional treatment modalities aimed at modulating inflammation have enjoyed limited success [331, 332]. Histological studies that focussed on symptomatic (late) biopsy components reveal few or absent inflammatory cells but rather implicated substantial degenerative changes comprising hypoxia, hyaline, mucoid or myxoid degenerations in over 85% of biopsy specimens [333, 334].

In contrast to the foregoing, recent studies implicate an early, important inflammatory component in disease processes. Molloy et al [74] performed microarray studies on a running rat supraspinatus tendinopathy model and showed upregulation of key inflammatory cell receptors and immunoglobulin's. Barbe and colleagues [335] using a cumulative trauma disorder rodent model showed increased infiltrating macrophages compared to controls. Human tissue biopsy samples from small rotator cuff tears taken at the time of surgery show a significant inflammatory infiltrate, consisting of macrophages and mast cells, compared to larger tears reflecting a more degenerative picture [66].

One of the major limitations of human studies is that tendon biopsies are usually obtained when patients are symptomatic and therefore biopsy material is likely to represent chronic, rather than early phase tendinopathy. We previously suggested that matched subscapularis tendon from patients with full thickness rotator cuff tears may be a model of early human tendinopathy based on histological appearances and significantly increased levels of cytokines and apoptotic markers in these tissues [137]. ***The purpose of this part of my thesis was to formally characterize inflammatory cell subtypes within this putative model.***

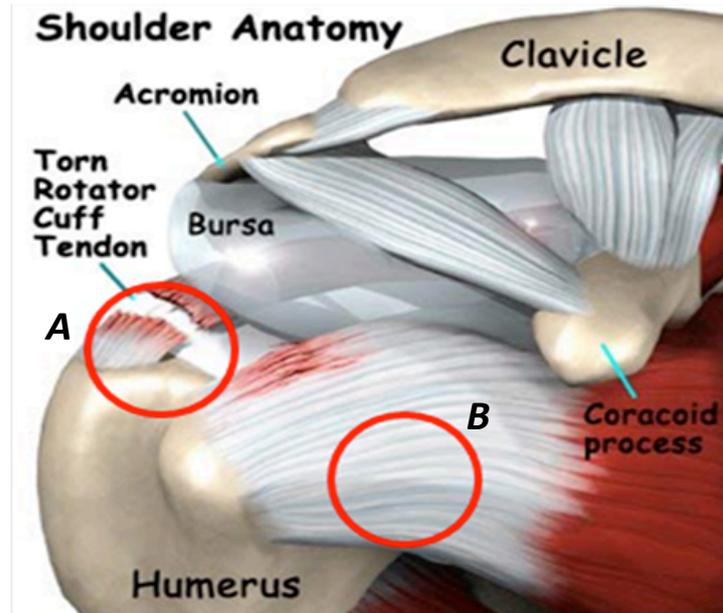
### 3.2) Inflammatory staining in human tendinopathy

Twenty supraspinatus tendon samples were collected from patients with rotator cuff tears undergoing shoulder surgery (Table 3-1). The mean age of the rotator cuff ruptured patients was 57 years (range, 39-75 years) - the mean tear size was 2.8cm<sup>2</sup>. Samples of the subscapularis tendon were also collected from the same patients. Patients were only included if there was no clinically detectable evidence of subscapularis tendinopathy on a preoperative MRI scan or macroscopic damage to the subscapularis tendon at the time of arthroscopy – by these criteria they represented a truly pre-clinical cohort. An independent control group was obtained comprising 10 samples of subscapularis tendon collected from patients undergoing arthroscopic surgery for shoulder stabilization without rotator cuff tears. The absence of rotator cuff tears was confirmed by arthroscopic examination. The mean age of the control group was 35 years (range, 20-41 years).

We obtained specimens through arthroscopic repair of the rotator cuff which was carried out using the standard three-portal technique as described by Xu et al [336]. The cross-sectional size of the rotator cuff tear was estimated and recorded as described previously [337]. The subscapularis tendon was harvested arthroscopically from the superior border of the tendon 1 cm lateral to the glenoid labrum. The supraspinatus tendon was harvested from within 1.5 cm of the edge of the tear prior to surgical repair (Figure 3-1) . For immunohistochemical staining the tissue samples were immediately fixed in 10% (v/v) formalin for 4 to 6 hours and then embedded in paraffin. Sections were cut to 5µm thickness using a Leica-LM microtome (Leica Microsystems, Germany) and placed onto Superfrost Ultra Plus glass slides (Gerhard Menzel, Germany). The paraffin was removed from the tissue sections with xylene, rehydrated in graded alcohol and used for histological and immunohistochemical staining per previously stated methodologies [338].

<b>Tear Size</b>	<b>Control</b>	<b>Small (<math>&lt;1\text{cm}^2</math>)</b>	<b>Medium (<math>&gt;1-3\text{cm}^2</math>)</b>	<b>Large (<math>&gt;3-5\text{cm}^2</math>)</b>	<b>Massive (<math>&gt;5\text{cm}^2</math>)</b>
Number of cases	10	6	7	4	3
Mean age in years(range)	35 (20-41)	51 (39-60)	57 (48-64)	55 (47-60)	63 (50-75)
Mean duration of symptoms in months (range)	8.3 (1-14)	7.8 (2-18)	7.0 (3-13)	8.8 (4-22)	6.3 (2-15)
Mean number of steroid injections	0	1.2	1.6	1.5	1.8

**Table 3-1 Patient demographics and rotator cuff tear size**



**Figure 3-1 Anatomical locations of biopsied tendon.**

Area A shows the torn edge of supraspinatus which is obtained at time of surgery. In the same patient a biopsy is taken from Area B, the matched subscapularis tendon, which has no changes on MRI and looks macroscopically normal at the time of arthroscopy. Control tendon is taken from a separate cohort of patients with no rotator cuff tear. These patients have a biopsy of area B taken at the time of instability surgery. (Reproduced with permission from Professor George Murrell, Orthopaedic Research Institute, Sydney Australia)

### **3.2.1) Establishment of human 'model' of tendinopathy**

Sections were stained with haematoxylin and eosin and toluidine blue for determination of the degree of tendinopathy as assessed by a modified version of the Bonar score [339] (Grade 4= marked tendinopathy, Grade 3 = advanced tendinopathy, 2= moderate degeneration 1= mild degeneration 0 = normal tendon). This included the presence or absence of oedema and degeneration together with the degree of fibroblast cellularity and chondroid metaplasia. We have previously described a novel 'early human model' of tendinopathy based on surprising findings when investigating the histology of matched subscapularis tendon samples [340]. We used the same biopsy techniques and scoring system as utilized in this previous study.

All torn supraspinatus samples showed Grade 4 changes consistent with marked degeneration, mucoid change and frank chondroid metaplasia. The massive tears had reduced fibroblast cellularity and greater chondroid metaplasia compared to all other tears ( $p < 0.05$ ). Despite their normal MRI and arthroscopic appearances, matched subscapularis tendon showed Grade 2-3 changes indicative of moderate-advanced tendinopathy (Figure 3-2). All control samples were classified as Grade 1 consistent with normal fibrotendinous tissue with large distinct collagen fibrils. There were no significant correlations between Bonar score [339] and the mean duration of symptoms or age of the patient cohort.

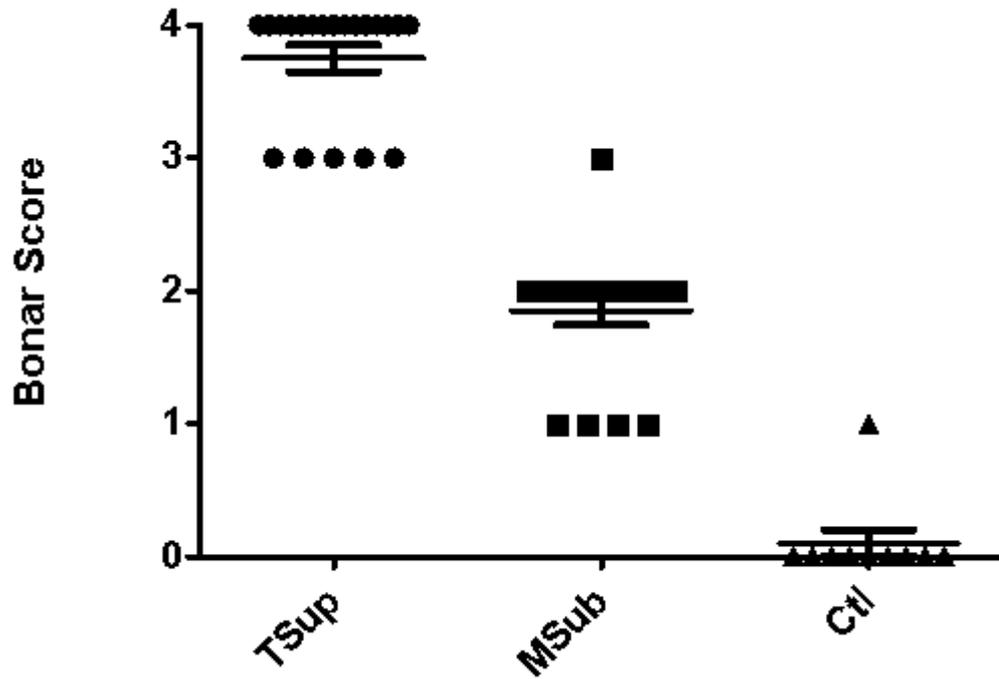


Figure 3-2 Bonar histological scoring for tendon biopsies.

The majority of torn tendons showed classical features of advanced to marked tendinopathy. Subscapularis tendon showed mainly features of mild-moderate tendon disease in keeping with an 'early model' of tendinopathy. Bonar score Grade 4= marked tendinopathy, Grade 3 = advanced tendinopathy, 2= moderate degeneration 1= mild degeneration 0 = normal tendon.

TSUP= Torn Supraspinatus, MSub =Matched Subscapularis tendon, Ctl= Control tendon.

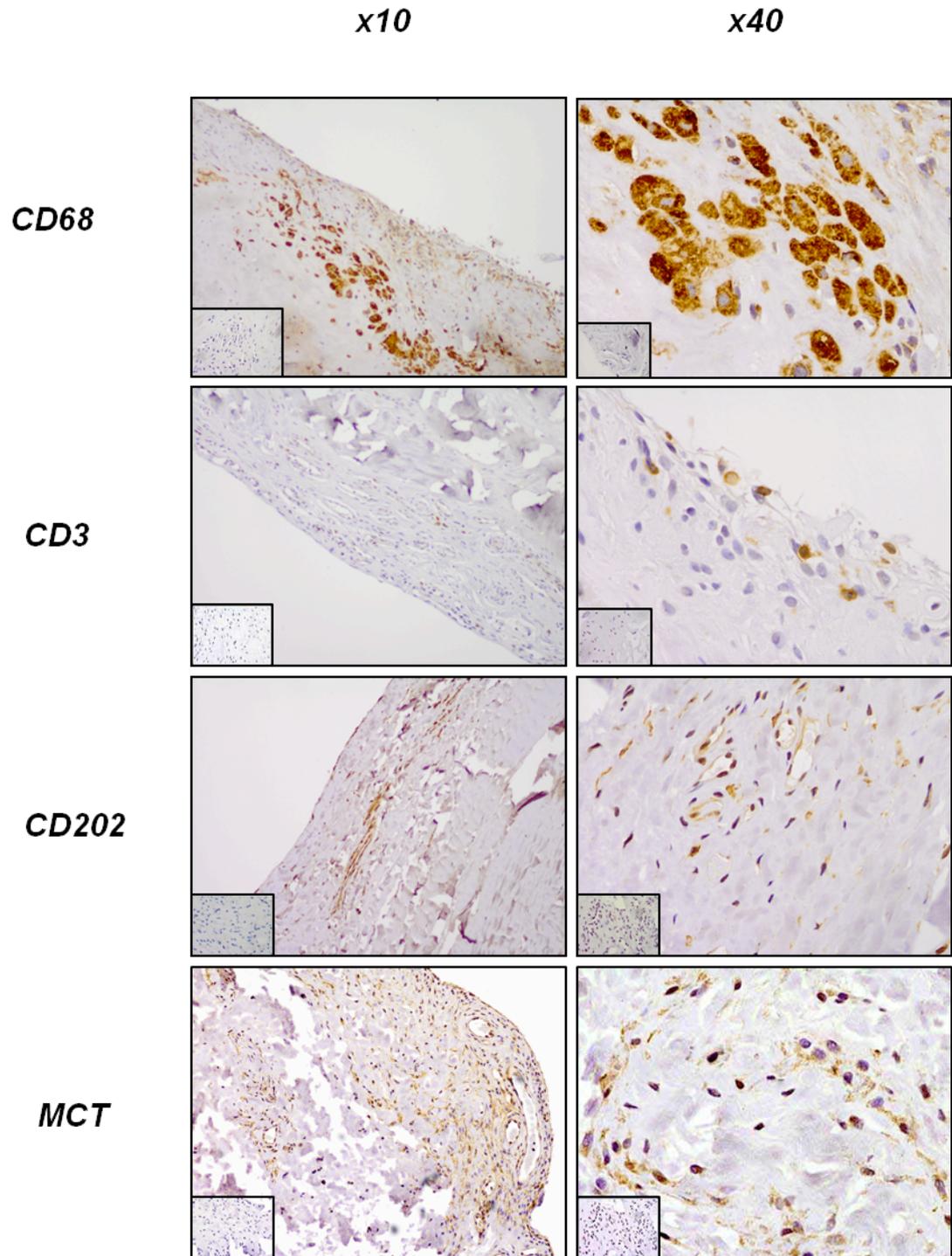
### 3.2.2) Early human tendinopathy shows an increased inflammatory infiltrate

We applied a novel scoring system based on previous methods[14] to quantify the immunohistochemical staining. Ten random high power fields (x400) were evaluated by three independent assessors. 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. In addition the blood vessel numbers were assessed in the same random high power fields. Intra-rater reliability was good reflected in  $r = 0.82$ . Additionally the same fields were photographed and evaluated by collaborators in Sydney, Australia. These included Dr Fiona Bonar, Consultant Pathologist, whose original scoring system [341] the above methodology was based on and is seen as one of the fields preeminent authorities on tendon pathology. The results were readily reproducible by these collaborators.

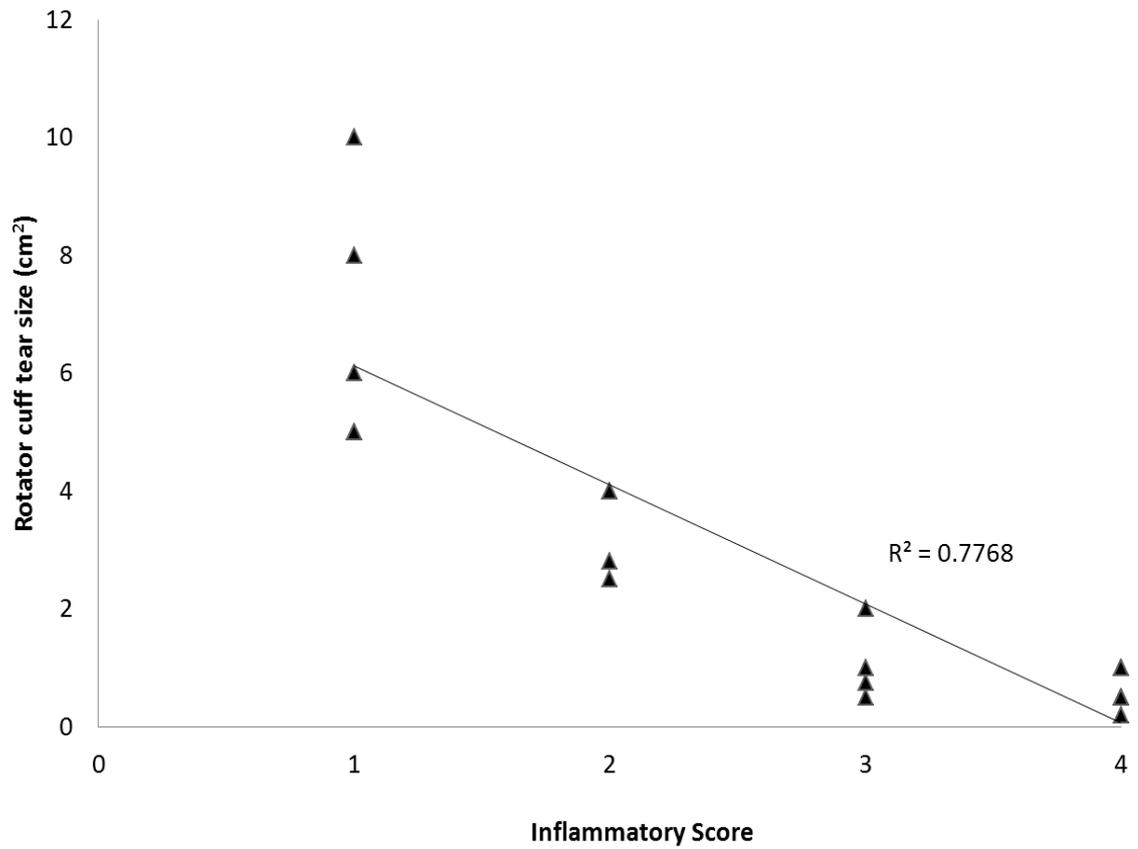
I initially chose to investigate the staining profile of mast cells and macrophages in each cohort due to previous reports of these cells in torn rotator cuff samples [66]. Following this I expanded the staining to include T cells and specially CD34 as a marker of endothelial/vasculature to specifically assess whether mast cell staining was co-localised around neovascularisation. Additionally due to parallel studies in the laboratory I chose CD206 as an M<sub>2</sub> macrophage marker in an attempt to stratify the macrophage infiltrate.

The cohort of subscapularis tendon samples exhibited significantly greater ( $p < 0.01$ ) staining for macrophages, mast cells and T cells compared to both matched torn supraspinatus samples and control tissue (Figure 3-3). Macrophages were scattered mainly throughout the subintimal layers although some were present in a peri-vascular cuff. The majority of mast cells were located around the vasculature with the remainder residing in the synovial lining or scattered throughout the tissue. Inflammatory cell infiltrate correlated inversely ( $r^2 = 0.8$ ,  $p < 0.01$ ) to rotator cuff tear size in the torn supraspinatus biopsies with larger tears showing a marked reduction in all cell types. In particular mast cells and M<sub>2</sub> macrophages were seen in specimens with increased fibroblast cellularity and decreased markedly in number as the fibroblast cellularity of the specimen

decreased (Figure 3-4). No significant associations were noted between inflammatory infiltrate and the age of patients or duration of symptoms.



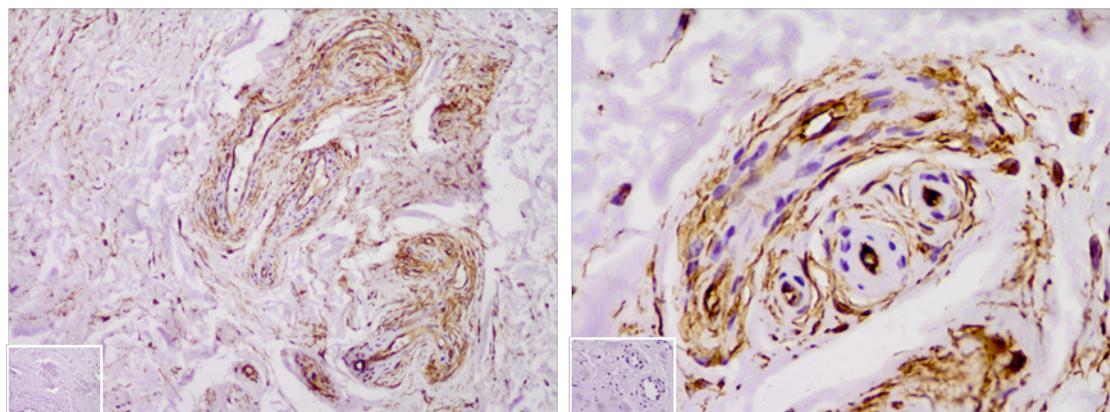
**Figure 3-3 Immunohistochemical staining for inflammatory cell surface markers. Staining for CD68 (pan macrophage) in sub intimal layers of tendon. CD3 (Tcells) are sparse but seem to be localised around lining layers and vascular regions. CD202 (M2 macrophages) are again sub intimal but are more readily localised to the vasculature. MCT (Mast cell tryptase) is highly expressed throughout the tissue with large expression patterns around vascular regions.**



**Figure 3-4 Correlation between tear size and inflammatory cell infiltrate.** Inflammatory infiltrate is graded Grade 0= no staining, Grade 1= <10% cells stained positive, 2= 10-20% cells stained positive, Grade 3= 21-50% cells positive, Grade 4 > 50% cells positive per high power field (HPF). Thus as tear size decreases there is an increase in inflammatory infiltrate.

### **3.2.3) Increased vascularity in tendinopathy**

CD34 positive vessels were found in the greatest quantity in matched subscapularis samples (Figure 3-5) compared to all other groups with a mean vessel count of  $38 \pm 2$  in subscapularis compared with  $15 \pm 3$  in matched torn biopsies or  $6 \pm 1$  in control tissues. As tear size increased in the supraspinatus samples, there was a significant ( $p < 0.05$ ) reduction in vascularity suggesting an inverse relationship. The mean vessel count was significantly ( $p < 0.01$ ) different between all tear groups (Table 3-2). There was a moderate but significant correlation between mast cells and CD34 expression ( $r = 0.4$ ,  $p < 0.01$ ).

**x10****X40**

**Figure 3-5 Immunohistochemical staining for CD34 Endothelial marker**

Feature	Control (n=10)	Matched subscapularis (n=20)	Torn Supraspinatus				
			Overall (n=20)	Small (n=6)	Medium (n=6)	Large (n=4)	Massive (n=4)
Mean vessel Count <sup>†</sup>	6 ± 1	38 ± 2	15 ± 3	28 ± 2	17 ± 2	6 ± 1	1 ± 1
Inflammatory cell count <sup>‡</sup>							
Macrophages	4 ± 1	30 ± 4	13 ± 2	23 ± 1	14 ± 2	5 ± 1	3 ± 1
Mast Cells	0 ± 0.5	25 ± 3	10 ± 3	18 ± 4	11 ± 1	4 ± 1	4 ± 2
M <sub>2</sub> Macrophages	2 ± 1	26 ± 3	9 ± 2	15 ± 2	13 ± 2	7 ± 2	2 ± 1
T cells	1 ± 1	12 ± 2	6 ± 2	9 ± 2	7 ± 1	3 ± 1	2 ± 1

**Table 3-2 Histological features in control, matched subscapularis and torn supraspinatus tendon samples**

<sup>†</sup> mean number of vessels in ten high power fields of view (magnification x400)

<sup>‡</sup> mean number of cells in ten high power fields of view (magnification x400)

### 3.3) Discussion and Conclusions

These experiments provide convincing evidence of an inflammatory cell infiltrate in early mild/moderate human supraspinatus tendinopathy. Our data are novel and therefore directly inform the controversy that surrounds the role for inflammation in the development of tendinopathies.

Experimental models provide good evidence of an early inflammatory response. A running rodent model of tendinopathy is associated with up regulation of key inflammatory modulators [74] including the 5-lipoxygenase activating protein (FLAP) and cyclo-oxygenase at early and intermediate time points [342]. In rabbit and equine models excessive mechanical load induces acute inflammatory cell infiltrates [93, 343]. Human studies are less convincing. Whereas some studies describe the presence of increased cytokine expression [112, 137, 344, 345] as proof of an inflammatory component, virtually all histological studies in human tissue have failed to demonstrate inflammatory cells in tendinopathic samples [82, 333, 334, 339, 346]. One of the previous limitations has been that such tissues were from patients with advanced disease, presumably dominated pathologically by chronic degenerative changes. Recent human biopsy work in smaller tears [66] with a less degenerative picture revealed a significant inflammatory infiltrate of mast cells and macrophages. Inflammation is also intimately linked to the Fas/Fas ligand system of apoptosis, which has been found in excessive amounts in a range of tendinopathies [76, 347, 348]. Our data provide convincing evidence that inflammation is indeed present and could therefore provide a molecular link to key pathological events in tendinopathy.

Our previous work on matched subscapularis tendon biopsies from patients with a full thickness supraspinatus tear produced unexpected results [340]. We found that despite normal MRI or arthroscopic appearances, at the histological level, the tendon had mild/moderate tendinopathic changes. These samples contained increased expression of proinflammatory cytokines compared to controls assessed at the mRNA level [137]. We have now extended these observations to include a detailed cellular analysis. Macrophages play a critical role in the initiation, maintenance and resolution of inflammation [349]. In response to cytokines and microbial products, mononuclear phagocytes develop specialized and polarized functional properties within functionally discrete M<sub>1</sub> or M<sub>2</sub> subsets [350]. M<sub>1</sub> macrophages are efficient producers of effector moieties, including

reactive oxygen and nitrogen intermediates and inflammatory cytokines and chemokines, whereas in general, M<sub>2</sub> macrophages act to dampen inflammatory responses and scavenge debris as well as promote angiogenesis. M<sub>2</sub> macrophages express fibronectin and IGF-1; key signals for tissue repair [351] and recent work has shown that defective M<sub>2</sub> polarization resulted in impaired muscle tissue repair mechanisms [352]. We detected a significant proportion of macrophages expressing CD206, compatible with an M<sub>2</sub> phenotype. Inflammation in early tendinopathy could in part reflect an attempt at tissue repair. The associated higher blood vessel density in back to back sections of tendon samples is compatible with this hypothesis.

Of particular interest is the large number of mast cells present in early tendinopathy. Mast cells play a key role in the inflammatory process - they rapidly release characteristic granules and various mediators that mediate the recruitment and activation of monocytes, neutrophils, dendritic cells, B and T cells [353, 354] and have recently been linked to neoangiogenesis [355]. They are associated with inflammatory diseases including atopic dermatitis [356], scleroderma [357] and rheumatoid arthritis [358]. Scott et al [359] found significantly increased mast cell numbers in human patellar tendinosis and correlated this with symptom duration and vascular hyperplasia while studies in rabbits have shown that mast cells and their mediators influence fibroblast activity and vascular permeability [360]. In keeping with these previous studies, our findings are suggestive of a pro-angiogenic role for mast cells in tendinopathies. Indeed mast cells could theoretically play a role in 'stress induced' tendinopathy by degranulating in response to mechanical stress thereby releasing mast cell tryptase and other vasoactive and angiogenic mediators which are important in the balance between repair and further degeneration. This observation warrants further mechanistic studies to understand how mast cells interact with tenocytes and regulate matrix synthesis.

Limitations exist in this set of experiments. Firstly, age-related changes within the tendon samples could contribute to the degenerative picture and inflammatory cell expression seen in the matched subscapularis tendons. However the lack of degenerative change on MRI and arthroscopic examinations suggests that the differences are truly at the cellular level as suggested by our work. Secondly, subscapularis tendon is functionally and organizationally distinct from supraspinatus and thus responds to mechanical loading in a different manner

which may alter its cellular profile. It is reassuring however that we found the same inflammatory and vascular cell subtypes in matched subscapularis tissue indicating that the inflammatory response may be uniform within joints subjected to tendon degeneration. In addition having subscapularis samples from the same patient eliminates bias that may result from variation between individuals and has been previously shown to be useful method in sampling of tissues.

In summary we have found a distinct inflammatory infiltrate in early human tendinopathy. This data suggests that inflammation may play a crucial role in tendon healing - better understanding of this inflammatory cascade may lead to the development of cell targeted treatment modalities for early supraspinatus tendinopathy. I next sought to characterise a plausible candidate mechanism that could promote the inflammatory changes that I observed in these studies, namely hypoxia.

## **Chapter 4 Hypoxia and Tendinopathy**

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

Millar NL, Reilly JH, Kerr SC, Campbell AL, Little KJ, Leach WJ, Rooney BP, Murrell GA, McInnes IB.

Hypoxia: a critical regulator in early human tendinopathy

Annals of Rheumatic Diseases 2012 Feb;71(2):302-10. Epub 2011 Oct 4.

## 4.1) Aims and introduction

The intrinsic pathogenetic mechanisms underlying the development of tendinopathies are largely unknown however excessive apoptosis (programmed cell death) and inflammation have recently been shown to be functionally important in several models systems [74, 342, 360]. In particular, data contained in my previous chapter clearly provide evidence of early inflammation in tendinopathy. The interaction between apoptosis and inflammation is critical to tissue homeostasis in a variety of tissues and is known to play a key role in inflammatory articular [361], neoplastic [362], neurodegenerative [363] and cardiovascular diseases [364].

Apoptosis may be induced by many stimuli, including hypoxia. The magnitude and duration of the hypoxic microenvironment largely determines whether cells become apoptotic or adapt and survive [365]. Hypoxia inducible factor 1 (HIF-1 $\alpha$ ), is a critical regulator in this process. Whereas HIF-1 $\alpha$  can initiate apoptosis by inducing high concentrations of proapoptotic proteins [366], such as caspase 3, during hypoxia, HIF-1 $\alpha$  induces antiapoptotic proteins [367], such as Clusterin and Bcl-2, while the proapoptotic protein Bax is downregulated. HIF-1 $\alpha$  is also recognised to control key aspects of inflammation particularly leukocyte recruitment and subsequent cytokine production [368].

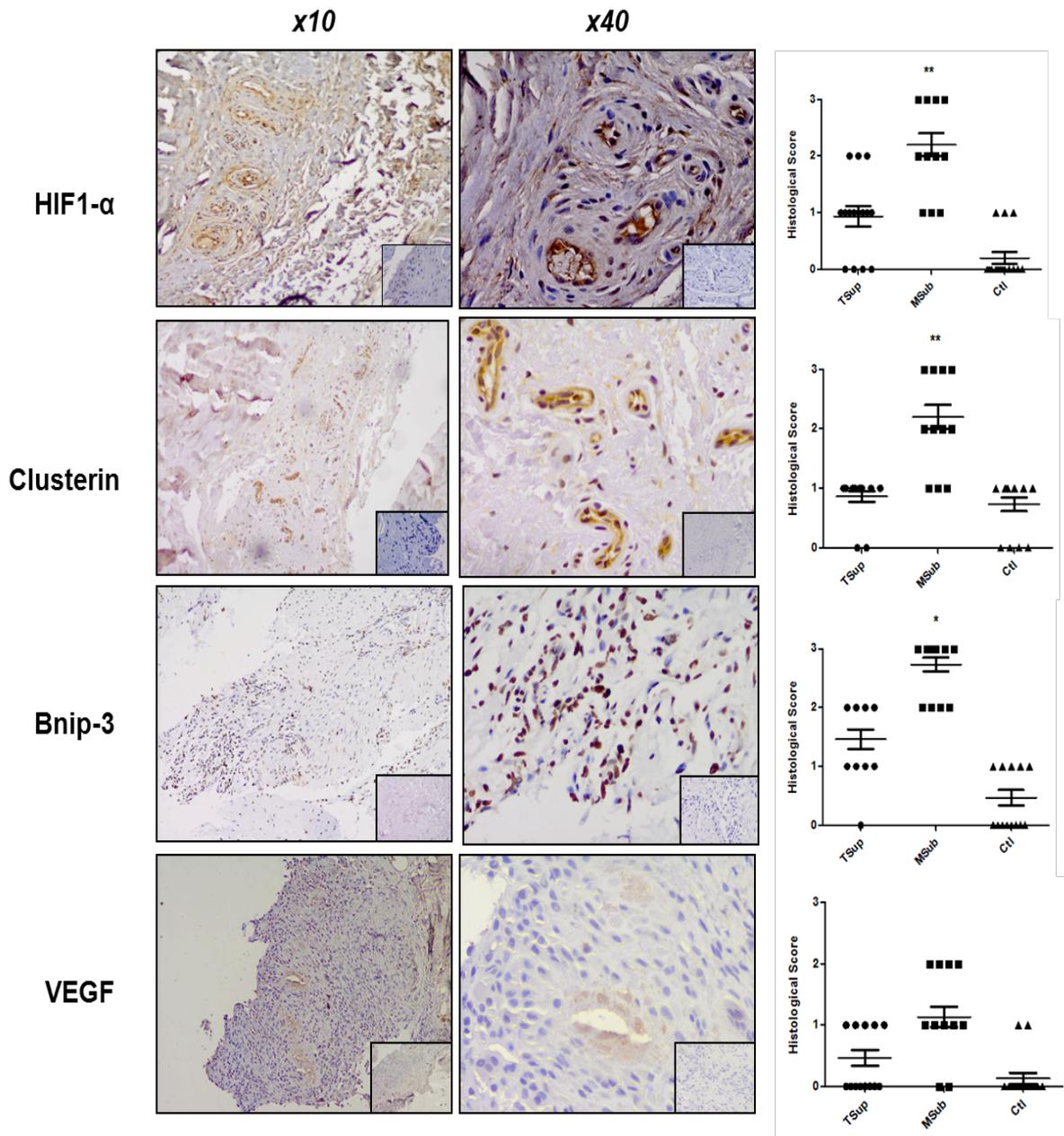
Biopsy and *in vivo* model studies suggest that tendon hypoperfusion [274] and subsequent hypoxia [275] are central elements of tendon failure but few data have addressed this at a mechanistic level in humans. In rodent models, cyclic strain regulates the expression of the HIF-1 $\alpha$  in rat tendon fibroblasts [117]. Increased hypoxia in the tenosynovium of rheumatoid arthritis patients is observed compared to controls [369], and recently Benson et al [68] described hypoxic damage throughout the spectrum of rotator cuff disease. To date no studies have explored the functional role of hypoxia in modulating apoptosis, inflammation and matrix regulation in tendinopathy.

One of the major limitations of human studies is that tendon biopsies are usually obtained when patients are symptomatic and therefore biopsy material likely represents chronic, rather than early phase disease. Medical intervention at this early stage may offer considerable therapeutic advantage over later surgical approaches. We previously demonstrated that matched subscapularis tendon from patients with full thickness rotator cuff tears represents a model of early human

tendinopathy based on histological appearances and significantly increased levels of cytokines and apoptotic markers in these tissues [137]. ***The purpose of this section of my thesis was to formally assess the presence and magnitude of expression of hypoxic markers within this early phase human model ex vivo and thereafter to explore the mechanistic outcome of tissue hypoxia on apoptosis, inflammation and matrix production in tenocytes in vitro.***

## **4.2) Early tendinopathy is associated with increased hypoxic and apoptotic markers**

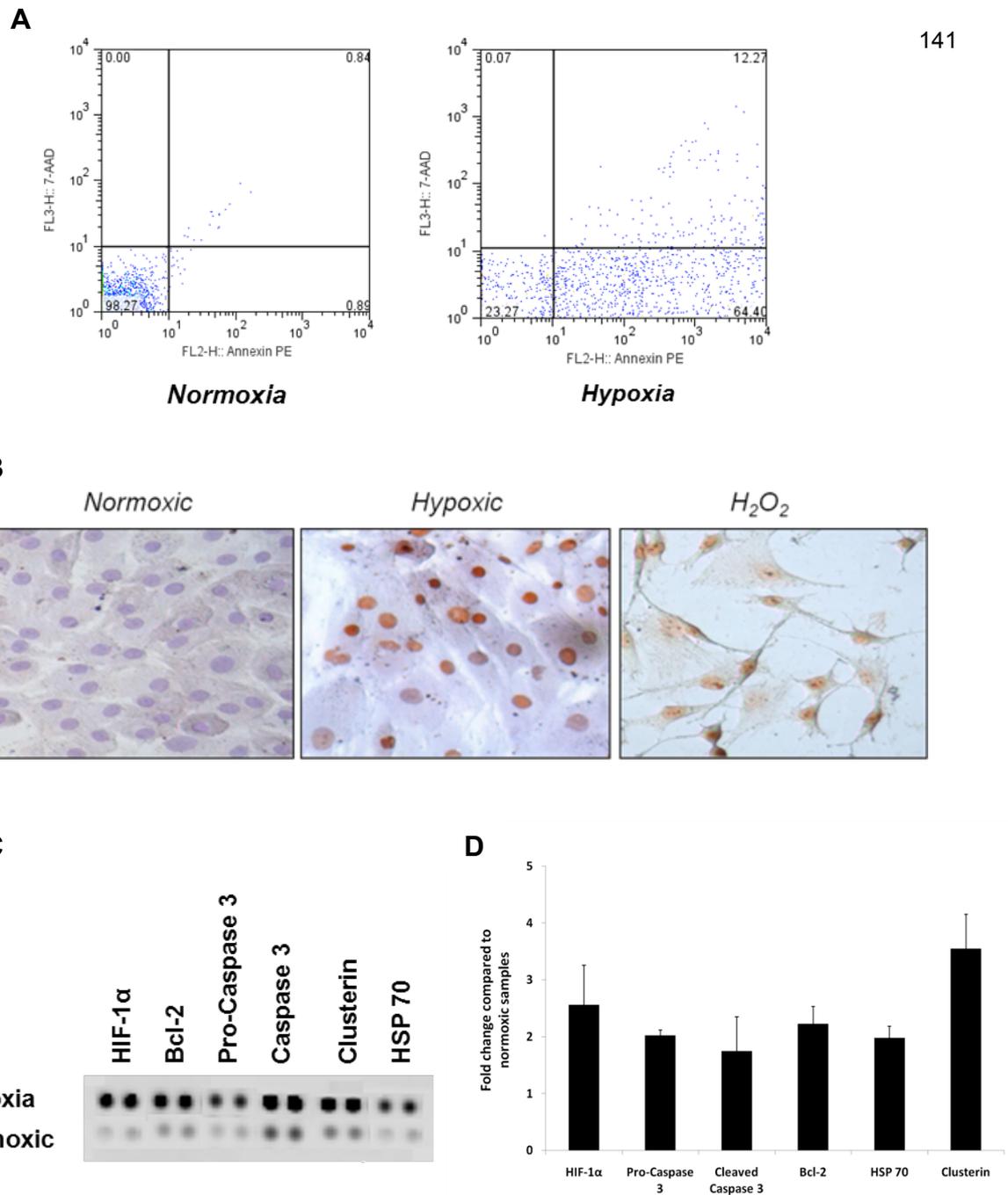
Torn supraspinatus samples exhibited marked degeneration, mucoid change and frank chondroid metaplasia (Grade 4) whereas matched subscapularis tendon biopsies had Grade 2-3 changes indicative of early tendinopathy. All control samples were classified as Grade 1 consistent with normal fibrotendinous tissue with large distinct collagen fibrils. There were no significant correlations between Bonar score and the mean duration of symptoms or patient age. However subscapularis tendon samples exhibited significantly greater staining for HIF 1 $\alpha$ , clusterin, Bcl-2, BNIP-3 and VEGF compared to either matched torn supraspinatus samples or control tissue (Figure 4-1). Subscapularis tendon samples exhibited significantly greater ( $p < 0.01$ ) numbers of macrophages, mast cells and T cells compared to either matched torn supraspinatus samples and control tissue. Inflammatory cell infiltrate and apoptosis markers correlated inversely ( $r = 0.4$ ,  $p < 0.01$ ) with rotator cuff tear size in the torn supraspinatus biopsies, with larger tears showing a marked reduction in inflammatory cell types and hypoxic/apoptotic markers.



**Figure 4-1 Apoptosis and hypoxic markers in tendon samples.** Subscapularis tendon stained for HIF-1 $\alpha$ , Clusterin, Bnip-3 and VEGF ( magnification x10 and x40). Isotype IgG in bottom right corner. Graphs illustrate relative expression of corresponding proteins in human tendon samples. Histological scoring system, 0= no staining, 1= <10% cells positive, 2= 10-20% cells positive, Grade 3 = >20 % cells positive. Data displayed as mean +/- SEM, n=15 for supraspinatus and matched subscapularis, n=10 for control group. (\*=p<0.05, \*\* =p<0.01)

### 4.3) Hypoxic induces apoptosis in tenocytes in vitro

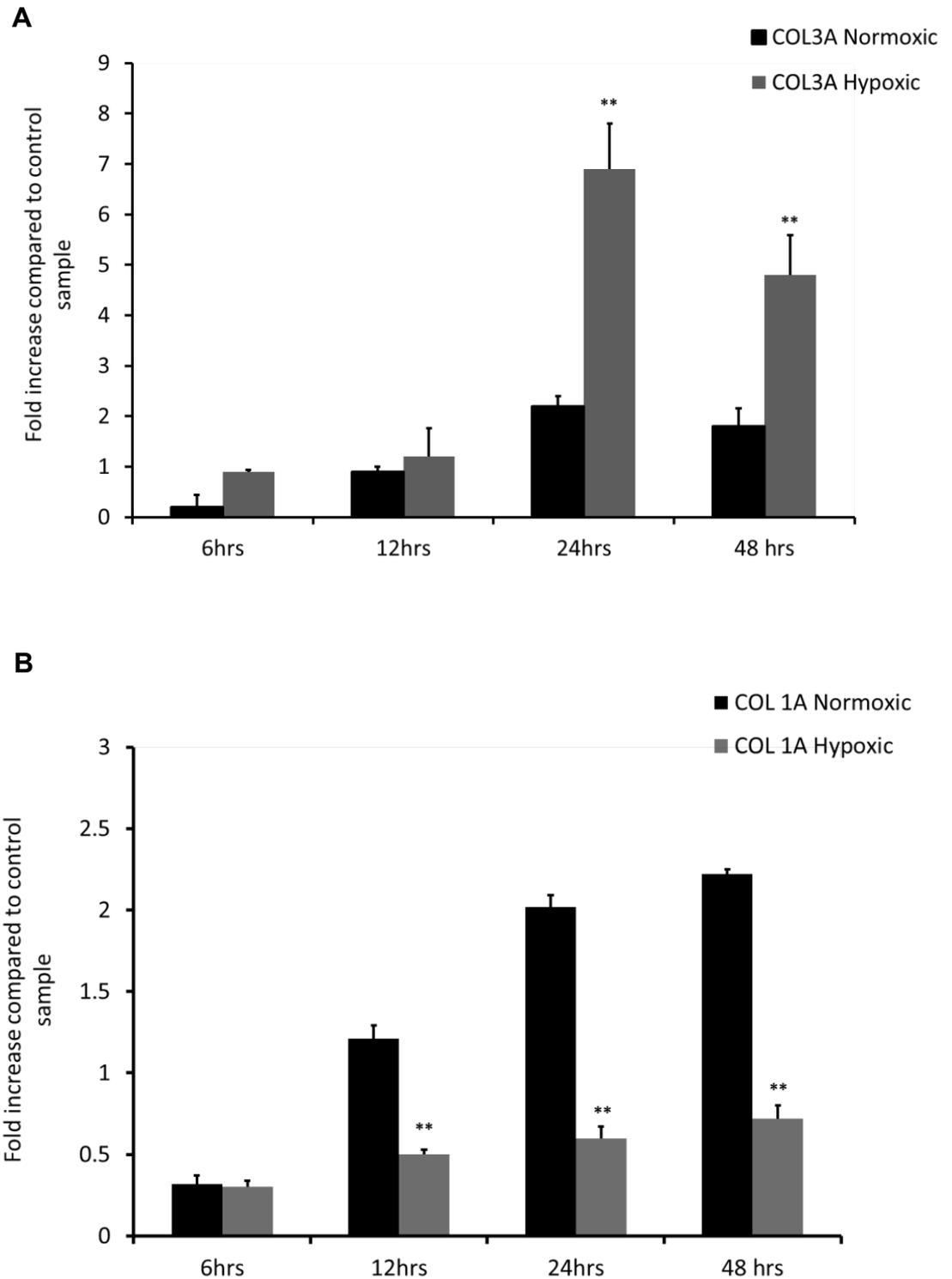
To explore the physiological significance of these findings, we first established an *ex vivo* primary human tenocyte culture system in which ambient oxygen concentrations could be meticulously controlled as required. Hypoxia significantly increased the number of cells undergoing apoptosis assessed by annexin V FACS staining at 12 hours (normoxia; mean  $5 \pm 2\%$  apoptosis, hypoxia; mean  $23 \pm 8\%$ ), 24 hours (normoxia; mean  $3 \pm 2\%$ , hypoxia; mean  $58 \pm 10\%$  and 48 hours (normoxia; mean  $8 \pm 4\%$ , hypoxia; mean  $60 \pm 15\%$ ) (Figure 4-2A). This was confirmed by TUNEL staining showing increased levels of apoptosis in 1% conditions (Figure 4-2B). Limited proteomic profiling revealed relative over expression of Bcl-2, HIF 1 $\alpha$ , clusterin, pro and cleaved caspase 3 and heat shock proteins 70, in hypoxic conditions (Figure 4-2C;  $p < 0.01$ ). These protein expression changes were confirmed at the mRNA level by quantitative PCR that confirmed significantly elevated expression of HIF 1 $\alpha$ , caspases 3 & 7, clusterin, Bcl-2 and HSP 70 compared to normoxic conditions (Figure 4-2 D).



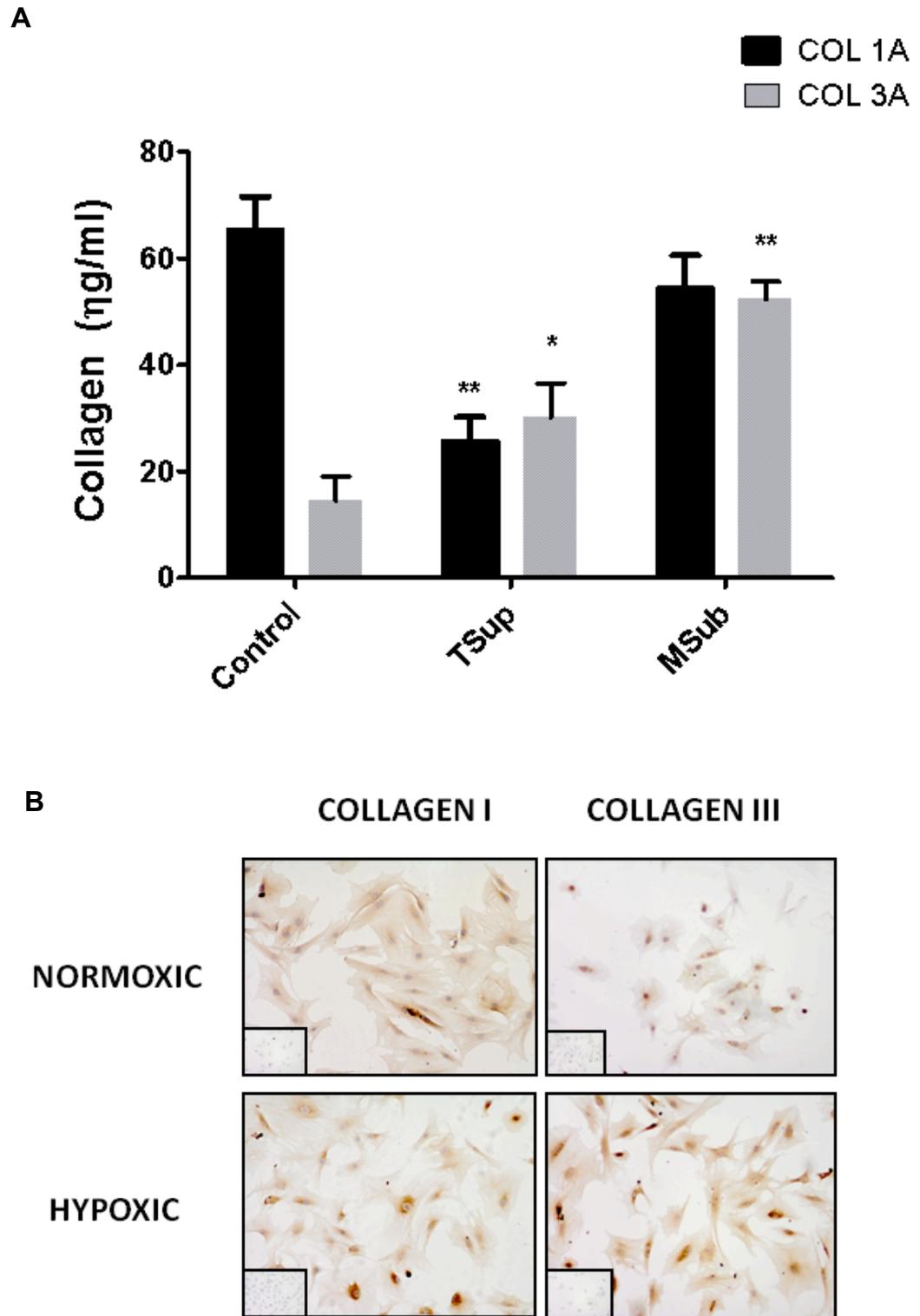
**Figure 4-2 (A) Cell death after exposure to hypoxic conditions.** Apoptosis was evaluated after treating tenocytes with 24h of normoxic (24% O<sub>2</sub>) and hypoxic (1% O<sub>2</sub>) conditions. Flow cytometry profile represents Annexin V-FITC staining in x axis and 7-AAD in y axis. (B) Cultured tendon fibroblasts underwent a TUNEL assay to detect nuclear DNA degradation, a feature of apoptotic cells. Cells with brown stained nuclei are positive for DNA degradation. Samples displayed include 24% O<sub>2</sub>, 1% O<sub>2</sub> and +ve controls treated with H<sub>2</sub>O<sub>2</sub>. Whole cell lysates from normoxic (24% O<sub>2</sub>) and hypoxic (1% O<sub>2</sub>) conditions were harvested and apoptotic markers evaluated using an apoptotic proteome profiler (C). The fold change of apoptotic proteins was determined by densitometry and normalised to the control sample on the array (D). Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

#### **4.4) Hypoxia alters tenocyte collagen matrix regulation**

Since disproportionate type III collagen expression is a characteristic feature of tendinopathy, we next considered whether hypoxic changes noted above might alter differential collagen synthesis by tenocytes and whether collagen matrix changes were present in tendon biopsies noted to have increased hypoxic markers. Collagen III was significantly ( $p < 0.05$ ) elevated in torn supraspinatus and matched subscapularis samples compared to controls (Figure 4-3 A). Collagen IA mRNA was significantly reduced at all time points in hypoxic compared to normoxic conditions. In contrast, collagen IIIA mRNA was significantly up-regulated at 24 & 48 hours in hypoxic compared to normoxic cultures (Figure 4-3 B&C). Commensurate with this at the protein level, hypoxia significantly elevated total collagen production compared to normoxia after 24 hours (mean  $328\mu\text{g/ml} \pm 81$  hypoxia;  $520\mu\text{g/ml} \pm 98$  normoxic) and 48 hours ( $712\mu\text{g/ml} \pm 66$  hypoxia;  $909\mu\text{g/ml} \pm 74$  normoxic). Semi-quantitative immunocytochemistry revealed no marked change in type I collagen protein expression in hypoxic compared to normoxic cultures, whereas collagen III staining was markedly increased at all time points by hypoxia (Figure 4-3 D).



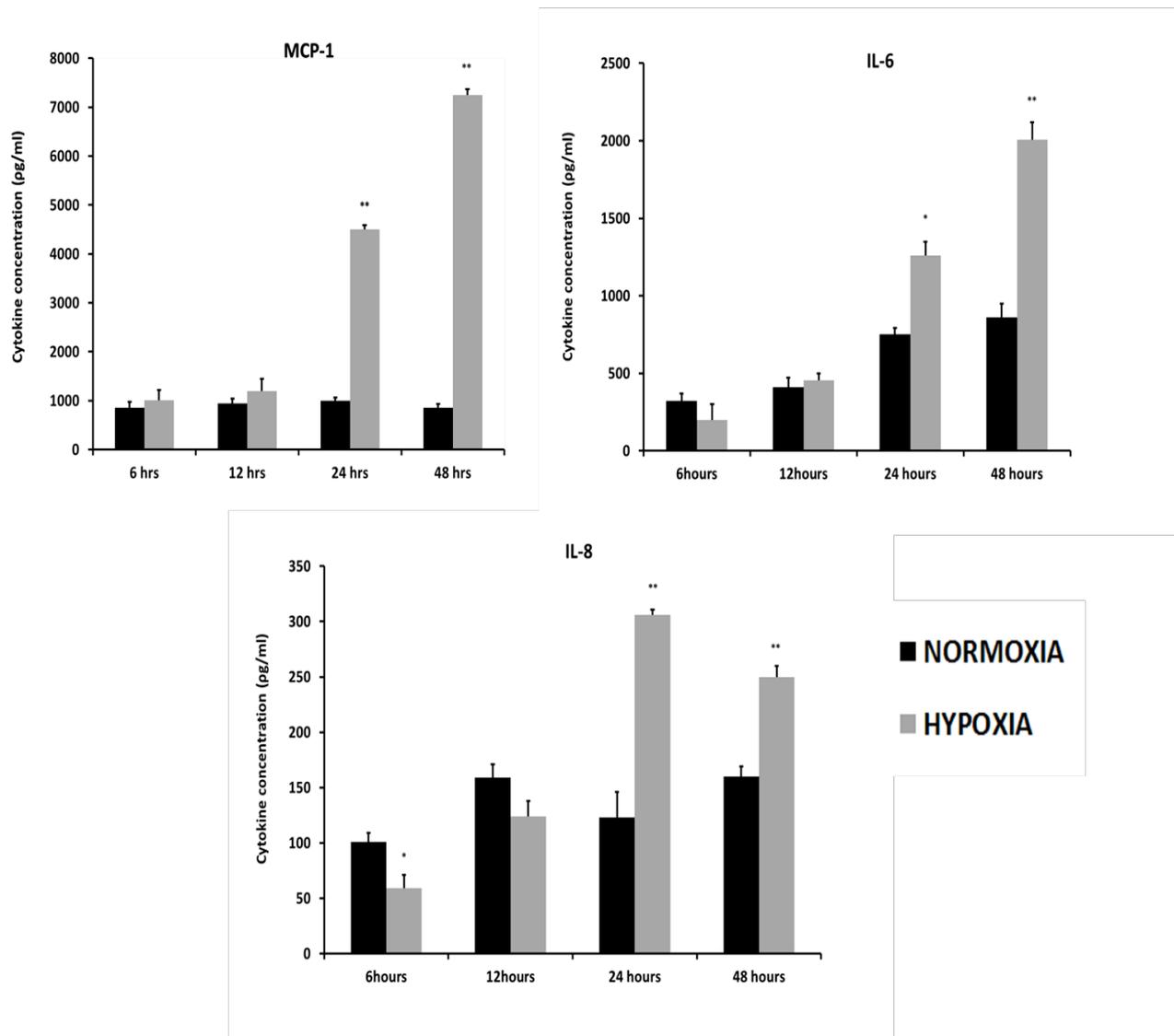
**Figure 4-3 Collagen I and III mRNA in hypoxic conditions**  
 (A) The levels of mRNA for Collagen type IIIA and (B) Collagen type IA were determined by real time PCR. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on five individual patient samples. \* $p < 0.05$ , \*\* $p < 0.01$  compared to normoxic



**Figure 4-4 Collagen I and III protein expression in hypoxic conditions**  
 (A) Collagen I and III concentrations (ng/ml) were assessed using ELISA. Data represents mean collagen production (ng/ml)  $\pm$  SD in 10 biopsies of torn supraspinatus, matched subscapularis and control tendon samples (B). Immunostaining for Collagen types I and III in normoxic and hypoxic conditions at 24 hours incubation. Cells were plated onto chamber slides and incubated in different oxygen conditions for 24 hours then fixed in methanol and stained with antibodies

## 4.5 ) Hypoxia promotes Cytokine production in tenocytes

Since our initial histological examination revealed a significant association between inflammatory infiltrate and hypoxic markers, we next explored the extent to which hypoxia could regulate the local cytokine milieu directly via modulating tenocyte behaviour. Hypoxic conditions rapidly induced significantly elevated production of IL-6, and of the chemokines IL-8 and MCP-1 commensurate with the notion that hypoxic tenocytes could initiate leukocyte recruitment (Figure 4-5). In contrast, we found no production over time of a range of other cytokines including IL-4, IL-5, IL-10, IL-12, IL-13, IL-15, TNF- $\alpha$ , IL-1 $\beta$  by tenocytes, suggesting that the prior detection of such entities in early tendinopathy likely reflects infiltrating leukocyte populations [137].

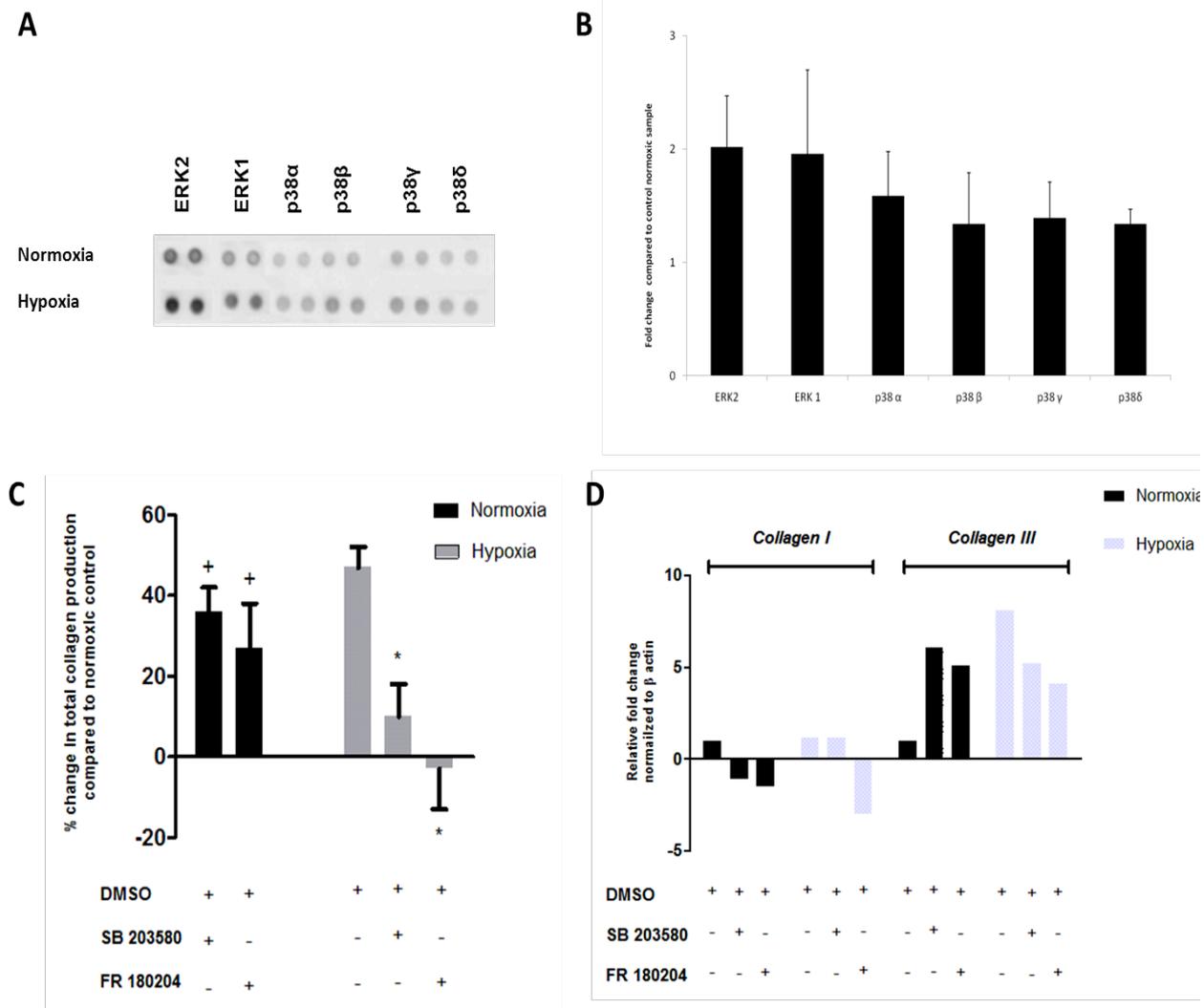


**Figure 4-5 Effects of hypoxia on cytokine production in cultured tenocytes.** Cultured tenocytes were incubated in a normoxic and hypoxic environment over a time course from 0-48 hours. Data shows levels of MCP-1, IL-8 and IL-6 in supernatants removed from culture at various time points analysed using Luminex. Data shown are as the mean  $\pm$  SD of triplicate samples and are representative of five individual experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared to normoxic samples.

## 4.6 ) Hypoxia, MAP Kinase expression and collagen synthesis

As expected, tenocytes cultured in a hypoxic environment exhibited a generalized increase in MAPK phosphorylation. In particular, we observed increased phosphorylation of ERK 1, ERK 2 and the  $\alpha$  &  $\beta$  isoforms of p38 (Figures 5 A&B). To explore the functional consequences of this, we employed ERK and p38 MAPK inhibitors in our tenocyte cultures. We observed partial but significant abrogation of the hypoxia-induced increase in total collagen production by tenocytes (24 hours DMSO  $592 \pm 32 \mu\text{g}$  collagen (mean  $\pm$  SD); p38 inhibitor  $461 \pm 35 \mu\text{g}$ ; ERK inhibitor  $390 \pm 38 \mu\text{g}$ ) (Figure 5C). In contrast, p38 or ERK inhibition in normoxic conditions resulted in an increase in total collagen production (24 hours DMSO  $402 \pm 33 \mu\text{g}$  collagen (mean  $\pm$  SD); p38 inhibitor  $541 \pm 35 \mu\text{g}$ ; ERK inhibitor  $509 \pm 55 \mu\text{g}$ ).

Collagen subtype synthesis was evaluated by RT-PCR. Both ERK and p38 inhibition abrogated the hypoxia-induced enhancement of collagen type III in tenocytes and significantly increased the relative expression of Collagen III in normoxic conditions (Figure 5D).

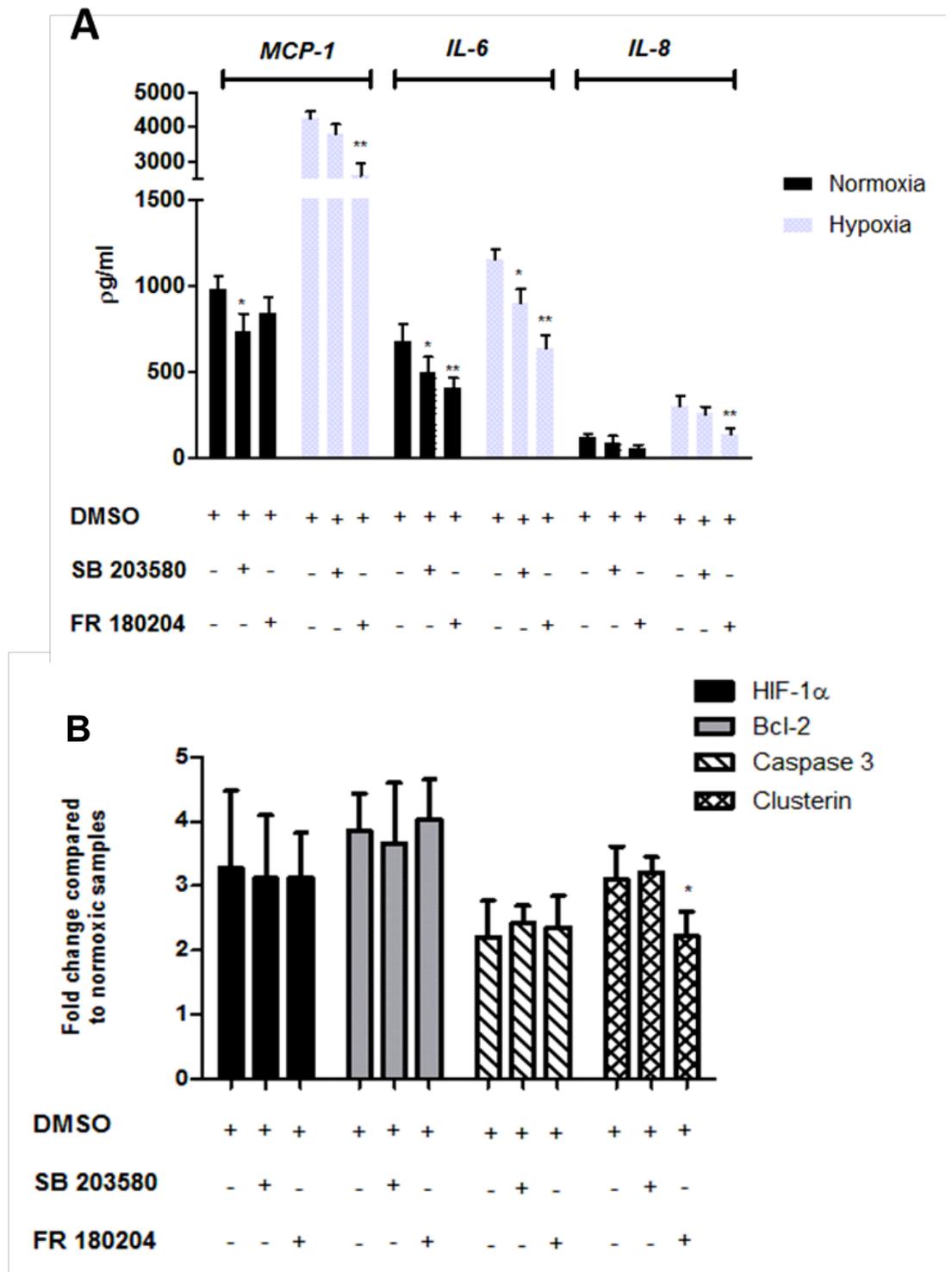


**Figure 4-6 Hypoxia induced phosphorylation of MAPK in cultured tenocytes and matrix response to p38 and ERK inhibition**

Whole-cell lysates were examined for the phosphorylation of ERK1, ERK2 and p38 isoforms at 1 hour after exposure to normoxic and hypoxic conditions (A). The fold change of MAPK's was determined by densitometry and normalised to the control sample on the array(B). Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue. Cells were preincubated for 24 h with specific inhibitors for p38 [SB 203580], or ERK (FR 180204), which also were included assay media. Total collagen production was assessed using Sircol assay(C). Data represents percentage change in total collagen production compared to normoxic control sample  $\pm$  SD in normoxic and hypoxic conditions over a 48 hour time course. \* $p < 0.05$ , \*\* $p < 0.01$  compared to normoxic samples. The levels of mRNA (D) for Collagen type IA and Collagen type IIIA were determined by real time PCR.

#### **4.7) ERK and p38 inhibitors reduce proinflammatory cytokine / chemokine production but do not modulate apoptosis in tenocytes**

Tenocyte production of IL-6, MCP-1 and IL-8 under normoxic and particularly hypoxic conditions was reduced by both p38 and ERK inhibition (Figure 6A). ERK appeared more important in this regard. Annexin V staining revealed a moderate reduction ( $p=0.06$ ) in the percentage of apoptotic cells in hypoxic conditions using either p38 (hypoxia control  $62 \pm 10\%$ , inhibitor  $55 \pm 10\%$ ) and ERK inhibitors (hypoxia control  $63 \pm 8\%$ , inhibitor  $52 \pm 12\%$ ). While ERK inhibition did induce a small but significant reduction in clusterin gene expression in hypoxic conditions neither p38 and ERK inhibition had significant effect on other key apoptotic genes in normoxic nor hypoxic environments suggesting that p38/ERK pathways do not significantly contribute to tenocyte apoptosis.



**Figure 4-7** The effects of ERK and p38 MAPK inhibition on cytokine production and apoptosis.

(A) Cells were preincubated for 24 h in a normoxic and hypoxic environment with specific inhibitors for p38 [SB 203580], or ERK (FR 180204), which also were included assay media. Data shows levels of MCP-1, IL-8 and IL-6 in supernatants removed from culture analysed using Luminex. Data shown are as the mean  $\pm$  SD of triplicate samples and are representative of five individual experiments.

(B) Whole-cell lysates were examined for the expression HIF 1 $\alpha$ , Caspase 3, Bcl-2 and Clusterin, at 24 hours after exposure to normoxic and hypoxic conditions with specific inhibitors for p38 [SB 203580], or ERK (FR 180204). Expression was determined by densitometry and normalised to the control sample on the array. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

## 4.8) Discussion

These experiments provide convincing evidence that hypoxia could operate as an initiator and thereafter regulator of early tendinopathy. Herein we demonstrate that hypoxia related proteins are present in early tendinopathy biopsies and thereafter in mechanistic studies demonstrate that hypoxia regulates inflammatory and apoptotic mediators in tendon cells associated with a significant shift in collagen matrix synthesis.

Approximately 95% of collagen in normal tendon is type I; type III is present in small amounts under physiological conditions [370]. Biopsies from normal and ruptured Achilles tendon demonstrate that ruptured tendons contain reduced quantities of type I collagen together with a significantly increased proportion of type III collagen [371] that in turn accounts for the decreased resistance of tendon to tensile forces and thereby subsequent rupture [372]. Hypoxic cell damage has long been considered a mechanism underpinning degenerative tendon disorders [4, 64]. Conventionally, stress responses such as oxidative damage are considered to operate through MAPK pathways [373]. These are up regulated by cyclical strain [79] and subsequently involved in apoptosis [78] in tendinopathy; however there remains limited investigation into signalling events involved in tendon biology. Our work suggests a functional role for ERK and p38 in the matrix and inflammatory response to hypoxia. Hypoxia not only increases the total collagen production but seems to 'switch' the production towards an increase in collagen III compared to type I suggesting a detrimental phenotypical change in the extracellular matrix. In particular ERK inhibition reduces the total collagen and collagen III production in hypoxia whilst has the opposite effect (increased collagen and collagen III) in normoxia suggesting that the ERK pathway is oxygen sensitive.

Experimental models provide good evidence for an early inflammatory response in tendinopathy [74]. A running rodent model of tendinopathy is associated with upregulation of key inflammatory modulators including the 5-lipoxygenase activating protein (FLAP) and cyclo-oxygenase [342] while rabbit and equine models show excessive mechanical load induces acute inflammatory cell infiltrates [93, 343]. Recently we have shown a distinct inflammatory infiltrate in early human tendinopathy [374] and increased levels of proinflammatory cytokine in torn supraspinatus samples [137]. Hypoxia has been shown to induce the expression of cytokines and proinflammatory molecules including PDGF, IL-6, IL-8

and PAF in several animal and human models [375-377]. Our data now demonstrate in human tenocytes that a hypoxic environment is capable of inducing key inflammatory cytokines that may ultimately disturb the balance between reparative and degenerative changes in the extracellular matrix. In particular increased levels of MCP-1, IL-6 and IL-8 which have previously been shown to effect matrix metalloproteinases production in fibroblasts [378] likely have downstream affects on tenocyte collagen matrix production. Inhibition of ERK in hypoxia significantly reduced cytokine production and thus may provide a mechanism for reducing proinflammatory. We and others have highlighted the key presence and therefore putative functional role of a large mast cell infiltrate in tendinopathy [66, 359, 374] and this combined with evidence from Gulliksson et al[379] demonstrating hypoxic induced IL-6 production in mast cells provides growing importance to the role of chemokines in the initiation and perpetuation of the inflammatory cascade in tendon biology (Figure 6C).

Dysregulated apoptosis has also been postulated as a primary cause of tendinopathies [107]. Several causal factors, including mechanical overuse [380], hypoxia [52] and oxidative stress [56] have been proposed. We have previously shown increased expression of pro-apoptotic genes in a rodent overuse models and in torn human supraspinatus tendons compared to controls [340]. Increased mechanical loading [77] and loss of homeostatic tension [380] increases tenocyte apoptosis in rodents and HIF-1 $\alpha$  is induced during cyclical strain of tenocytes [274]. Increased HIF-1 $\alpha$  has been shown in rotator cuff tears although since these represent by definition advanced disease it is difficult to evaluate its relative role in early disease [68]. Our study now reveals HIF 1 $\alpha$  expression in *early* tendinopathy biopsies. Clusterin [381] and Bcl-2 [382] have been functionally implicated in DNA repair, cell cycle regulation, while both have a prosurvival role during cell death [383]. Their abundant expression in early tendinopathy biopsies and over expression under hypoxic conditions at both the protein and mRNA level strongly supports the concept of hypoxia as a regulator of apoptosis. Moreover parallel changes were noted in collagen subtype synthesis in our studies. Thus the equilibrium between pro/anti apoptotic markers during hypoxic conditions in a tissue rescue/wound healing process may play a critical role in determining the functional outcome. The Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs) have been implicated in tenocyte apoptosis [79]and whilst a moderate increase was noted in JNK/SAPKs in our culture system this did not

prove significant. At a mechanistic level we could not identify a functional role for ERK and p38 in the apoptotic response to hypoxia which may suggest a mitochondrial regulatory pathway as that in synovial fibroblasts [384].

**On the basis of these results we propose hypoxia as a critical pathophysiological regulator in tendon healing (Figure 4-8) - better understanding of its pathological cascade should lead to the development of cell targeted treatment modalities for early supraspinatus tendinopathy.**

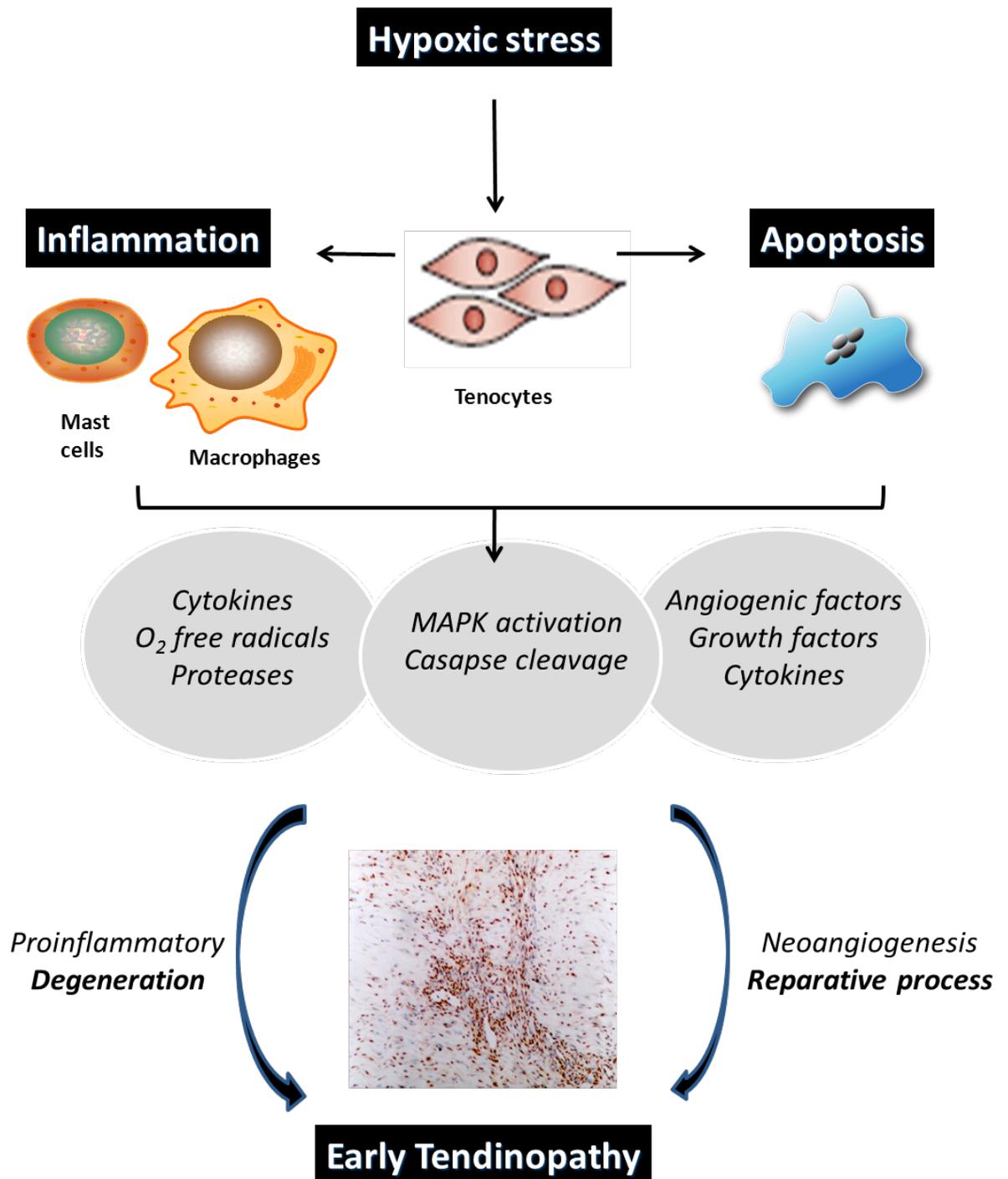


Figure 4-8 Schematic diagram illustrating the manner in which early tendinopathy may arise due to hypoxic injury.

An increase in hypoxic stress that a tendon cell experiences results in the release of various inflammatory mediators with associated MAPK activation that interact to drive the tendon matrix toward a degenerative or reparative process.

## **Chapter 5 Interleukin 17 in tendinopathy**

Content of this chapter has been presented as abstracts at the following meetings:

Orthopaedic Research Society Annual Meeting New Orleans 2010

***Shortlisted as NIRA Prize finalist***

American Shoulder and Elbow Surgeons Speciality Day San Diego 2011

## 5.1) Aims and Introduction

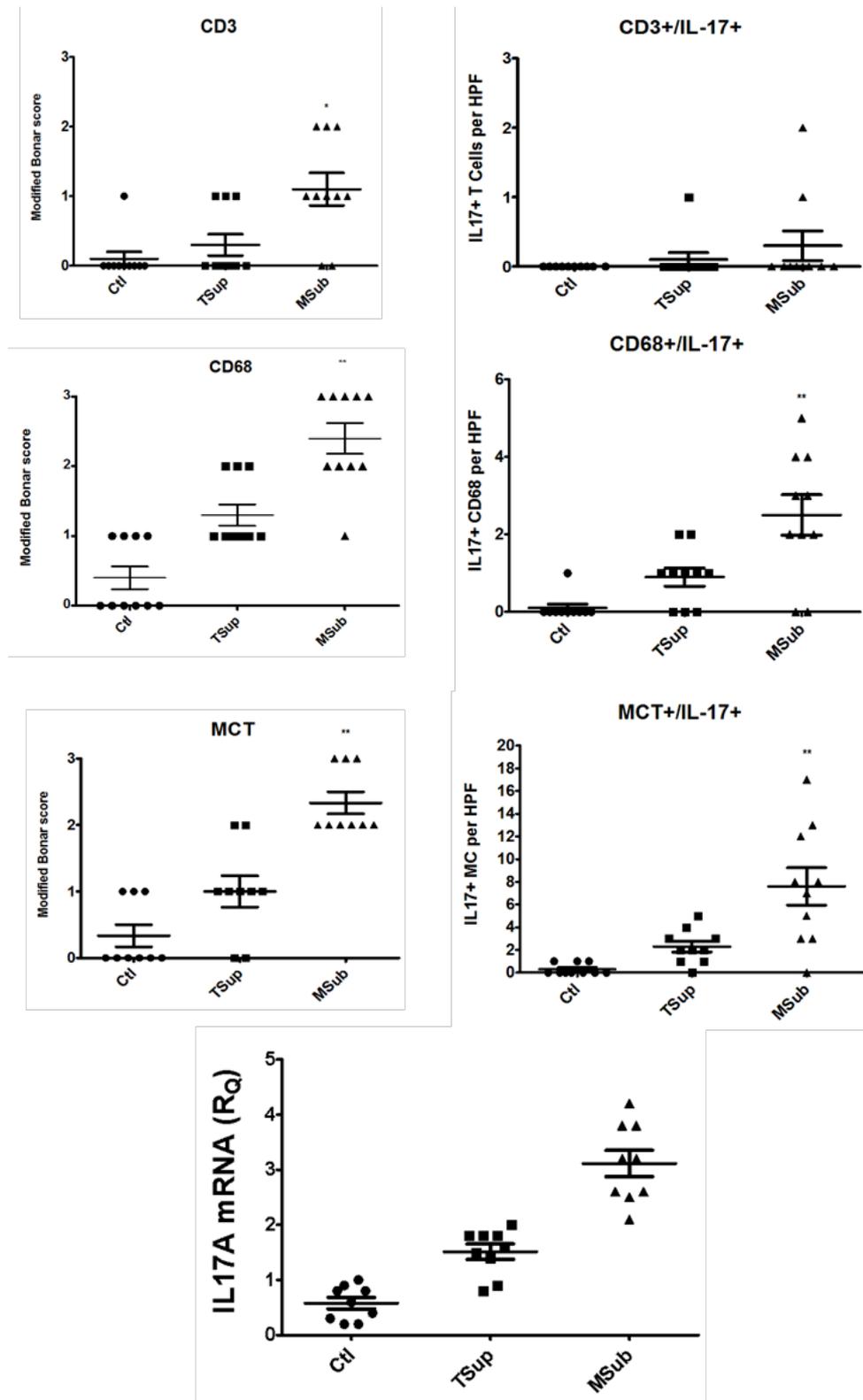
Cytokines are critical in the response of soft tissue to injury and wound healing and have been shown to be involved in the regulation of matrix turnover in tendinopathy[42]. Endogenous increased expression of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, VEGF and TGF $\beta$  has been demonstrated in tenocytes [111-114] while the mechanical properties of healing tendons in IL-6 -/- mice were inferior compared with normal controls. Cytokines also play a key role in oxidative stress-induced cellular apoptosis[385] which is mediated by the activation of a variety of caspases. IFN- $\gamma$  or TNF- $\alpha$  increased, whereas with TGF- $\beta$  or IL-10 decreased caspase 8 driven Fas mediated apoptosis in dermal fibroblasts[386] while IL-1 $\beta$  and TNF- $\alpha$  induce mitochondrial DNA damage, and mitochondrial transcription with a subsequent induction of apoptosis in human chondrocytes[387]. By this means an inflammation / apoptotic cascade of molecular events promotes tissue degeneration[348].

Interleukin-17 is a pro-inflammatory mediator increasingly implicated in a variety of immune and inflammatory disease states. IL-17A expression is reported in several cell types including Th17 cells[388],  $\gamma\delta$ T cells[389] and some non lymphocyte lineages[162]; its receptor is ubiquitously expressed on many cell types, including myeloid cells, epithelial cells and fibroblasts[390]. IL-17A exerts various biological activities that could promote tissue destruction and degeneration during inflammation. In particular, it induces the production of cytokines, including IL-1, IL-6, TNF- $\alpha$ , chemokines, inducible NO synthase, and matrix metalloproteinases (MMPs) by fibroblasts, macrophages, and endothelial cells[177]. Experimental models provide good evidence for an early inflammatory response in tendinopathy[78, 93, 342]. Recently we have shown a distinct inflammatory infiltrate in early tendinopathy[374] and increased levels of proinflammatory cytokines including TNF- $\alpha$ , IL-6, in torn supraspinatus samples[137]. Based on these observations and the plausible biological profile exhibited by IL-17A, we hypothesized that IL-17 may play a role in tendinopathy.

**The purpose of this section of my thesis was to formally assess the expression of IL-17 within the human model of tendinopathy previously described and thereafter explore the mechanistic outcome of IL-17 on inflammation, apoptosis and matrix production in tenocytes in vitro.**

## **5.2) Early tendinopathy shows increased IL-17A expression with localization particularly to mast cells**

I first examined mRNA expression in my tissue cohort. Subscapularis tendon samples exhibited significantly ( $p < 0.01$ ) greater IL-17A mRNA levels than torn supraspinatus or control tendon. Additionally immunohistochemical staining for IL-17A, macrophages, mast cells and T cells exhibited significantly greater levels of expression for each marker in subscapularis compared to both matched torn supraspinatus samples and control tissue (Figure 1). To formally identify these IL-17A+ cells in human tendinopathy, we performed co localisation studies and calculated the proportion of IL-17A+ cells contained in each cellular subset. Although occasional CD3+IL-17A+ cells were identified, the majority of IL-17A+ cells were CD3-negative (<1%). Only 1% of IL-17A-expressing cells were found to express CD3. CD4 cells were extremely limited in number throughout the samples and we identified no co-expression of IL-17. Up to 20% of IL-17A+ cells were CD68+. The majority of IL-17A+ cells double-stained strongly with MCT (50–100%) of IL-17A-expressing cells per tissue area across patients, clearly demonstrating that mast cells are key producers of IL-17A in human tendinopathy (Figure 5-3). The validity of the antibody was assured by staining in rheumatoid arthritis samples which were previously noted to be positive in the laboratory.



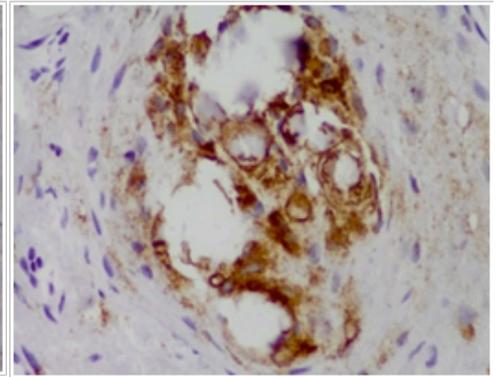
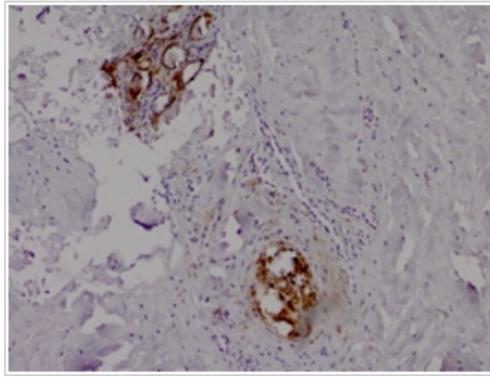
**Figure 5-1**Quantification of IL-17A in tendon biopsies

Scoring for T cells (CD3), macrophages (CD68) and mast cells (MCT) in tendon biopsies using Modified Bonar score. Double positive cells per high power field (mean of 5 HPF's) showed the greatest concentration to be IL-17/MCT cells. Extremely low numbers of IL-17 positive T cells were detected. The levels of IL-17 mRNA was determined by real time PCR. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on ten samples of control tendon, torn supraspinatus and matched subscapularis tendon biopsies.

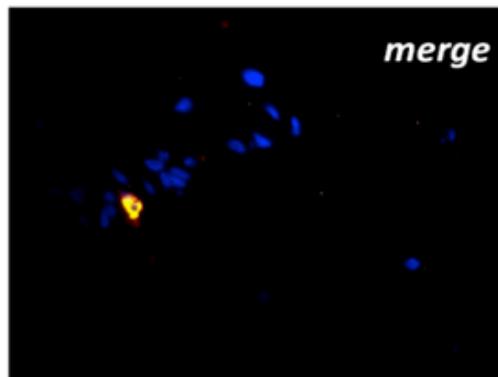
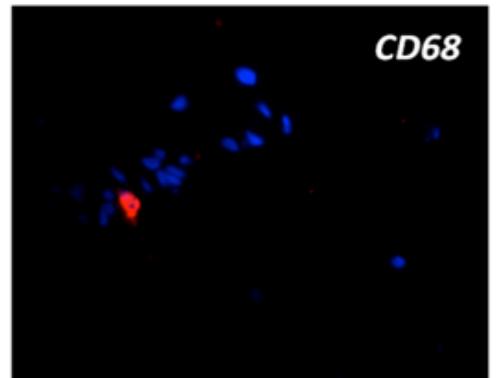
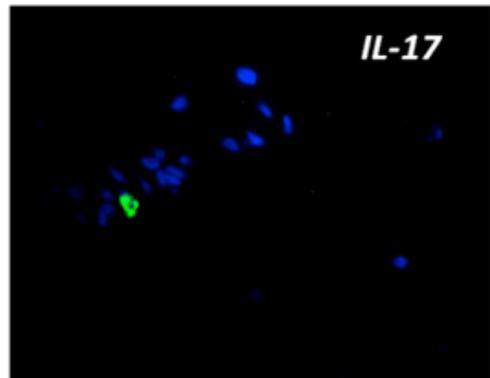
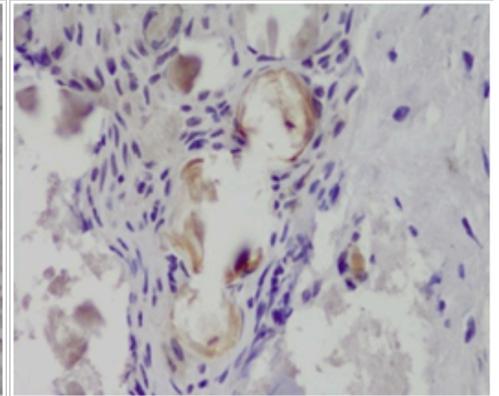
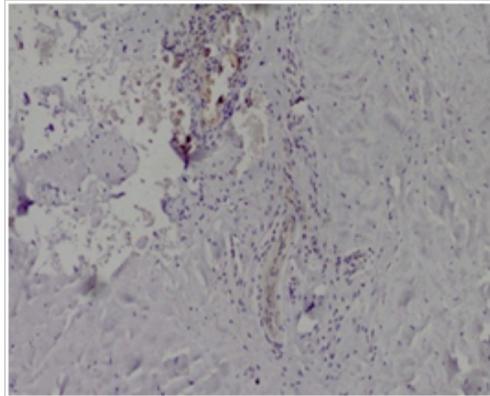
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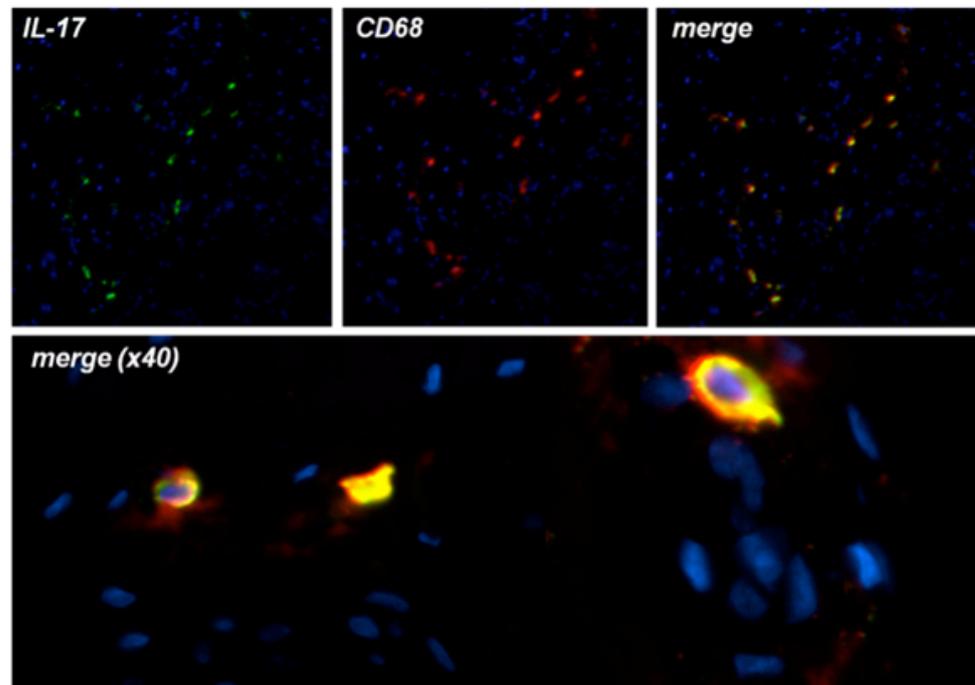
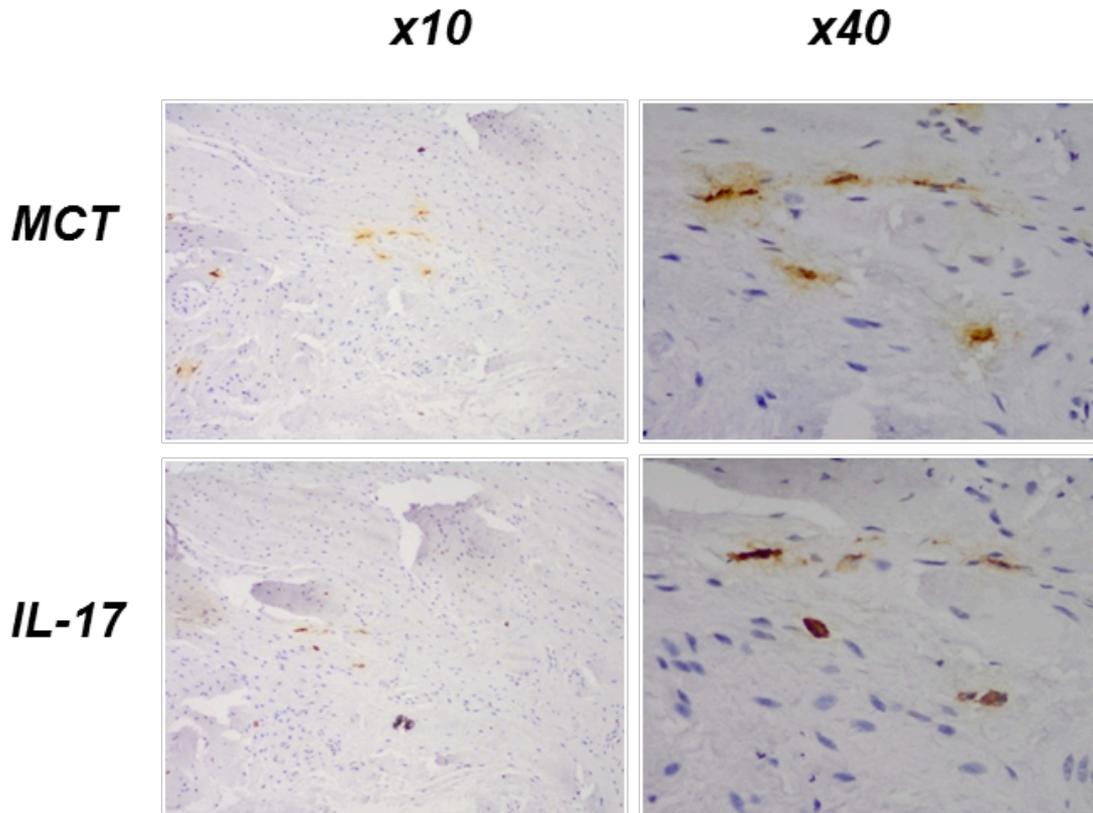
**CD68**



**IL-17**



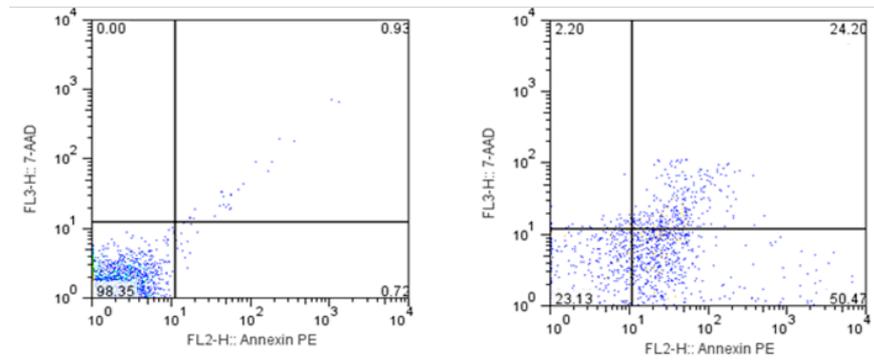
**Figure 5-2 Immunohistochemistry and double immunofluorescence for IL-17A and CD68**  
Large areas of CD68 positive areas with single light microscopy suggesting double positive cells. Confirmed on immunofluorescence. Sections were counterstained with DAPI (blue). Bottom row show merged staining. Images are shown at 40x magnification.



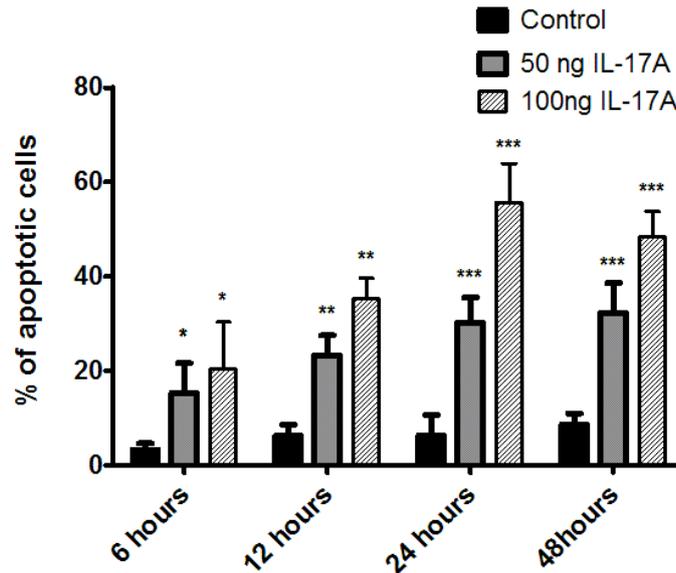
**Figure 5-3 Immunohistochemistry and double immunofluorescence for IL-17A and Mast cell tryptase**  
 Large areas of MCT positive areas with single light microscopy suggesting double positive cells. Confirmed on immunofluorescence. Sections were counterstained with DAPI (blue). Bottom row show merged staining. Images are shown at 60x magnification.

### **5.3) IL-17A induces apoptosis in tenocytes in vitro**

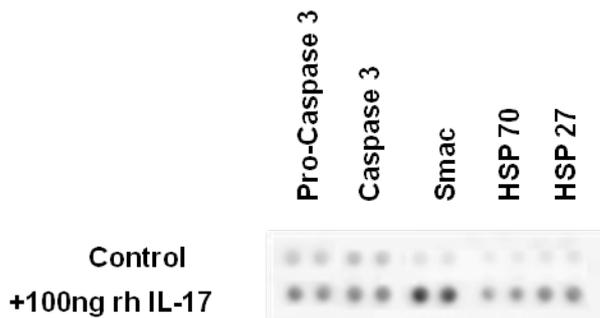
We next wished to examine the functional implications of IL-17A expression in early tendinopathy. Addition of 50ng or 100ng of rhIL-17A to human tenocyte cultures significantly increased the number of cells undergoing apoptosis assessed by Annexin V staining at 24 hours (control mean  $6\pm 2\%$ , IL-17A mean  $56\% \pm 8\%$ ) and 48 hours (control  $9\pm 4\%$ , IL-17  $48\% \pm 5\%$ ) (Figure 5-4A&B). Limited proteomic profiling of these cells after 24 hours, revealed relative over expression of pro and cleaved caspase 3, with the most profound effects mediated upon expression of heat shock proteins (HSP) 27, 70 and second mitochondria-derived activator of caspases (Smac) ( $p < 0.01$ ) (Figure 5-4C). These protein expression changes were confirmed at the mRNA level by quantitative PCR that confirmed elevated expression of caspase 3, HSP 27, HSP 70 and Smac ( $p < 0.01$ ) in tenocytes (Figure 5-4D).



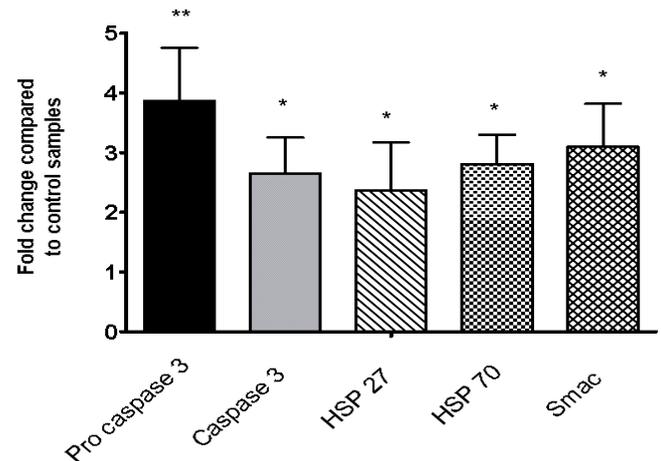
B



C



D

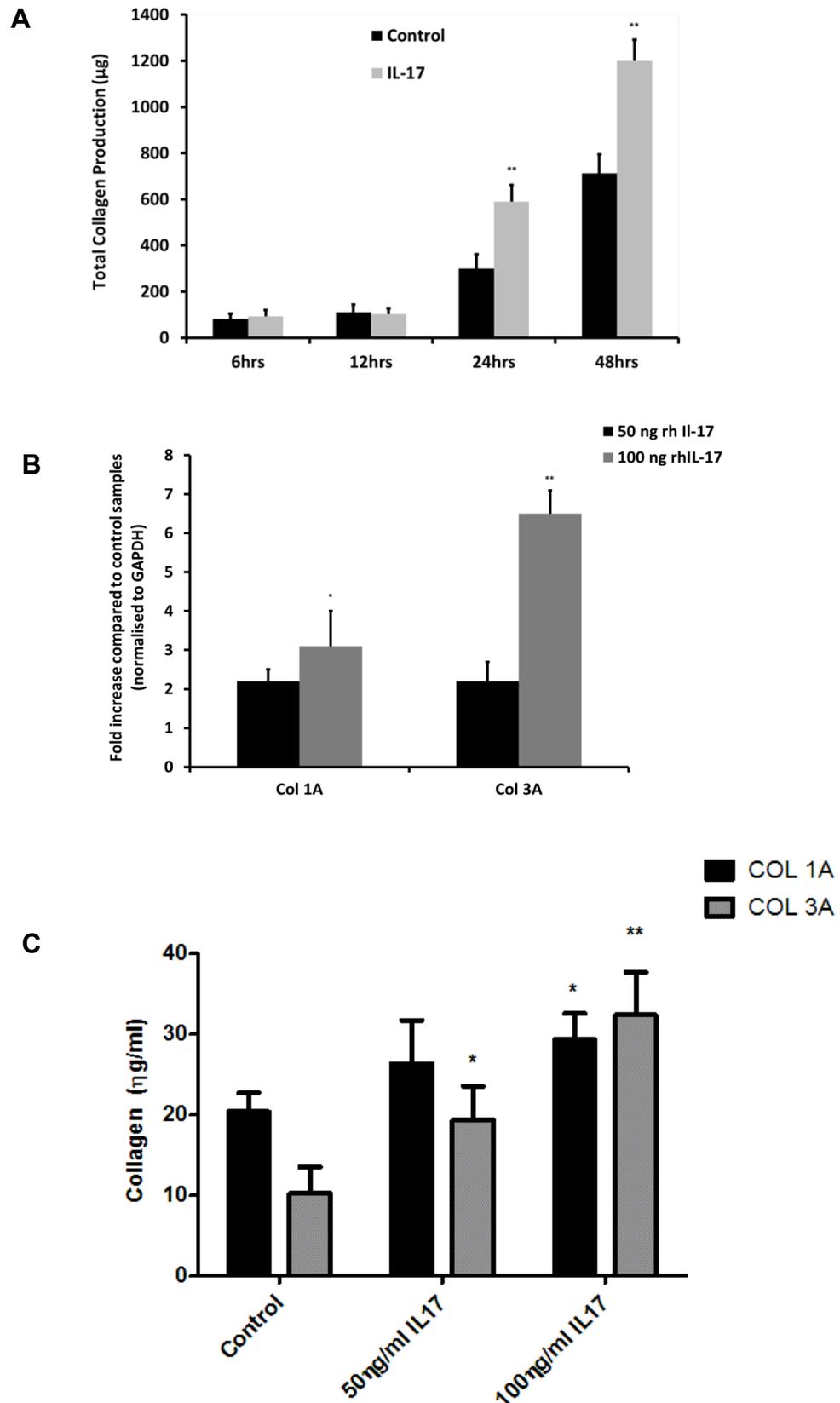


**Figure 5-4 Apoptosis and IL-17A**

(A) Apoptosis was evaluated after 24h control conditions IL-17 stimulation. Flow cytometry profile represents Annexin V-FITC staining in x axis and 7-AAD in y axis. (B) % of apoptotic cells at various time points and concentrations of rh IL-17. (C) Whole cell lysates from control and IL-17 conditions were harvested and apoptotic markers evaluated using an apoptotic proteome profiler (D). The levels of mRNA Caspase 3 and HSPs were determined by real time PCR. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on five discrete patient samples.

#### **5.4) IL-17A alters tenocyte collagen matrix regulation**

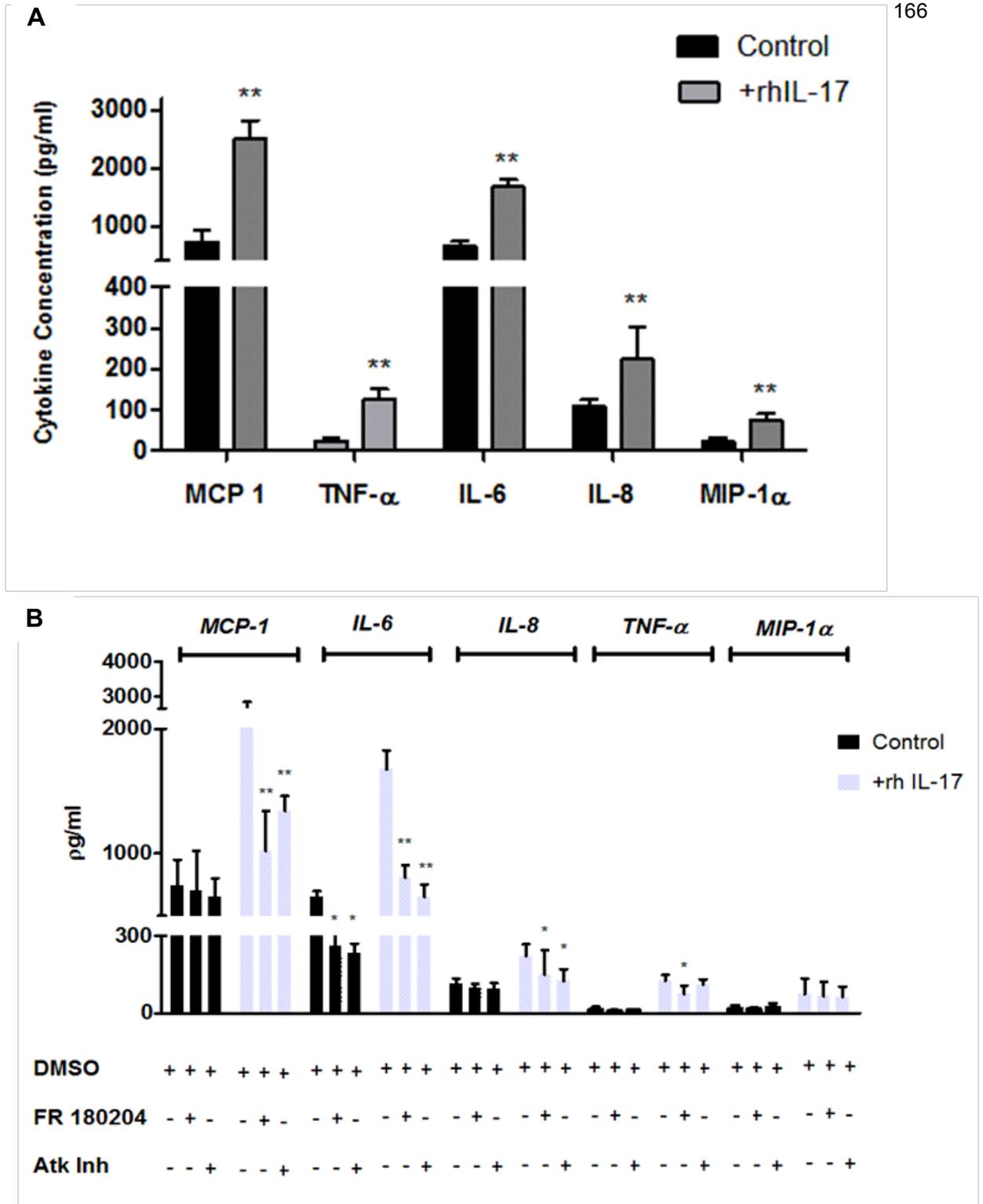
Since type III collagen expression is a dominant feature of tendinopathy, we next considered whether IL-17A might alter differential collagen synthesis by tenocytes. rhIL-17A significantly elevated total collagen production (Figure 4A) compared to untreated cells at 24 hours (mean  $300\mu\text{g} \pm 62$  control;  $590\mu\text{g} \pm 71$  IL-17A) and 48 hours ( $712\mu\text{g} \pm 82$  control;  $1200\mu\text{g} \pm 92$  IL-17). Collagen 1A mRNA levels were unchanged at 12, 24 and 48 hours whereas in contrast, Collagen 3A mRNA was significantly up-regulated at 24 & 48 hours by rhIL-17A compared to controls. Immunocytochemistry revealed no change in expression of type 1 collagen in rhIL-17 treated samples at all time points compared to controls whereas collagen 3 staining demonstrated a significant increase 48 hours post incubation with rhIL-17A (Figure 4C). This was confirmed by ELISA which revealed a significant increase in collagen III (mean  $\eta\text{g} \pm$  control;  $\eta\text{g} \pm$  IL-17A) production at 24 hours and 48 hours post treatment with rhIL-17A.



**Figure 5-5 The effect of IL-17A on collagen matrix production**  
**(A)**Total collagen production assessed using Sircol assay(A). Data represents mean collagen production( $\mu\text{g}$ )  $\pm$  SD in control and IL-17 treated cultures over a 48 hour time course. \* $p < 0.05$ , \*\* $p < 0.01$  compared to control samples. **(B)** The levels of mRNA for Collagen type IA and IIIA were determined by real time PCR. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on five individual patient samples. \* $p < 0.05$ , \*\* $p < 0.01$  compared to control samples.**(C)** Collagen I and III (C) Collagen I and III protein levels as determined by ELISA. Data shown are mean  $\pm$  SD of five individual patient samples.

## **5.5) IL-17A promotes cytokine production in tenocytes**

We next explored the extent to which IL-17A could regulate the local cytokine milieu directly via modulating tenocyte behaviour. Incubation with rhIL-17A resulted in significantly elevated production of TNF- $\alpha$ , MIP1 $\alpha$ , IL-6, IL-8 and MCP-1 at 24 and 48 hours post incubation by tenocytes (Figure 5-6). In contrast we found no production over time of a range of other cytokines including IL-4, IL-5, IL-10, IL-12, IL-13, and IL-15, consistent with our expectations of the known cellular sources of such cytokine production.



**Figure 5-6 IL-17 induced cytokine production in cultured tenocytes**

(A) Cultured tenocytes were incubated in with recombinant IL-17 over 24 hours. Data shows levels of MCP-1, TNF-α, IL-8, IL-6 and MIP-1α in supernatants removed from culture at 24 hrs analysed using Luminex.

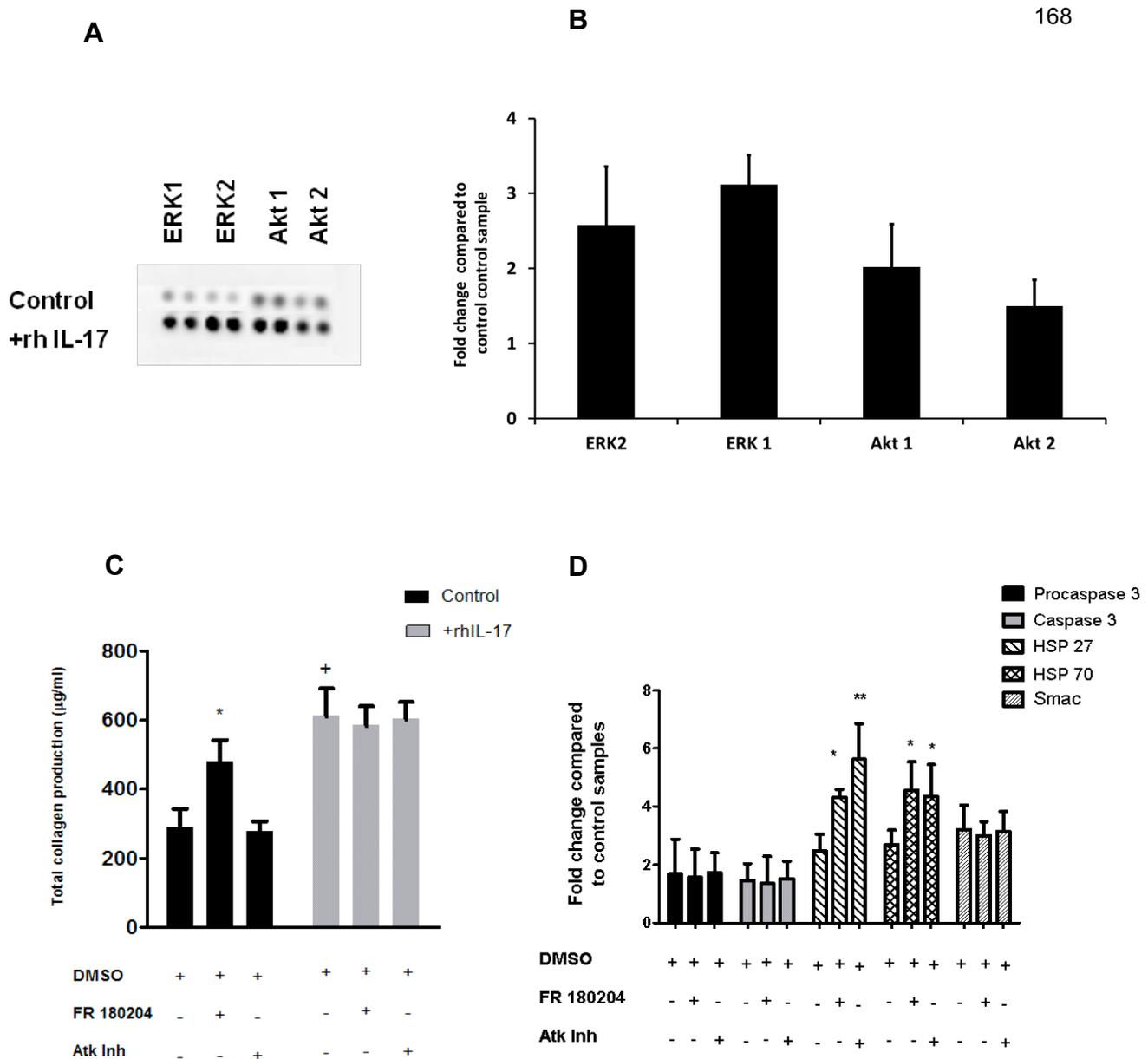
(B) Cells were preincubated for 24 h with 100ng recombinant IL-17 and specific inhibitors for ERK (FR 180204) and Atk, which also were included assay media.

Data shown are as the mean ± SD of triplicate samples and are representative of five individual experiments. \*p<0.05, \*\*p<0.01 compared to normoxic samples

## **5.6) MAP Kinases mediate some of the effects of IL-17A in tenocytes.**

Tenocytes cultured with rhIL-17A exhibited a generalized increase in MAPK phosphorylation. In particular, we observed increased phosphorylation of ERK 1, ERK 2 and Akt 1 & 2 (Figure 5-7A). To explore the functional consequences of this, we employed ERK and Akt inhibitors in our tenocyte cultures using doses previously shown to offer optimal inhibition at minimal off target effect. We observed significant reduction in production of MCP-1, IL-6, and IL-8 with both ERK and ATK inhibition, while TNF- $\alpha$  production was reduced by ERK inhibition and not Atk inhibition and no change was noted in MIP-1 $\alpha$  production after addition of either inhibitor (Figure 5-6 B).

To assess if MAP kinases were involved in an IL-17A induced apoptotic mechanism tenocytes were treated with 100ng of IL-17A with and without the aforementioned MAPK inhibitors. ERK (7% mean reduction in apoptosis compared to IL-17 treated cells alone) and Atk (9% mean reduction compared to IL-17 treated cells alone) inhibition caused a generalised decrease in tenocyte apoptosis which approached significance ( $p=0.06$ ). Interestingly both ERK and Atk inhibition resulted in a significant increase in HSP 27 and 70 gene expression with no associated changes in caspase 3, or Smac (Figure 5 D) suggesting that ERK/Atk pathways may be at least involved in modulating part of the apoptotic response while having little ultimate phenotypical effect on ultimate tenocyte apoptosis.



**Figure 5-7 IL-17 induced phosphorylation of MAPK in cultured tenocytes and matrix response to ERK and Akt inhibition**

Whole-cell lysates were examined for the expression of ERK1, ERK2 and Akt 1 & 2 at 24 hours after exposure to 100ng rh IL-17 (A). The fold change of MAPK's was determined by densitometry and normalised to the control sample on the array (B). Data are shown as the mean fold change ± SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

Cells were preincubated for 24 h with specific inhibitors for ERK (FR 180204), or Akt which also were included assay media. Total collagen production was assessed using Sircol assay(C). Data represents percentage change in total collagen production compared to normoxic control sample ± SD in normoxic and hypoxic conditions over a 48 hour time course. \*p<0.05, \*\*p<0.01 compared to normoxic samples. The levels of apoptotic proteins following incubation with ERK and Akt inhibitors (D) was determined by densitometry and normalised to the control sample on the array. Data are shown as the mean fold change ± SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

## 5.7) Discussion and Conclusion

This section provides evidence that IL-17 could operate as a key cytokine modulator of early tendinopathy. The experiments herein demonstrate that IL-17 is present in early tendinopathy biopsies and thereafter in mechanistic studies demonstrate that IL-17 regulates inflammatory and apoptotic pathways in tendon cells associated with a significant shift in collagen matrix synthesis.

These data suggest in human tenocytes that IL-17 is capable of inducing key inflammatory cytokines that may ultimately disturb the balance between reparative and degenerative changes in the extracellular matrix. In particular increased levels of IL-6 and IL-8 which have previously been shown to modulate matrix metalloproteinases production in fibroblasts [378] likely have downstream effects on tenocyte collagen matrix production. We and others have highlighted the potential role of the large mast cell infiltrate in tendinopathy [66, 359, 374]. This combined with recent evidence demonstrating that significant proportion of IL-17 producing cells in RA synovium [391, 392], psoriatic skin lesions [34] and psoriatic spondyloarthritis [393] are in fact mast cells provides growing emphasis on the role of the mast cell / cytokine / chemokine axis in the initiation and perpetuation of the inflammatory cascade in tendon biology. Indeed these results add growing credence to the idea that innate immune cells are an early source of IL-17 in response to stress and injury [162]. Strategies to interfere with mast cell activation/degranulation and the ability of ERK/Atk inhibitors to reduce IL-17 associated cytokine production may provide novel targets for human tendinopathy.

We have previously shown increased expression of pro-apoptotic genes in a rodent overuse models and in torn human supraspinatus tendons compared to controls [340]. These experiments now reveal IL-17 expression in early tendinopathy biopsies. The addition of IL-17 to tenocyte cultures causes over expression of caspases at both the protein and mRNA level strongly supporting the concept of IL-17 as a regulator of apoptosis. This work also shows the most significant change in small molecules regulating the mitochondrial pathway of apoptosis. HSP 27 indirectly interferes with cell death due to its ability to modulate intracellular glutathione, a parameter that is also regulated by exercise while HSP 70 interacts with Apaf-1 thereby preventing its interaction with the caspases preventing apoptosis [394, 395]. Smac binds to inhibitor of apoptosis proteins (IAPs) and deactivates them, preventing the IAPs from suppresses caspase

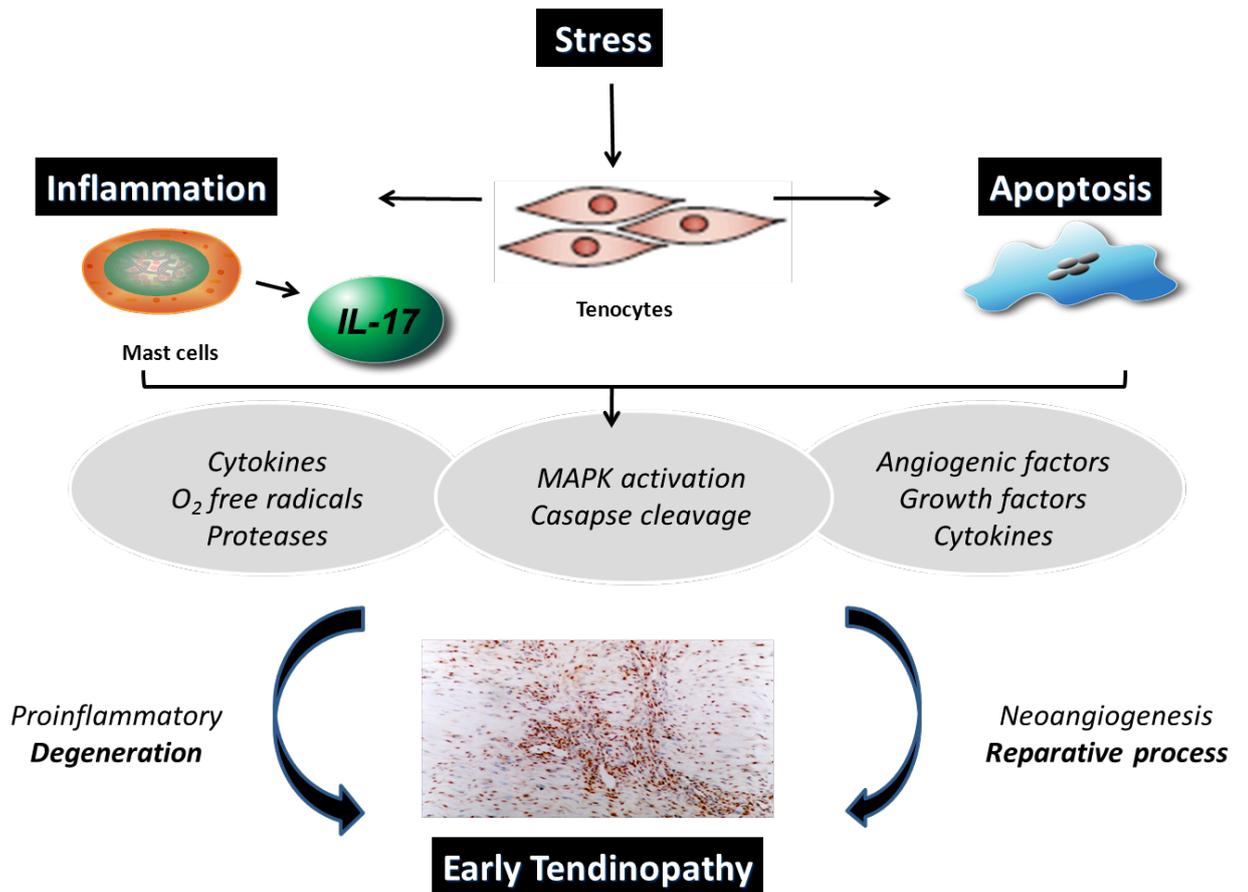
activity [396]. We have previously suggested heat shock proteins as modulators of apoptosis in early tendinopathy [340] and our mechanistic studies now emphasize the concept of that these small molecular chaperones as potential apoptotic regulators in tendinopathy.

My experiments reveal that IL-17 not only increases the total collagen production but seems to 'switch' the production towards an increase in synthesis of collagen type III compared to collagen type I suggesting that IL-17 may cause detrimental phenotypical changes in the extracellular matrix – at least in terms of force conduction and functional strength in practice. Inhibition of ERK/Atk signalling had no effect on IL-17 induced matrix production suggesting an alternative pathway must operate. Further investigation is required to understand the signalling biology of IL-17 on collagen synthesis and thus fully appreciate any translation targets.

Many questions remain to be explored. In particular the ability of mast cells to actively produce IL-17 in vitro. This has recently been demonstrated in mast cells cultured from skin biopsies and treated with IL-1 $\beta$  and IL-23 [397]. We are currently addressing this in our own experiments. We have isolated CD133+ cells from human buffy coats and are stimulating them toward a mast cell lineage. Once confirmed to be mast cells with FACS staining for Fc $\epsilon$ RI I plan to undertake experiments to reproduce mast cell IL-17 production in vitro. Secondly the literature still regards the main source of IL-17 to be derived from T cells whilst my results suggest a greater role for the innate mast cell and macrophage. Other groups have shown similar staining patterns to those presented above using the same goat polyclonal antibody however this does increase the possibility of cross reactivity to other epitopes. I am currently undertaking further IL-17 immunostaining using a mouse monoclonal antibody to confirm my findings. The fact that tendon biopsies contain relatively few T cell infiltrates does however provide a useful ex vivo tissue to safely interpret IL-17 innate producing cells. A final question is the interaction between tenocytes and mast cells both in vitro and in vivo. The former is to be tested in vitro using transwell cell contact experiments where various concentrations of mast cells will be incubated with tenocytes and both matrix and inflammatory changes noted. Our group has previous experience with mast cell-deficient Kit(W)/Kit(W-v) mice and I intend to run the patellar tendon injury model in these mice to ascertain an in vivo changes. This should provide

novel insight into how targeting of mast cells may influence the outcome of tendon injury/tendinopathy.

**On the basis of these results we propose IL-17 as a pathophysiological regulator in tendon healing (Figure 5-8) - better understanding of its pathological cascade may lead to the development of cell targeted treatment modalities for early supraspinatus tendinopathy.**



**Figure 5-8** Schematic diagram illustrating the manner in which early tendinopathy may arise due to IL-17 induction.

An increase stress that a tendon cell experiences results in the release of various inflammatory mediators such as IL-17 that interact to drive the tendon matrix toward a degenerative or reparative process.

## **Chapter 6 Interleukin 33 and related pathways in the pathogenesis of tendinopathy**

## Introduction

My foregoing experiments clearly show an important role for inflammatory changes in the early tendinopathy lesion. This may be regulated in part and may in turn regulate apoptosis operating at least partially through hypoxia dependent pathways. In other tissue systems an important role has been demonstrated for alarmins in such circumstances. I therefore investigated a potential role for a novel IL-1 superfamily member, IL-33 that is a postulated alarmin in the context of a variety of human pathologies.

Interleukin 33, similar to IL-1 $\beta$  and IL-18, is a member of the IL-1 family that has a major role in innate immune responses driven through its receptor ST2. IL-33 is expressed in endothelial cells and fibroblasts and is found associated with chromatin in the nucleus [189]. IL-33 is released by these cells following necrotic cell death, hence this cytokine has been referred to as an 'alarmin'; proteins that are rapidly released from cells in response to infection or tissue damage, alarming the immune system by promoting activation of innate and adaptive immunity [233]. MicroRNA has recently been discovered to be a new class of post transcriptional regulators of gene expression. The miRNA mediate this regulation by binding to partially complementary sites in the 3'UTR of target messenger RNA. The formation of MiRNA-mRNA complexes leads to mRNA degradation or translational repression. Recent evidence has emerged of a novel role of the miRNA 29 family in the post transcriptional regulator of collagen synthesis in several human pathologies [324, 398-400] which is extremely pertinent to collagen matrix changes associated with tendon disorders.

Herein, based on the key role of fibroblast-derived IL-33 that is emerging in inflammatory and fibrotic disorders and previous investigations showing increased mast cells in torn rotator cuff tendons [66] I investigated the role of the IL-33/ST2 signalling pathway on tendon pathology in animal and human models of tendinopathy. In addition due to an emerging role for miRNA in post transcriptional collagen matrix regulation I show that miRNA 29a is a key regulator of IL-33 induced collagen matrix changes through its direct targeting of soluble ST2. This data collectively suggests that IL-33 may function as an endogenous DAMP in tendon disease and that miRNA 29a is critical regulator of collagen matrix changes and thus may be a novel translational target for tendon disorders.

## 6.2) IL-33 and ST2 is expressed in human tendinopathy

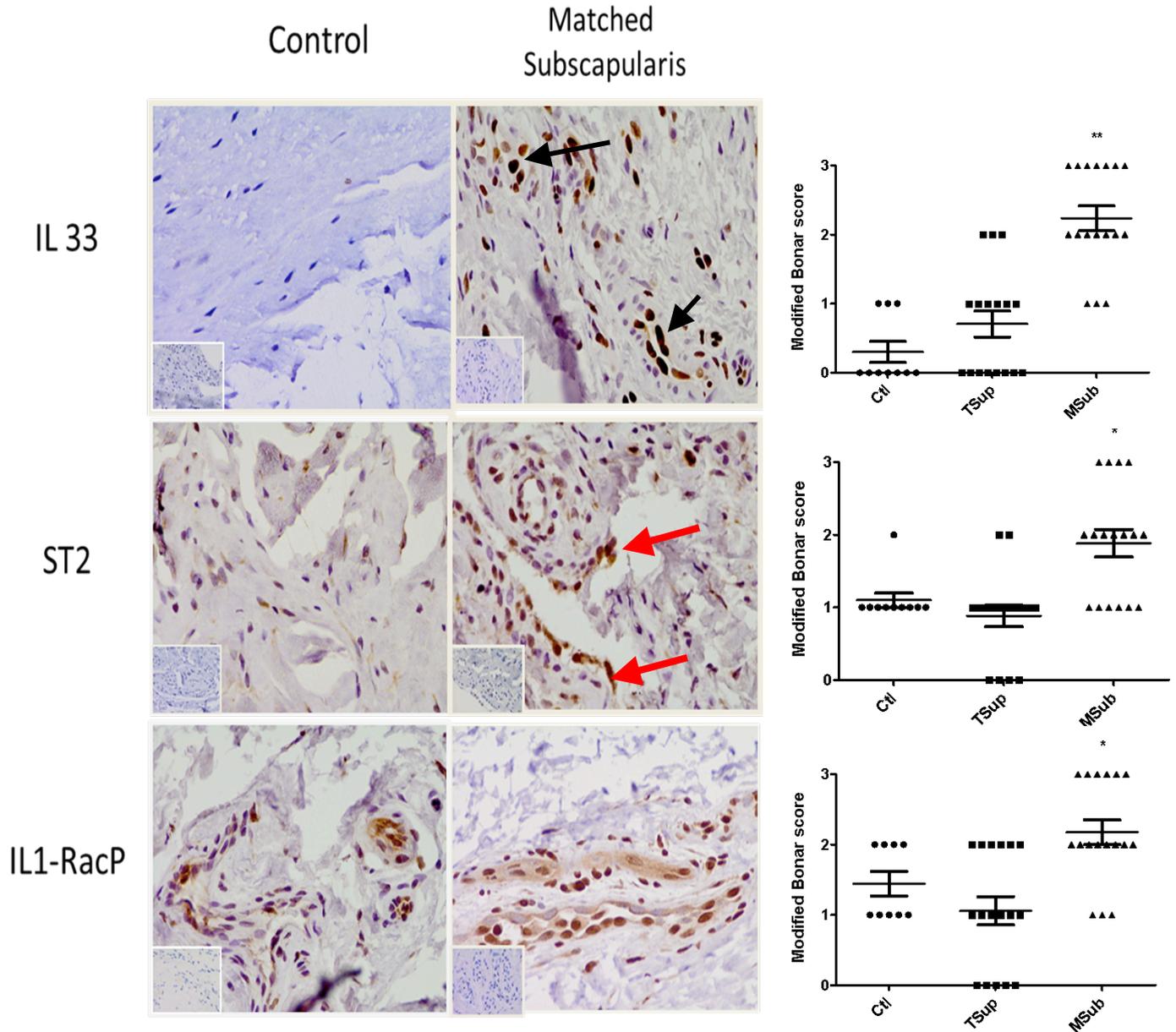
### 6.2.1) Expression of IL-33, ST2 and IL-1RAcP in tendon biopsies

IL-33 has been reported to be expressed in cells of body barriers but also in associated inflammatory tissues for example tonsils. The aim of the following experiments was to investigate IL-33 expression in human tendinopathy. To evaluate IL-33 expression, IHC staining was performed on tonsil tissue serving as a positive control. Consistent with previous reports, IL-33 was detected in the nuclei of endothelial cells. By topography and their morphology, these cells could also be fibroblast reticular cells described by Moussion [401]. After establishing the methodology for IHC of IL-33, tendon from healthy individuals, torn supraspinatus and matched subscapularis was investigated. Strong nuclear staining was detected in endothelial cells (black arrows), but also weak staining of IL-33 was noted in the cytoplasmic compartment (red arrows) (Figure 6-1).

Next, the expression of the IL-33 receptor, ST2 was determined in tendon biopsies. Positive cells were detected in the endothelium (black arrows) but also on numerous fibroblast like tenocytes cells (red arrows). The cellular staining pattern was associated with membrane and cytosolic localisation, but unexpectedly, was also partly nuclear. Controls demonstrated no non-specific binding of the isotype antibody. To complete the staining of the known molecular components of IL-33 and its signalling complex, we next evaluated IL-1RAcP expression in tendon. In a similar pattern to that observed for ST2 staining, positive cells were mainly endothelial and fibroblast like in terms of phenotype. However, in marked contrast to IL-33 and ST2 which were found in lower levels in control tissue, but commensurate with its reported homeostatic role and global distribution, IL-1RAcP was noted to be expressed in high levels in normal healthy tendon.

Together these data suggest a predominate staining pattern of IL-33/ST2/IL-1RAcP in subscapularis tendon. This finding is centrally important and it strongly suggests that IL-33/ST2 interaction is important during the early tendinopathy stages which many studies have failed to explore in human samples. In particular we have previously shown this tissue to be representative of early 'stressed' tendinopathy [340] and promotes the concept of IL-33 as a mechanically response cytokine particular in the mechano-sensitive tenocyte.

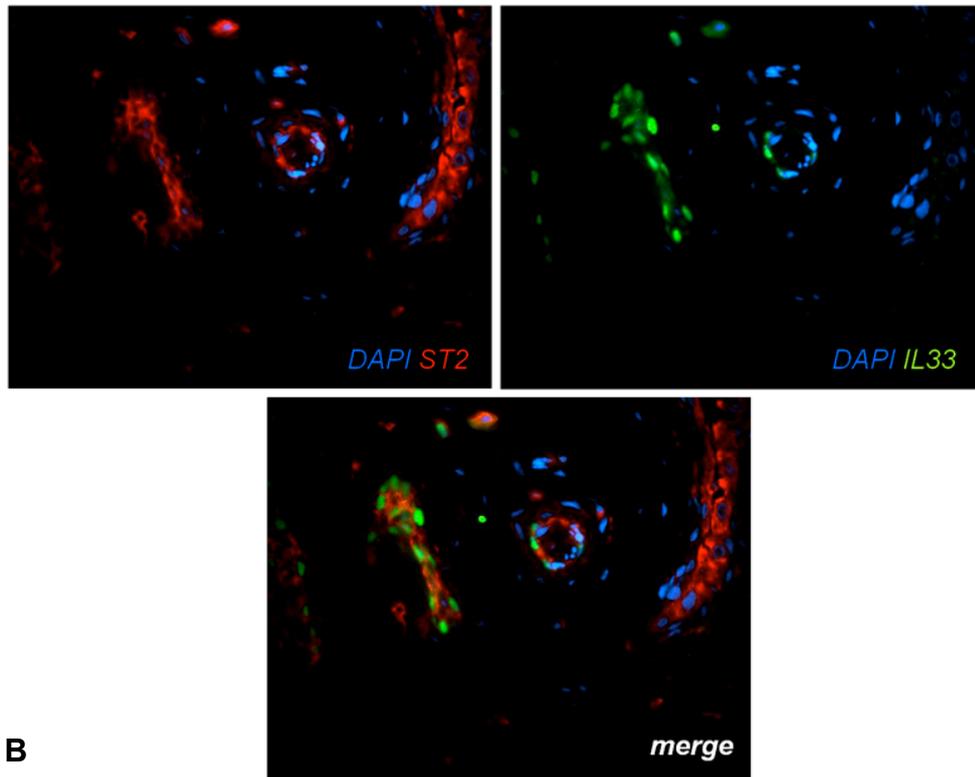
Thus further investigation of the IL-33/ST2 axis was performed due to its proposed role as a tissue alarmin, which could prove important in early tendinopathy.



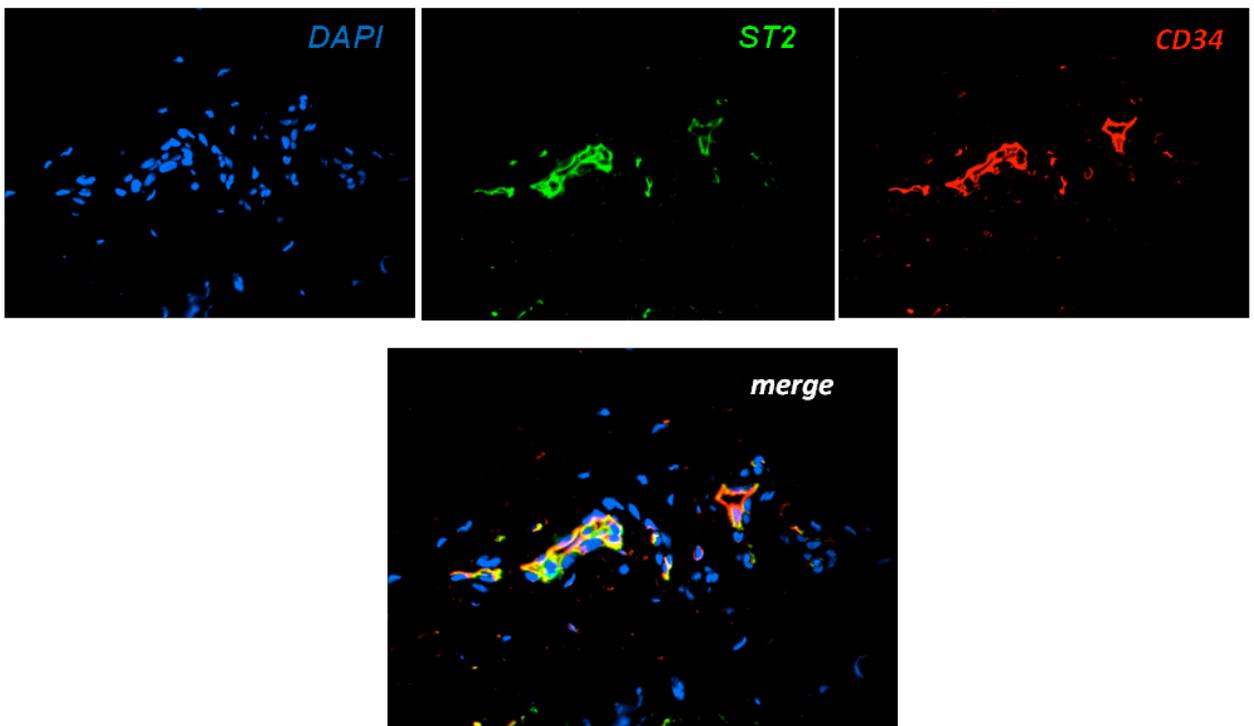
**Figure 6-1 IL-33, ST2 and IL1-RacP immunostaining**  
 Staining in control (Ctl), Torn supraspinatus (TSUP) and Matched Subscapularis (MSub). Black arrows show nuclear staining for IL-33 and red arrows show staining for ST2. Graphs illustrate Bonar scoring for samples of tendon with mean and SEM shown. n= 10 for control tendon, n=15 for torn supraspinatus and matched subscapularis. Scoring system depicts mean score per sample based on 10 high power field. 0= no staining, 1=<10%, 2=10-20%, 3= > 20% +ve staining of cells per high power field.

### **6.2.2) Co-localisation of IL-33/ST2 and endothelial markers**

To definitively define the expression pattern, double staining was established using fluorescence microscopy. With this technique exact overlay is possible and thus the confidence in true cellular identification is enhanced. Cells were stained as described in the methods chapter. Expression of IL-33 was compared initially with ST2 and CD34 as a endothelial marker. IL-33 stained with FITC is shown in green in figure 6.2 where cellular markers are in red. The IL-33 staining pattern observed confirmed previous IHC appearances with nuclear expression located predominantly around vascular structures. To visualize nuclei DAPI (in blue) was employed. Merged pictures clearly show distinct cells that exhibit IL-33 and ST2/CD34 co-expression.



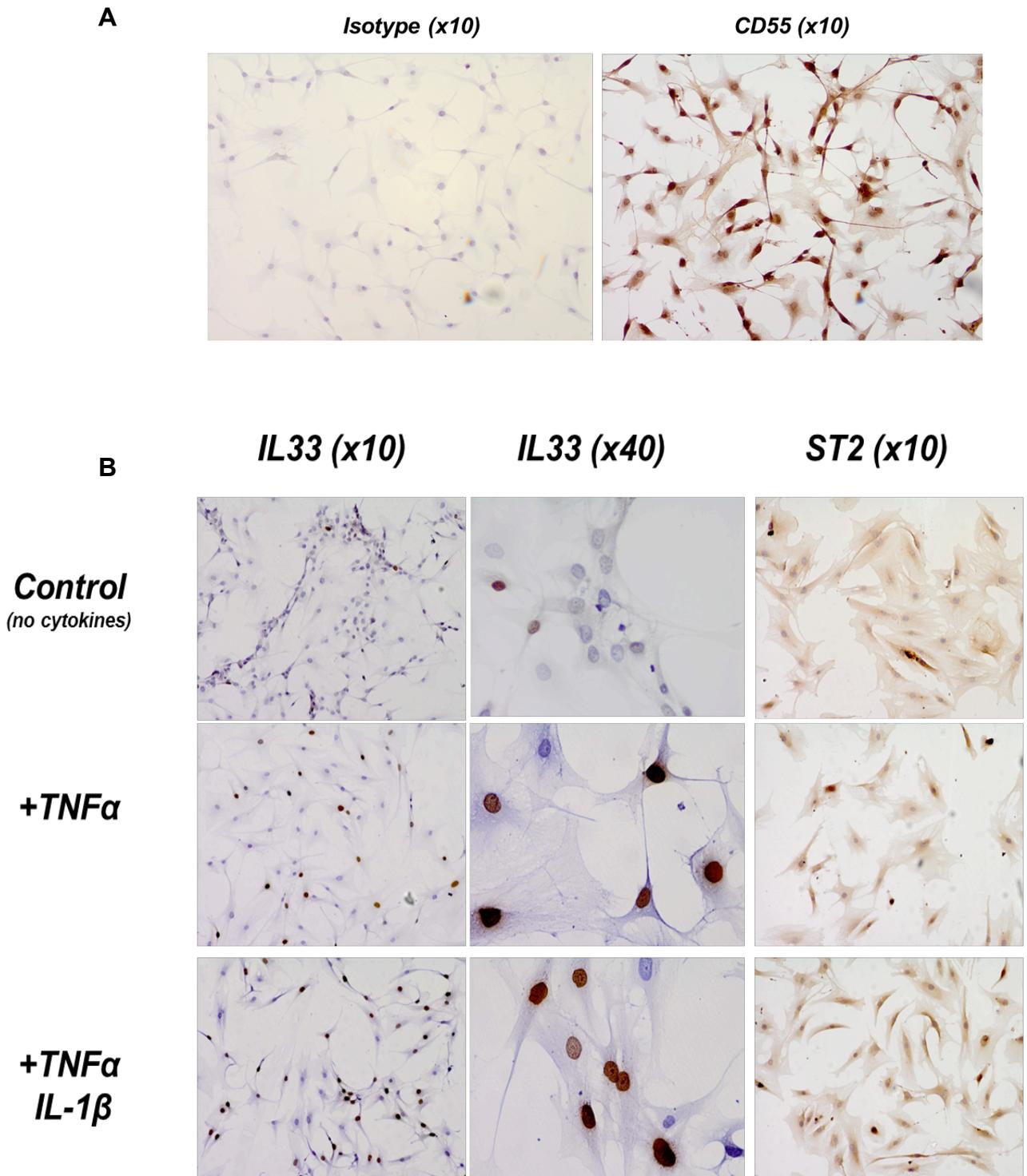
B



**Figure 6-2 Double immunofluorescence staining for IL33, ST2 and CD34 in tendon biopsies**  
 Tendon samples were stained for IL33, ST2 and CD34 in ten patients. (A) ST2 localised around perivascular channels in subscapularis tendon. Nuclear IL-33 staining appears to be in some endothelial cells and also in cytoplasm. Merge shows double staining in vascular region. There were very few double stained fibroblast like tenocytes in all tendon samples with the majority around neovascularised regions. (B) To further clarify the staining pattern of ST2 double staining with ST2/CD34 (red) was undertaken. Sections were counterstained with DAPI (blue). Images are shown at 40x magnification.

### 6.3) Induction of IL-33 in tenocytes

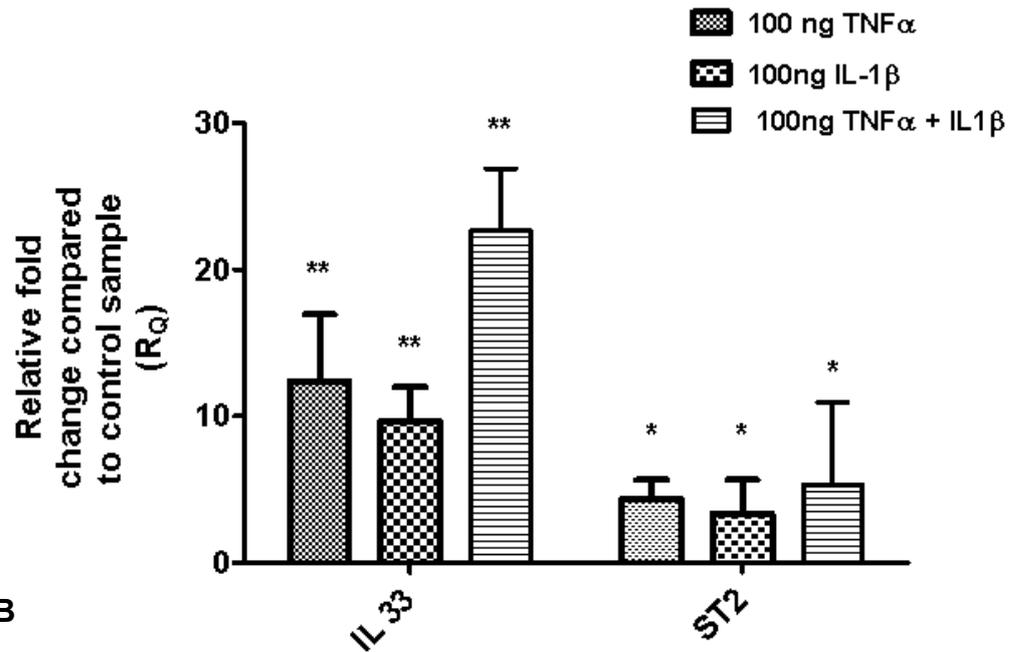
IL-33 has been reported to be expressed in fibroblasts derived from synovial tissue. Therefore, a logical progression from this was to examine expression in tenocytes. Human tendon derived fibroblasts were examined for their expression of IL-33 by IHC. Tendon fibroblasts were cultured until 80 % confluency and then split and plated on chamber slides. Although no specific cell surface markers exist for tenocytes we chose the surface marker CD55 that has been used by a collaborating laboratory (Prof P.P.Tak, AMC, Amsterdam) as a fibroblast marker to assure ourselves that cultured cells were indeed of fibroblast lineage. CD55 staining proved positive for >95% of the cultured cells. Additionally immunostaining for scleraxis was also performed as a marker of tenogenic cells as previously described [17]. Subsequently tendon fibroblasts were stimulated with or without TNF- $\alpha$  and IL-1. Twenty-four hours later cells were fixed and stained for IL-33 and ST2 using the same protocol as for synovial tissue staining previously described by the laboratory. As expected, IL-33 was detected in a predominantly nuclear expression pattern in *in vitro* cultured tenocytes and up regulated with stimulation by pro-inflammatory cytokines (Figure 6-3). ST2 staining revealed constitutive expression of ST2 in un-stimulated fibroblast like tenocytes in contrast to IL-33. Stimulation with IL-1 and TNF showed no significant increase in protein expression in ST2. Quantitative PCR confirmed significant up-regulation of IL-33 at the mRNA level while also showing that total ST2 message was significantly elevated with stimulation with TNF $\alpha$  and IL-1 $\beta$  (Figure 6-4). Finally FACS staining was undertaken to evaluate the constitutive expression of membrane ST2 on tenocytes and whether cytokine stimulation up regulated the IL-33 receptor. FACS showed approximately 20-30% of tenocytes to express membrane ST2 in normal culture conditions and showed no up regulation of membrane ST2 with TNF or IL-1 stimulation (Figure 6-4B).



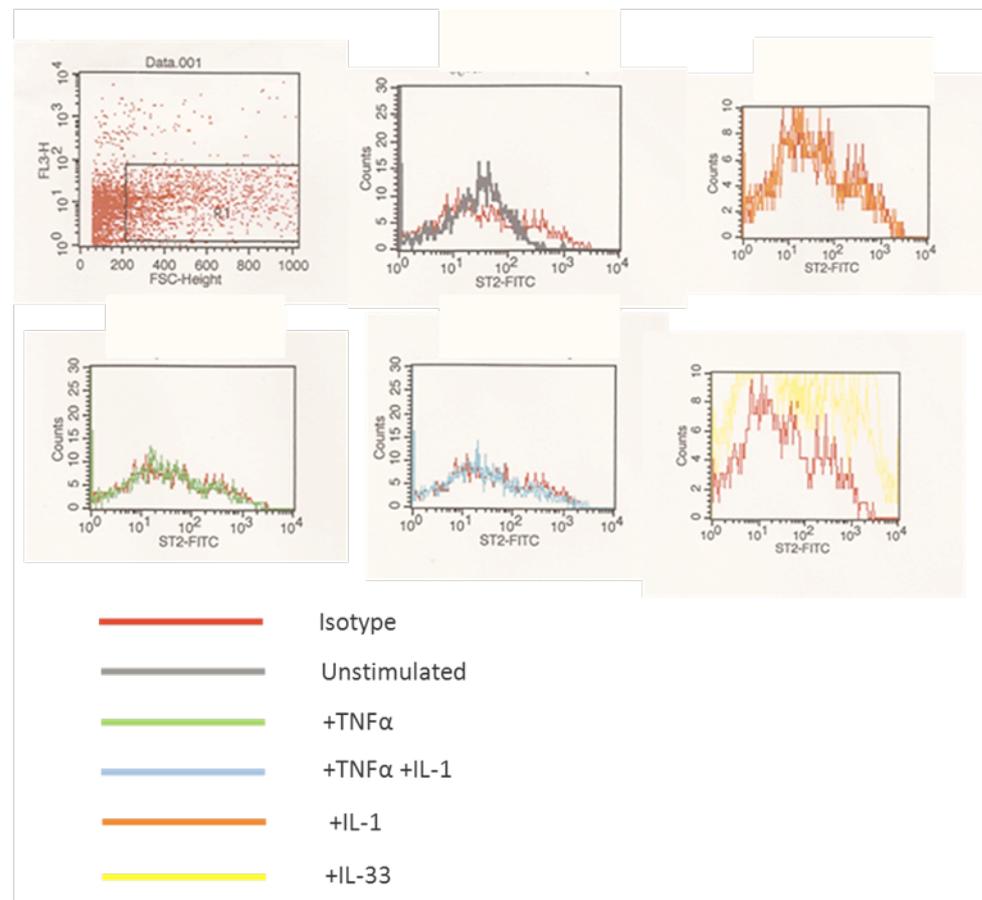
**Figure 6-3. Immunostaining of explant cultures.**

(A) CD55 marker used to verify that cells are of fibroblast lineage. (B) IL-33 and ST2 staining in explant cultures of tenocytes with no stimulation, 100ng/ml TNF $\alpha$  and 100ng/ml TNF $\alpha$  and IL-1 $\beta$  showing upregulation of nuclear IL-33 expression 24 hours post incubation. No definite changes were noted in ST2 expression.

A



B



**Figure 6-4 Expression of IL-33 and ST2 with TNF/IL-1 stimulation**

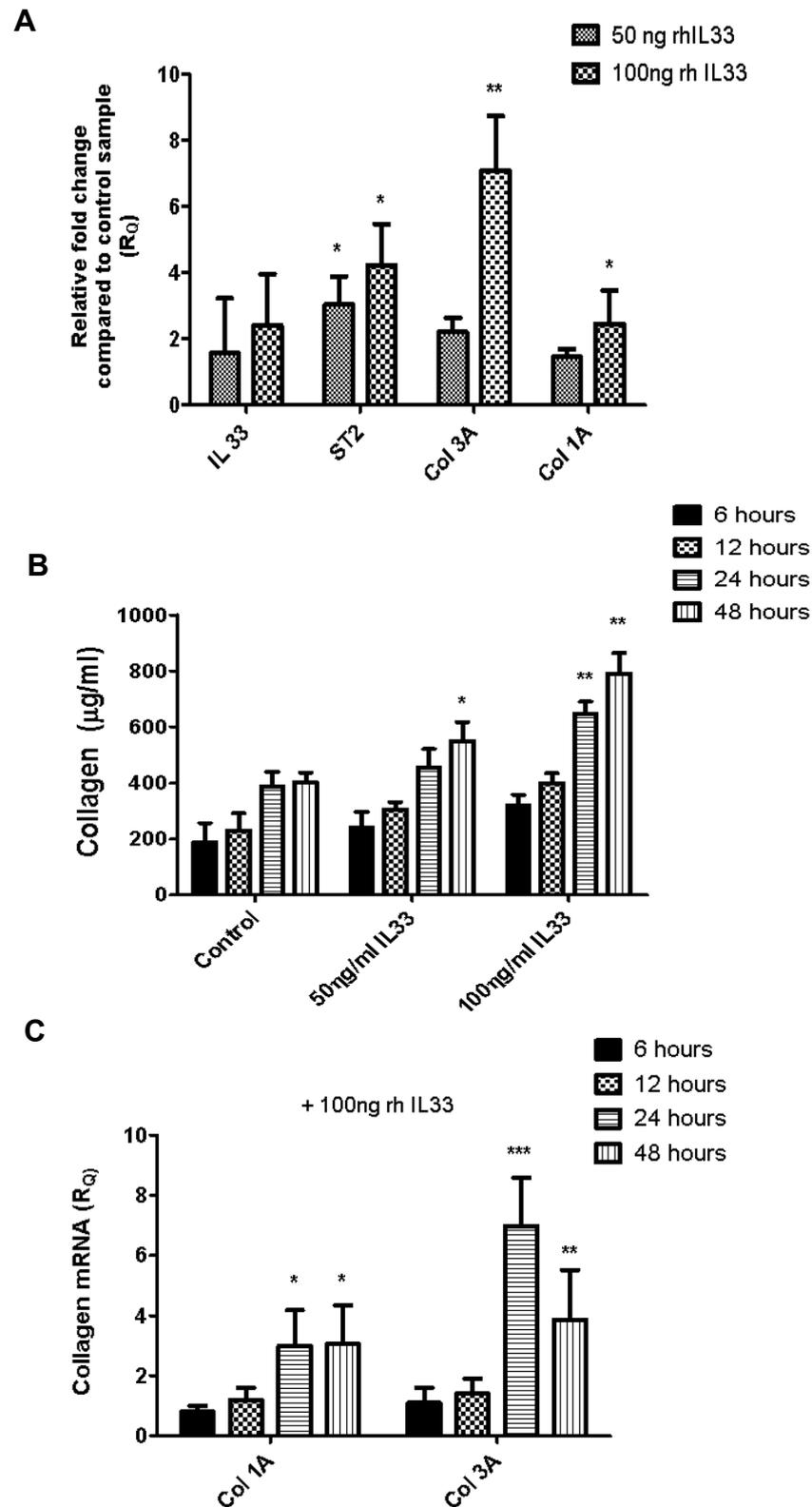
(A) Fold change in gene expression of IL-33, and ST2, 24 hours post incubation with respective doses of TNF $\alpha$  alone, IL-1 $\beta$  alone and in combination. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on three individual patient samples.

\*p<0.05, \*\*p<0.01 compared to control samples

(B) FACS analysis for ST2L in tenocytes treated with a variety of cytokines. No up regulation of ST2L was noted with TNF $\alpha$  alone, IL-1 $\beta$  alone and in combination. Addition of IL-33 resulted in a small but significant shift of the curve to the right indicating up regulation of ST2L through IL-33 stimulation

## **6.4) IL-33 induces collagen matrix changes and increased production of proinflammatory cytokines in vitro**

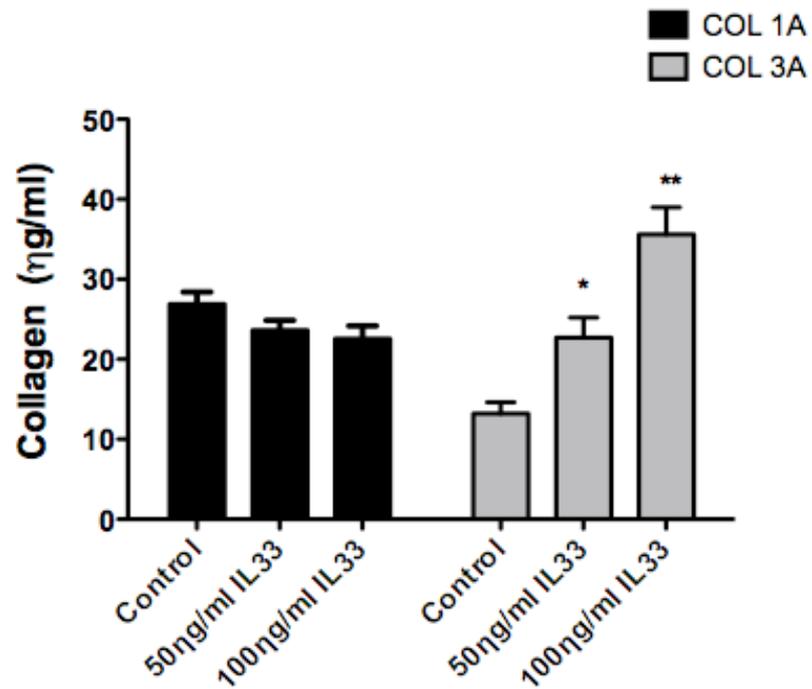
As matrix dysregulation is a key phenotypic change in tendinopathy we next sought to investigate the effect of addition of recombinant IL-33 to tenocytes in vitro. Concentrations of 50 and 100ng/ml of rhIL-33 were chosen based on previous investigation within the host laboratory. Initial quantitative PCR data revealed significant up regulation of Collagen I ( 50ng/ml mean fold change  $\pm$  SEM,  $1.45 \pm 0.2$ , 100ng/ml,  $2.45 \pm 1.0$ ) and III ( 50ng/ml,  $2.2 \pm 0.4$ , 100ng/ml  $7.08 \pm 1.7$ ) mRNA at 24 hours post incubation with rhIL-33, with by far the greatest change observed in collagen III mRNA expression (Figure 6-5 A). A time course experiment using 100ng/ml of rhIL-33 was then employed to examine collagen mRNA production over a 48 hour period. The most striking change was seen in Collagen III mRNA at 24 hours with an almost 7 fold increase which then was reduced to 4 fold change (relative to baseline) at 48 hours. In keeping with findings at the mRNA level, total collagen protein production was significantly increased at 24 ( $648\mu\text{g/ml} \pm 42$ ) and 48 hours ( $790 \mu\text{g/ml} \pm 74$ ) post incubation with 100ng/ml of rhIL-33 (Figure 6-5 B). The addition of rhIL-33 caused a significant increase in protein expression of Collagen III (control  $13.2\text{ng/ml} \pm 1.4$ , 50ng/ml rhIL-33,  $22.7\text{ng/ml} \pm 2.5$ , 100ng/ml rhIL-33  $35.6 \pm 3.4$ ) at 24 hours post incubation with no significant changes in Collagen I protein expression observed. Thus IL-33 seems to switch collagen matrix production toward a type III phenotype at early time points.



**Figure 6-5 IL-33 induced matrix changes in tenocytes.**

(A) Fold change in gene expression of IL-33, ST2, COL I and III with 50 and 100 ng/ml rhIL-33 24 hours post incubation (B) Total collagen production over time course following incubation with 50 and 100 ng/ml rhIL-33. (C) Fold change in COL I and III gene expression following incubation with 100 ng/ml rhIL-33 and (D) Collagen I and III protein expression 24 hours post incubation with respective rhIL-33. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

A



B

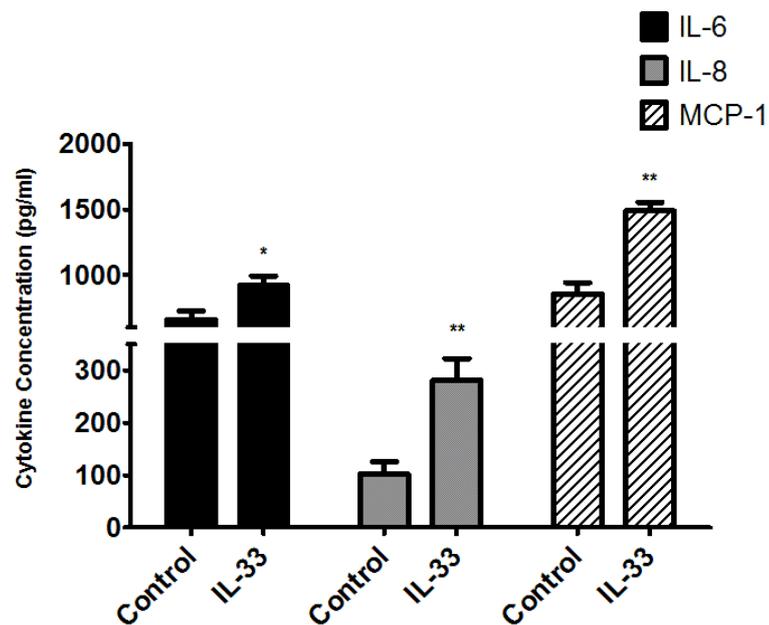


Figure 6-6 IL-33 induced COL I and III protein changes and cytokine production in cultured tenocytes

(A) Collagen I and III protein expression 24 hours post incubation with respective rhIL-33. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

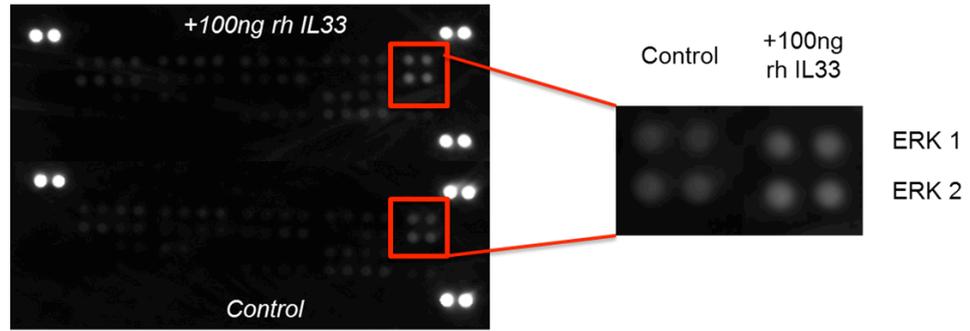
(B) Cultured tenocytes were incubated in with recombinant IL-33 over 24 hours. Data shows levels of IL-6, IL-8 and MCP-1, in supernatants removed from culture at 24 hrs analysed using Luminex.

## **6.5) Signalling mechanisms mediating rhIL-33 effects manifest in tenocytes in vitro**

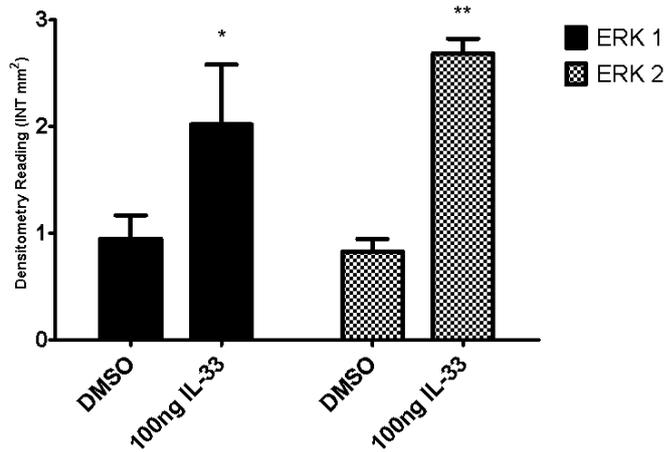
Next, in an attempt to elucidate the mechanistic pathway involved in IL-33 mediated matrix changes, I decided to analyse the phosphorylation of key downstream signalling targets, including particularly ERK1/2 and NFkB. Previous time courses in our laboratory using synovial fibroblasts revealed that 100ng/ml of rhIL-33 produced maximal effects on MAPK phosphorylation between 30 mins to 1 hour post stimulation[244]. I subsequently undertook a time course looking at the MAPK array results at 30,40,50 and 60 mins post incubation and noted maximal effects at 50 and 60 minutes post stimulation. Conversely I undertook a time course for NFkB at 5,10,15 and 20 minutes post incubation as again previous in house experiments had shown maximal effects between these time points. Ultimately NFkB. Activation was maximal 15 minutes post incubation with 100ng/ml of rhIL-33. These experiments analysed the phosphorylation status of mitogen-activated protein kinases (MAPKs), extracellular signal regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38 isoforms using the Human Phospho-MAPK Array (R & D Systems Europe, UK). In addition, NFkB activation status was assessed using the NFkB p65 ( Total/Phospho) InstantOne™ ELISA ( eBioscience, UK).

Although a generalised increase in phosphorylation was noted in the array, the only significant changes noted were manifest in ERK1/ERK2 phosphorylation after the addition of 100ng/ml of rhIL-33 one hour post treatment. Stimulation of tenocytes with IL-33 resulted in a significant down regulation of total NFkB while caused a significant up regulation of phosphorylated NFkB. TNF $\alpha$  stimulated cells were use as a positive control to assure the assays validity.

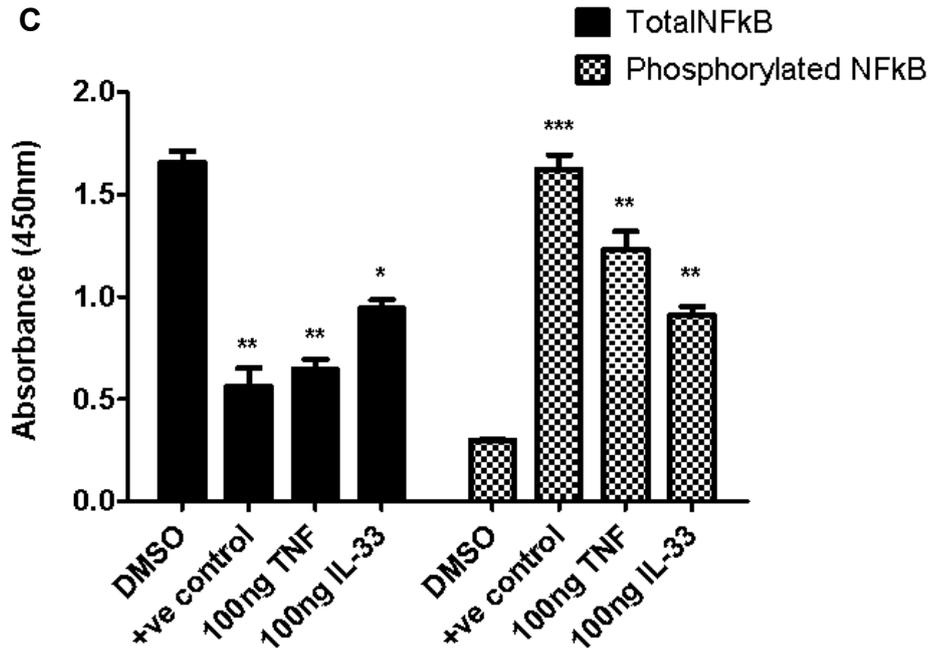
A



B



C



**Figure 6-7 ERK and NFkB phosphorylation in IL-33 treated tenocytes**

(A) Whole-cell lysates were examined for the phosphorylation of MAPK's at 1 hour after exposure to 100ng/ml rhIL-33 (A). The fold change of MAPK's was determined by densitometry and normalised to the control sample on the array(B). Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using five individual donors of tendon explant tissue.

(B) Whole-cell lysates were examined for the total NFkB and phosphorylated NFkB in tenocyte cultures treated with 100ng TNF and IL-33. Data shown are mean  $\pm$  SD of three individual experiments.

## **6.6) ERK and NFκB inhibition abrogates collagen changes and reduces IL-33 induced proinflammatory changes**

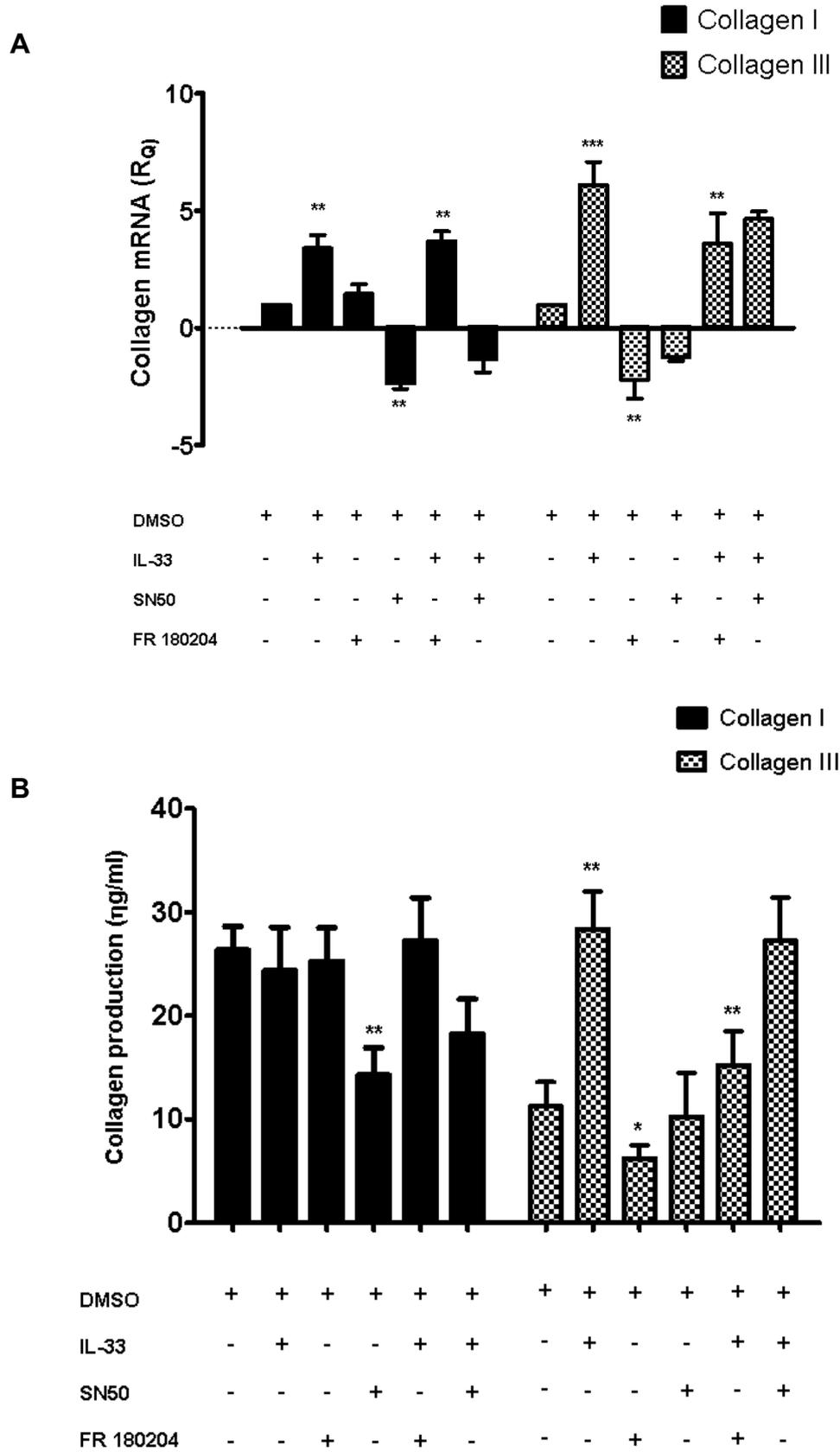
To understand if downstream IL-33 signalling influenced collagen matrix production I next sought to investigate whether inhibition of the previously noted ERK and NFκB activation mediated any effect on the changes in collagen synthesis patterns that I had observed..

The ERK (FR180204, 50μM) and NFκB (SN050, 18μM) inhibitors were applied alone or in combination with 100ng rhIL-33. The rationale for choosing these inhibitors was based on experiments carried out by Dr Kurowska-Stolarska, within our laboratory group on IL-33 in macrophages[244] where she had noticed significant reductions in IL-33 induced ERK and NFκB activity at IC<sub>50</sub>. The protocol from these set of experiments was followed.

SN050 significantly reduced collagen I mRNA compared to control while FR 180204 had no effect on Collagen I. Following incubation with rhIL-33, SN050 was able to significantly ( $p < 0.05$ ) reduce Collagen I message compared to IL-33 alone ( $\Delta\Delta\text{CT} \pm \text{SEM}$ , IL-33  $-3.4 \pm 0.6$ , SN050 +IL-33  $-1.3 \pm 0.6$ ). FR180204 had no significant effect on IL-33 induced Collagen I message. Collagen III message was significantly reduced by FR18024 treatment alone and significantly reduced in IL-33 treated tenocytes.

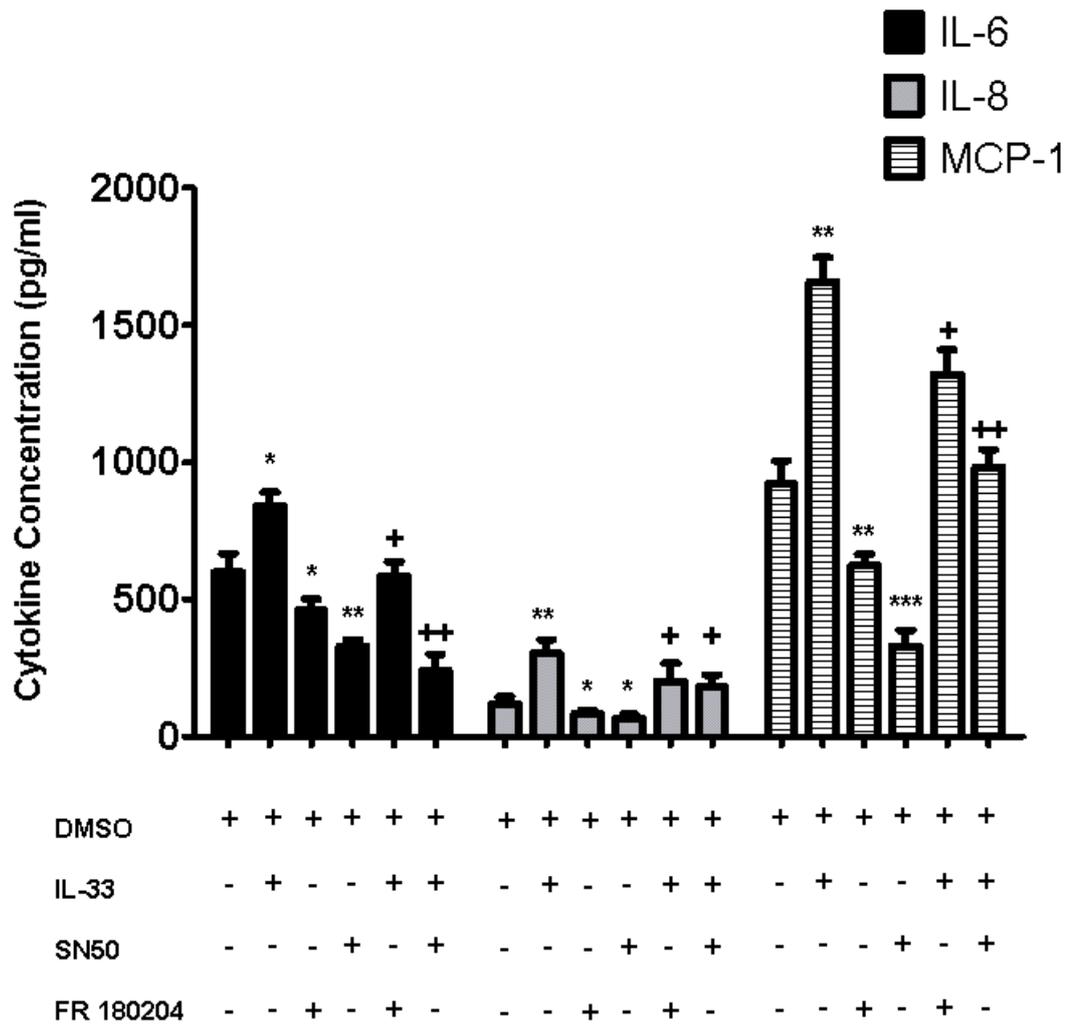
These changes were confirmed at the protein level in experiments in which SN050 was able to significantly reduce collagen I alone and in combination with IL-33. Again FR18024 was able to reduce collagen III alone and in combination with IL-33(Figure 6-8B).

rhIL-33 incubation resulted in significantly ( $p < 0.05$ ) reduce IL-6, IL-8 and MCP-1 production by tenocytes in the control environment and in the presence of 100ng rhIL-33. Thus both NFκB and ERK pathways appear to be involved in IL-33 induced cytokine production by tenocytes in vitro.



**Figure 6-8 ERK and NFκB inhibitors effects on collagen synthesis**

(A) The levels of mRNA for Collagen type IA and IIIA were determined by real time PCR. (B) Collagen I and III protein levels as determined by ELISA. ERK inhibition significantly affected type III collagen synthesis alone and in combination with IL-33 while NFκB significantly affected collagen I synthesis in a similar manner. Data shown as the mean  $\pm$  SD of triplicate samples and represent experiments on five individual patient samples. \* $p < 0.05$ , \*\* $p < 0.01$  compared to control samples



**Figure 6-9 ERK and NFκB inhibitors effects of cytokine production in tenocytes**  
 Cells were preincubated for 24 h with specific IL-33 and inhibitors for ERK (FR 180204) or NFκB (SN050) which also were included assay media. Data shows levels of MCP-1, IL-8 and IL-6 in supernatants removed from culture analysed using Luminex. Data shown are as the mean ± SD of duplicate samples and are representative of five individual experiments.

## 6.7) IL-33 in tendon healing *in vivo*

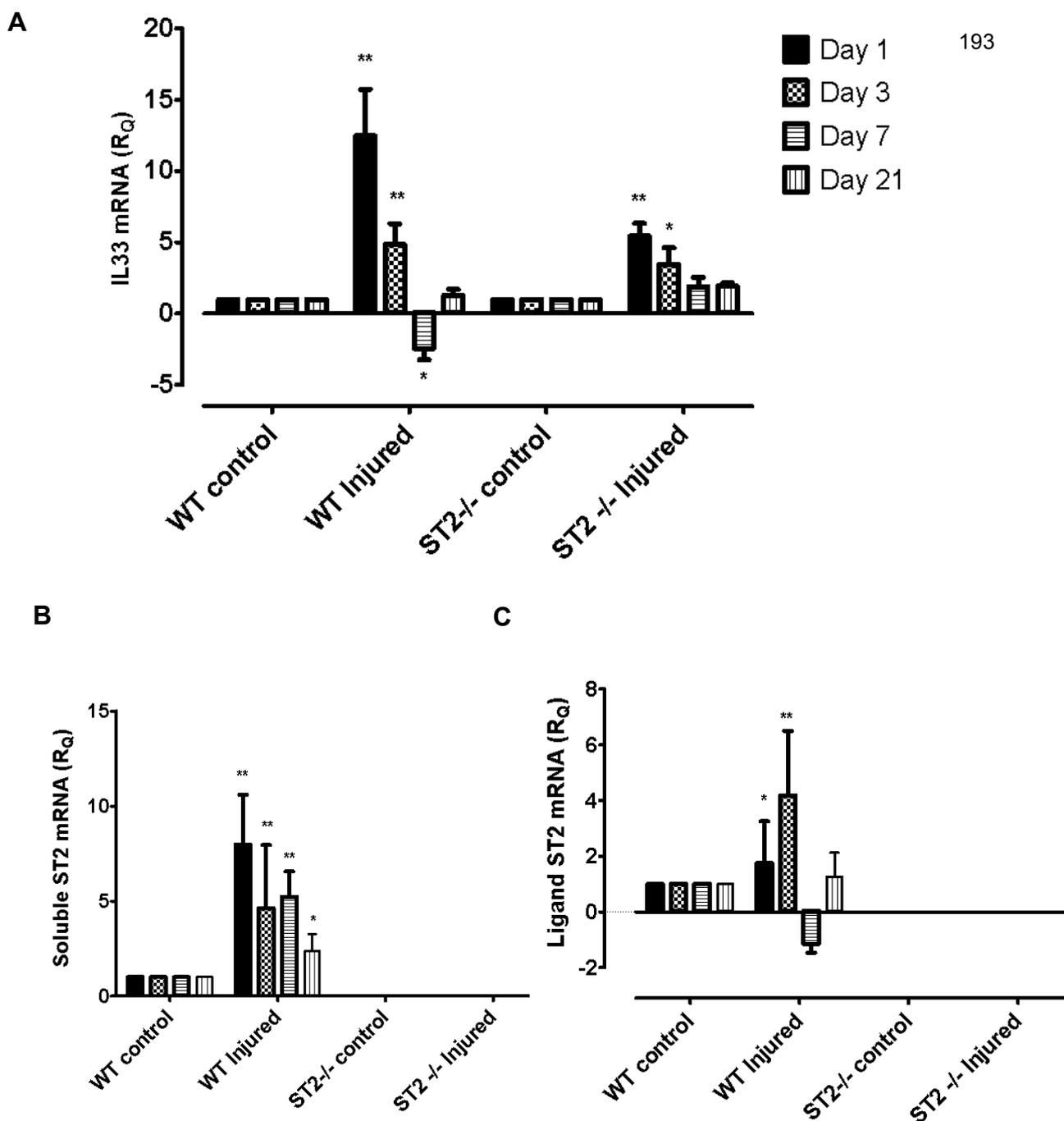
Having identified expression and a potential functional role for IL-33 in tissue biopsies and subsequent *in vitro* work, I next sought to explore the functional relevance of this in an *in vivo* model. The host laboratory was the first to discover ST2L and since then have been at the forefront of discovery of its biology. As such the next logical step was to investigate tendon pathology in an animal model. At the time of designing experiments IL-33 knockout mice were not available to the group and indeed had been shown to be embryonically lethal (personal communication, Dr D Gilchrist, University of Glasgow). However the laboratory had used ST2<sup>-/-</sup> mice in numerous occasions and these were readily available. Thus it was decided to explore the functional relevance of IL-33 in ST2 knockout mice. As discussed in section 1.3 the use of a running rodent model has not translated well to mouse experiments mainly through lack of convincing running by mice and subsequent pathological changes. With this in mind I chose to investigate tendon healing in ST2<sup>-/-</sup> with the use of the patellar tendon healing model previously discussed in the methods section.

### 6.7.1) Increased expression of IL-33/ST2 in early tendon healing

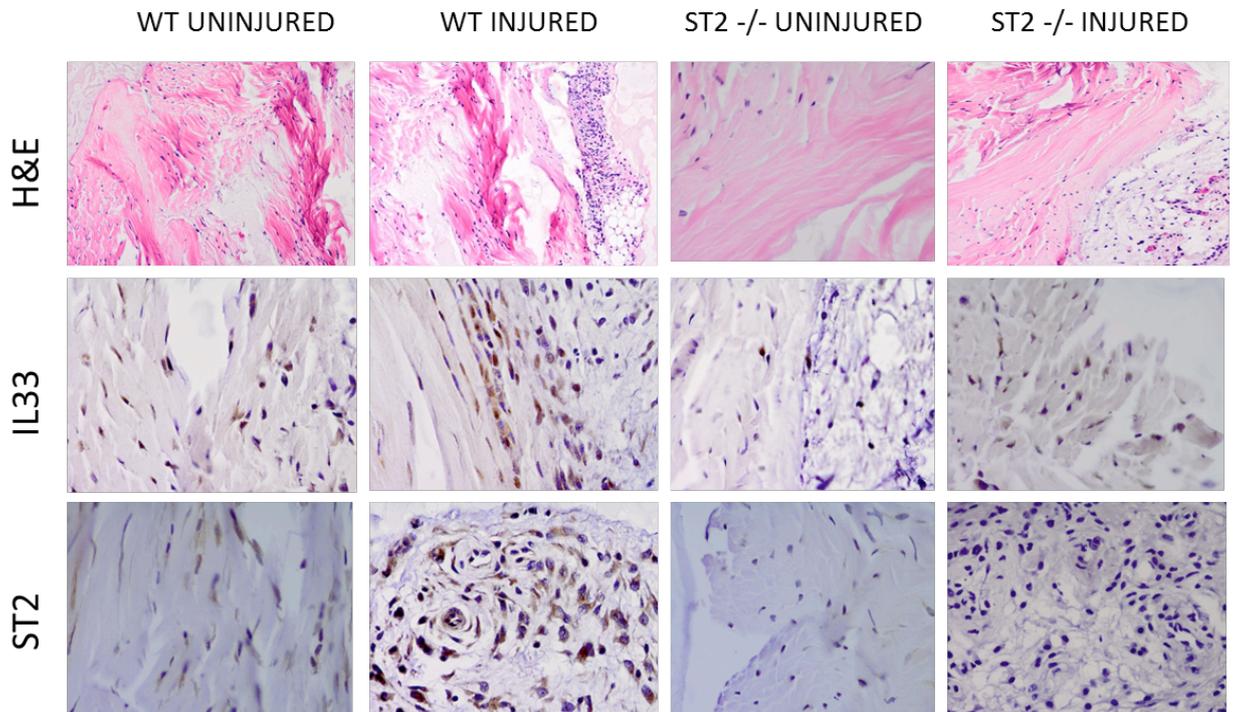
I first induced tendon healing using a well established tendon injury model as described in chapter 2. I examined expression of relevant factors in the area of the damaged and healing tendon at proscribed time points after the induction of the injury. Quantitative PCR revealed significant up regulation of IL-33 on days 1 (mean  $\Delta\Delta\text{Ct}\pm\text{SEM}$  to uninjured,  $12.5\pm 3.3$ ) and 3 ( $\Delta\Delta\text{Ct}$   $4.9\pm 1.4$ ) post tendon injury in WT mice (Figure 6-10 A). While injured ST2<sup>-/-</sup> mice on days 1 ( $\Delta\Delta\text{Ct}$   $5.4\pm 0.9$ ) and 3 ( $\Delta\Delta\text{Ct}$   $3.4\pm 1.2$ ) also showed a significant fold increase compared to controls this was markedly less when compared to the WT mice.

I next analysed the expression of soluble versus membrane bound ST2 in vivo. Soluble ST2 was significantly up regulated at all time points (mean  $\Delta\Delta\text{Ct}\pm\text{SEM}$ , Day 1,  $8.0\pm 2.6$ , Day 3,  $4.6\pm 3.3$ , Day 7,  $5.2\pm 1.3$  and Day 21  $2.4\pm 0.9$ ) post injury in WT mice compared to uninjured controls (Figure 6-10 B). Message for membrane ST2 only showed significant changes on Day 3 ( $\Delta\Delta\text{Ct}$   $4.2\pm 2.3$ ) post injury with no other significant changes noted (Figure 6-10 C).

Immunohistochemical analysis confirmed similar findings for IL-33 protein in WT mice at early time points (Figure 6-11). No significant changes in IL33 or ST2 message or protein expression was found in WT mice at days 7 or 21 post injury. This was also true for IL-33 expression in ST2<sup>-/-</sup> mice suggesting that IL-33 production  $\pm$  release is an early hit phenomenon in keeping with a possible 'alarmin' type role in tendon injury/repair.



**Figure 6-10. Relative expression of IL33, soluble and receptor ST2 in vivo**  
 (A) IL-33 gene expression on Days 1, 3, 7 and 21 post injury. The most significant increase is noted in WT injured mice on Day 1 post injury with globally reduced IL-33 message in ST2<sup>-/-</sup> mice. Soluble and receptor ST2 gene expression on days 1,3,7 and 21 post injury. The most significant increase is noted in WT injured mice on Day 1 and 3 post injury suggesting this an early mediator in tendon healing. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.



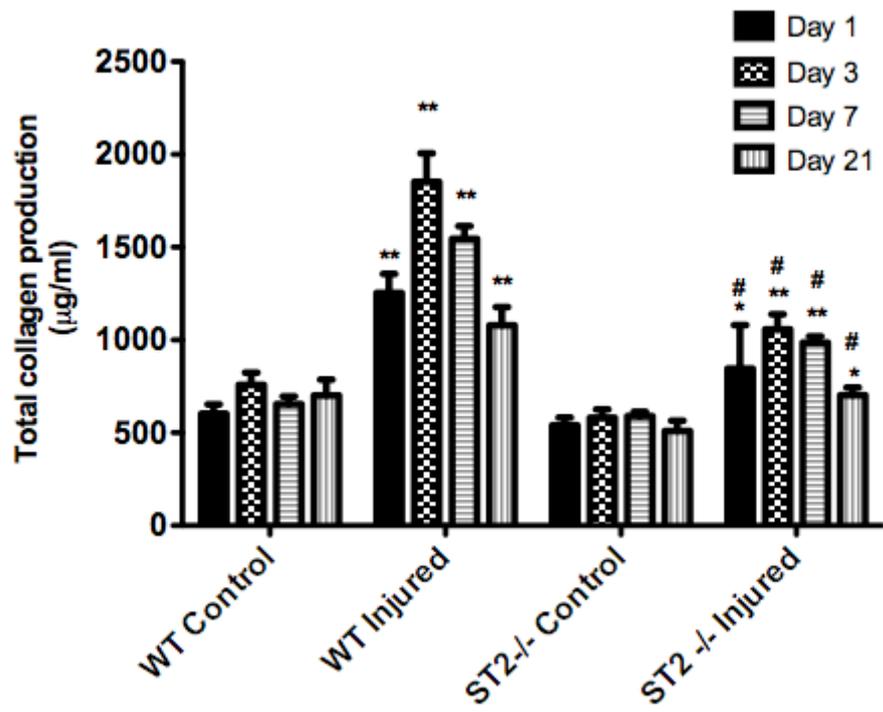
**Figure 6-11. Immunohistochemistry of IL-33 and ST2 expression in vivo.** IL-33 and ST2 immunostaining in WT and ST2<sup>-/-</sup> injured and uninjured mice on Day 1 post injury highlighting increased IL-33 and ST2 staining in WT injured and uninjured mice compared to ST2<sup>-/-</sup> controls.

### 6.7.2) Altered collagen synthesis responses in ST2<sup>-/-</sup> mice

Total collagen production was significantly ( $p < 0.01$ ) increased in WT injured (mean collagen production  $\pm$  SEM, Day 1; 1254  $\mu\text{g/ml} \pm 102$ , Day 3; 1852  $\mu\text{g/ml} \pm 154$ , Day 7; 1545  $\mu\text{g/ml} \pm 68$  and Day 21; 1078  $\mu\text{g/ml} \pm 98$ ) and ST2<sup>-/-</sup> injured (Day 1; 845  $\mu\text{g/ml} \pm 412$ , Day 3; 1056  $\mu\text{g/ml} \pm 82$ , Day 7; 986  $\mu\text{g/ml} \pm 33$  and Day 21; 702  $\mu\text{g/ml} \pm 41$ ) mice at all-time points compared to uninjured WT mice (Day 1; 602  $\mu\text{g/ml} \pm 50$ , Day 3; 758  $\mu\text{g/ml} \pm 65$ , Day 7; 654  $\mu\text{g/ml} \pm 41$  and Day 21; 701  $\mu\text{g/ml} \pm 85$ ) and ST2<sup>-/-</sup> uninjured (Day 1; 845  $\mu\text{g/ml} \pm 412$ , Day 3; 1056  $\mu\text{g/ml} \pm 82$ , Day 7; 986  $\mu\text{g/ml} \pm 33$  and Day 21; 702  $\mu\text{g/ml} \pm 41$ ). The patellar tendons of WT mice however had significantly ( $p < 0.05$ ) greater total collagen content at Days 1, 3 and 7 post injury compared to ST2<sup>-/-</sup> injured mice at the same time points.

Sub analysis of collagen type at the mRNA level revealed that WT mice showed significantly greater expression of collagen III at days 1 ( $\Delta\Delta\text{Ct } 14.7 \pm 6.5$ ), 3 ( $\Delta\Delta\text{Ct } 8.5 \pm 1.6$ ), 7 ( $\Delta\Delta\text{Ct } 5.8 \pm 2.4$ ) and 21 ( $\Delta\Delta\text{Ct } 4.2 \pm 1.4$ ) post injury compared to uninjured controls and injured ST2<sup>-/-</sup> mice at the same time points. Collagen I was initially down regulated at day 1 ( $\Delta\Delta\text{Ct } -3.6 \pm 2.2$ ) and day 3 ( $\Delta\Delta\text{Ct } -4.6 \pm 1.6$ ) in WT injured mice which then recovered to be relatively up regulated on Days 7 ( $\Delta\Delta\text{Ct } 2.3 \pm 1.0$ ) and 21 ( $\Delta\Delta\text{Ct } 4.6 \pm 0.5$ ) (compared to baseline). ST2<sup>-/-</sup> injured mice showed down regulated Collagen I on days 1 ( $\Delta\Delta\text{Ct } -3.3 \pm 1.7$ ), 3 ( $\Delta\Delta\text{Ct } -1.1 \pm 0.6$ ) and 7 ( $\Delta\Delta\text{Ct } -1.5 \pm 0.7$ ) which returned to baseline on Day 21 ( $\Delta\Delta\text{Ct } 1.2 \pm 0.4$ ). These results suggest that ST2<sup>-/-</sup> mice are unable to mount the same collagen healing response compared to WT controls implicating IL-33 as an early modulator of collagen changes in tendon injury.

A



B

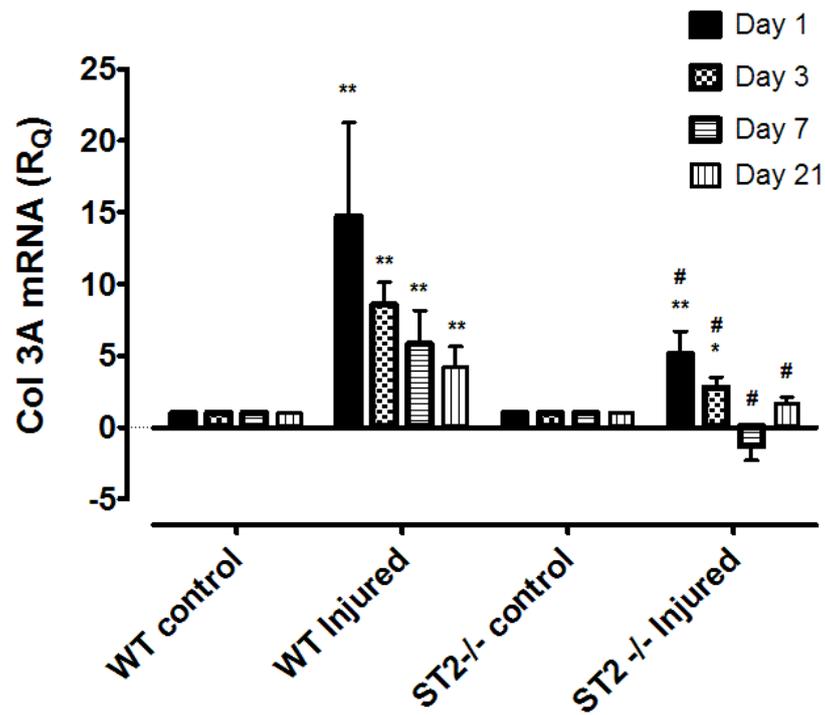
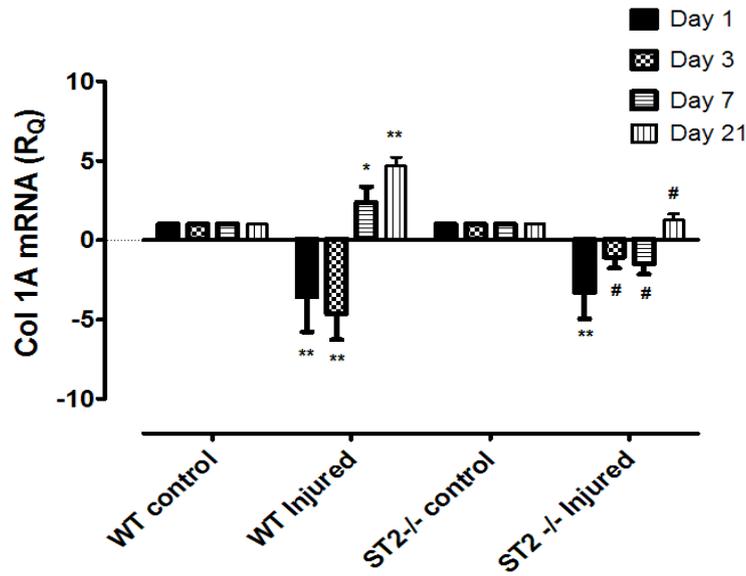


Figure 6-12. Total collagen production and Collagen III mRNA expression in vivo (A) Total collagen production (µg/ml) in WT and ST2<sup>-/-</sup> mice post injury. (B) Collagen III message levels in WT and ST2<sup>-/-</sup> post injury. Collagen III is significantly up regulated at all-time points post injury which is significantly abrogated in ST2<sup>-/-</sup> mice. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

A



197

B

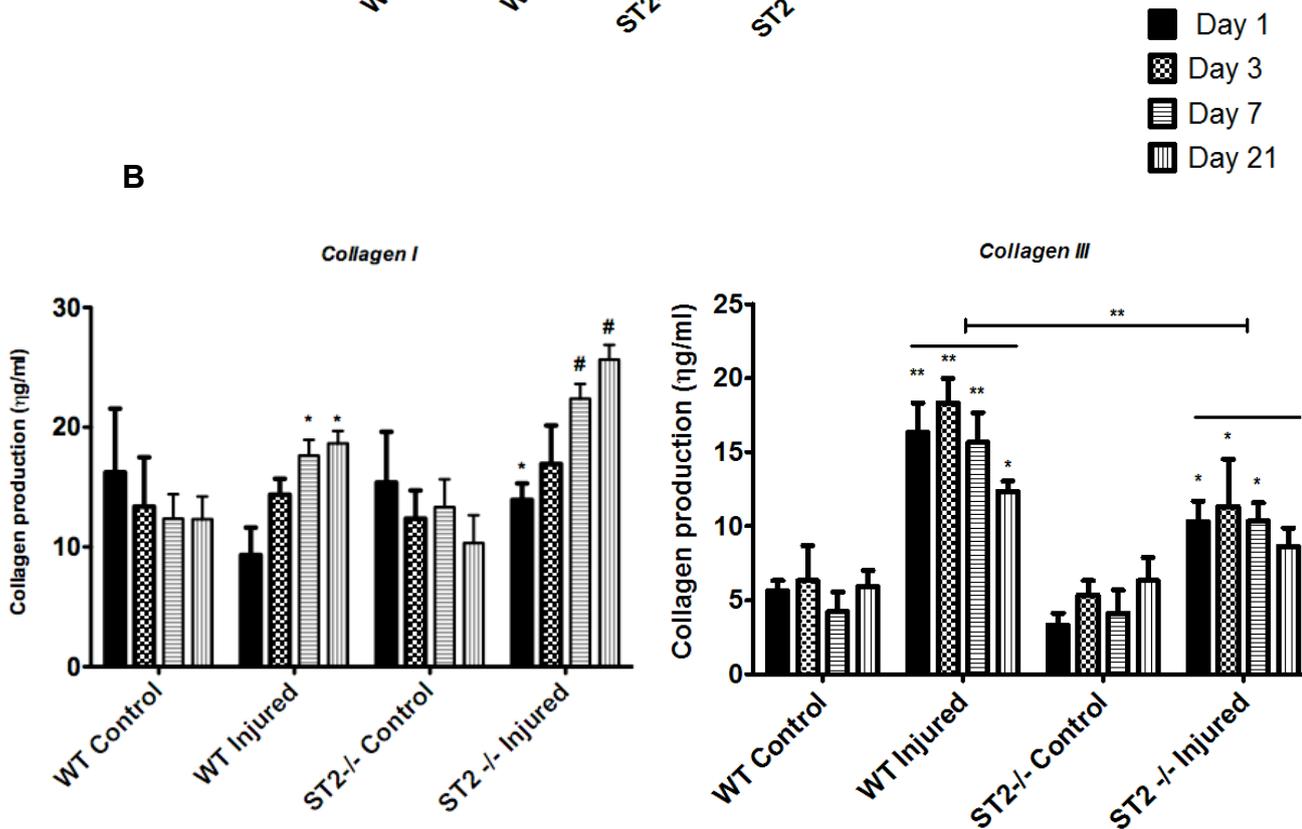


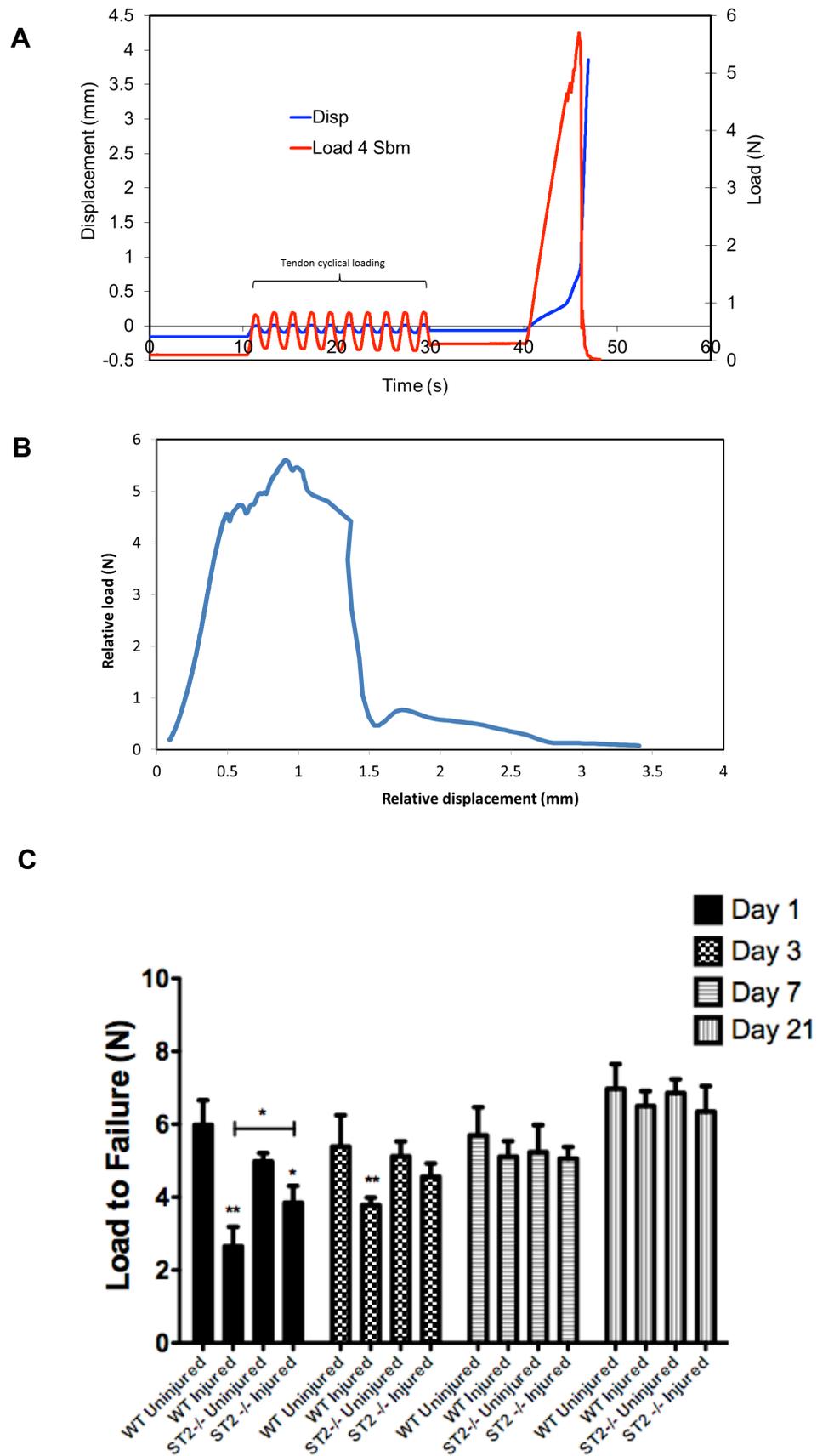
Figure 6-13 Collagen changes in vivo

(A) Collagen I message levels in WT and ST2<sup>-/-</sup> post injury. Collagen I is significantly down regulated at day 1 & 3 in WT injured mice and day 1 in ST2<sup>-/-</sup>. (B) Collagen I and III protein levels at time points post injury. WT mice show significantly greater production of Collagen I at days 7 and 21 post injury compared to WT injured tendons. ST2<sup>-/-</sup> mice produce significantly more Collagen I on days 1, 7 and 21 compared to WT injured mice. There is also a significant difference ( $p < 0.01$ ) in the lack of collagen III production in ST2<sup>-/-</sup> mice at all-time points post injury compared to the injured WT group. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

### **6.7.3) WT and ST2<sup>-/-</sup> mice showed reduced biomechanical tendon strength at early time points post injury**

Whilst changes at the molecular level may provide novel insight into pathophysiological mechanism, I next sought to evaluate the biomechanical strength implications of such molecular changes in the injured and uninjured tendons. This was designed to ultimately assess whether this line of experimentation could have any clinical relevance. This work was undertaken with the help of collaborators (Dr A Deakin, Dr P Riches) at the University Of Strathclyde Department Of Bioengineering.

Four tendons from each group (WT injured/uninjured and ST2<sup>-/-</sup> injured/uninjured) were analysed at all-time points as discussed in Materials and Methods. Injury to WT tendons resulted in a significant decrease in load to failure or ultimate strength at Days 1 and 3 post injury which recovered by day 7 and 21. ST2<sup>-/-</sup> uninjured mice showed no significant difference between WT uninjured mice at all-time points but there was a trend toward a lower tensile strength compared to WT uninjured. ST2<sup>-/-</sup> injured mice had a significantly reduced reduced load to failure on day 1 post injury compared to ST2<sup>-/-</sup> uninjured mice but interestingly had a greater tensile strength than WT injured mice on day 1. At all subsequent time points the ST2<sup>-/-</sup> injured mice showed a trend toward a decreased load to failure compared to ST2<sup>-/-</sup> uninjured mice but this was not significant. This suggests that the collagen matrix changes seen in vivo in the ST2<sup>-/-</sup> mice have no detrimental effect on the ultimate strength of injured tendons and indeed ST2<sup>-/-</sup> mice. These data are consistent with the notion that IL33/ST2 signalling could represent a novel pathway of early matrix and inflammatory changes in tendon healing/ tendinopathy with a direct effect on the ultimate strength of the tendon and subsequent clinical rupture or damage.



**Figure 6-14 Biomechanical testing of tendon injury model in WT and ST2-/- mice**  
**(A)** Load/Displacement curve for full loading cycle of tendon showing preconditioning of tendon during cycling and then ramp to failure load and displacement. **(B)** Load to failure data for WT and ST2-/- injured and uninjured tendons over time course. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

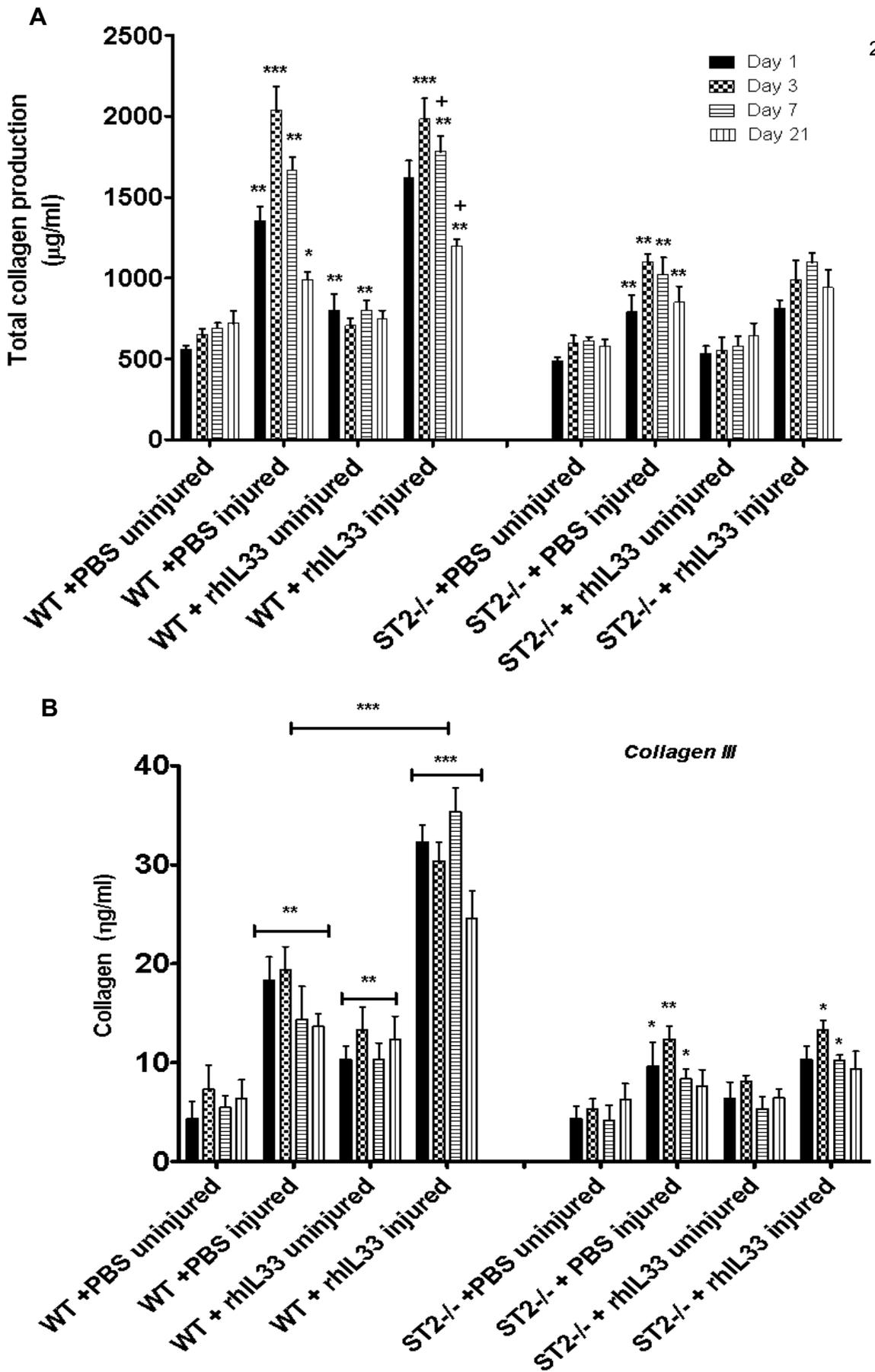
#### **6.7.4) Addition of rhIL-33 induces collagen matrix changes in vivo and reduces biomechanical strength**

Due to the observation that ST2<sup>-/-</sup> mice were unable to mount a similar genotypic and phenotypical collagen response in tendon healing, I next sought to ascertain if the addition of recombinant IL-33 would have any effect on matrix changes in vivo in WT and ST2<sup>-/-</sup> mice.

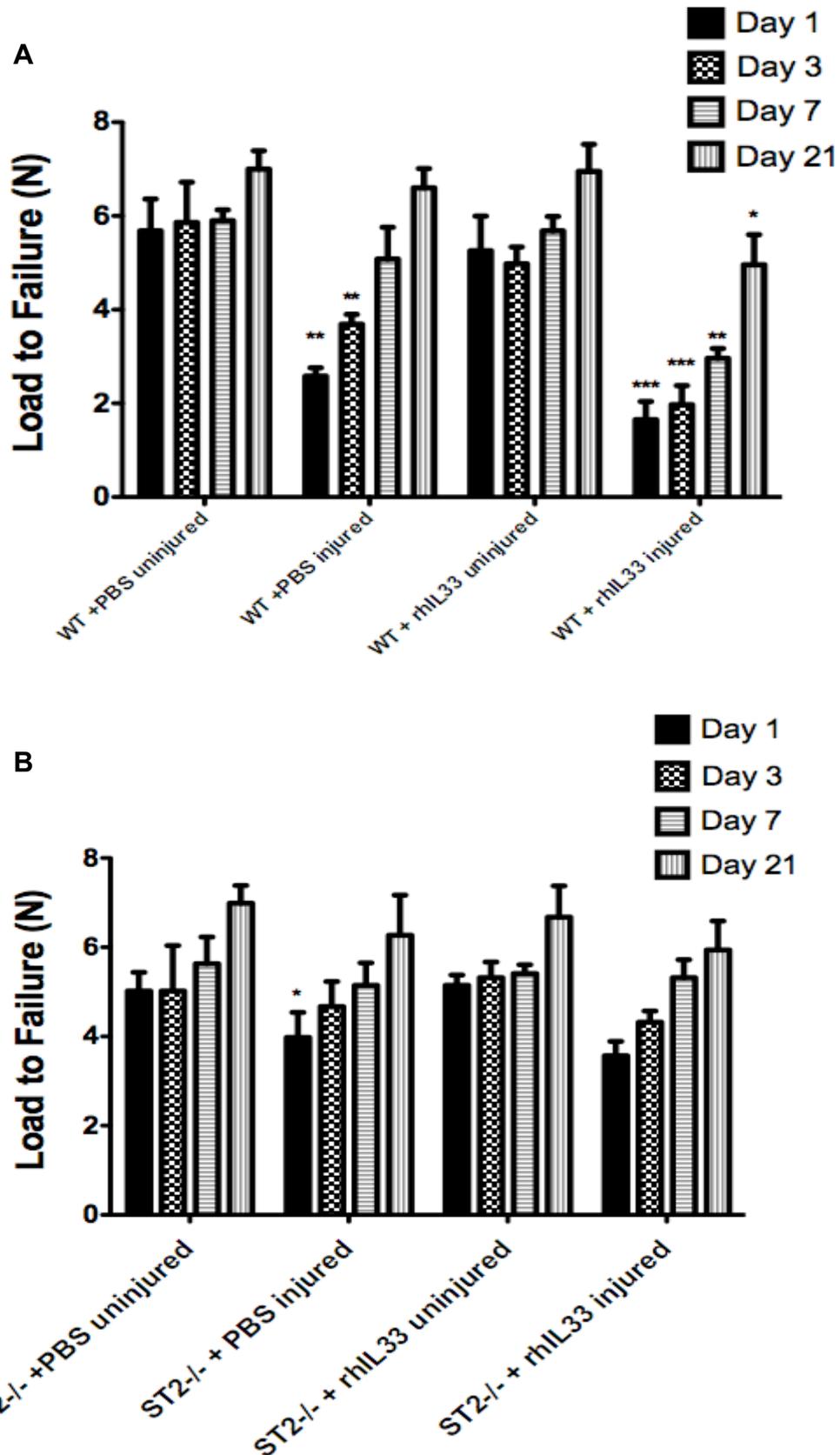
rhIL-33 was injected on day -3, -2, -1 and 0 prior to injury. Following this, tendons were harvested from the respective group at days 1, 3, 7 and 21 post injury for analysis of total collagen production, collagen I and III message and protein levels. Total collagen production was increased in WT injured and ST2<sup>-/-</sup> mice as previously mentioned with a significant reduction in the knockout group. Addition of rhIL-33 significantly increased total collagen on day 7 (mean collagen production $\pm$  SEM, WT injured; 1668  $\mu$ g/ml $\pm$ 80 WT, WT injured +rhIL-33 1782  $\mu$ g/ml $\pm$ 95) and 21 (mean collagen production $\pm$  SEM, WT injured; 988  $\mu$ g/ml $\pm$ 49, WT injured +rhIL-33 1200  $\mu$ g/ml $\pm$ 40) in WT injured mice compared to PBS injected controls. Addition of rhIL-33 significantly increased the production of Collagen III at both the mRNA and protein level in uninjured but most noticeably in injured tendons.

As a specificity control, we also demonstrated that IL-33 treatment did not affect the changes in collagen matrix synthesis or ultimate tensile strength of the healing tendon in ST2<sup>-/-</sup> mice confirming that IL-33 acted via an ST2-dependent pathway.

The addition of IL-33 significantly reduced the strength of tendons at all time points post injury compared to WT injured tendons injected with PBS (Figure 6-16A). The removal of IL-33's ligand in ST2<sup>-/-</sup> mice reduced the loss of strength significantly (Figure 6-16B) strongly implicating the loss of strength directly to the addition of IL-33 and its associated collagen matrix changes.



**Figure 6-15 rhIL-33 and collagen matrix changes in vivo**  
**(A)** Total collagen production and **(B)** Collagen III protein in WT and ST2<sup>-/-</sup> mice treated with rhIL-33. The most significant shift was in the production of Collagen III protein in WT mice treated with rhIL-33 at all-time points post injury. Data are shown as the mean fold change ± SD of duplicate samples and are representative of experiments using four mice per group.



**Figure 6-16 Biomechanical testing of WT and ST2<sup>-/-</sup> mice treated with rhIL-33**  
 (A) rhIL-33 resulting in a significantly decreased load to failure in WT uninjured mice on Days 1 and 3 of the treatment protocol. More significantly WT injured mice had 45% less load to failure if treated with rhIL-33 on days 1 and 3 post injury with a 20% decrease in strength at 21 days post injury compared to PBS injection alone. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

## 6.8) A role for micro RNA 29 in tendon healing

Due to parallel studies of mir-155 in rheumatoid arthritis alluded to before and in an effort to accelerate my understanding of some of the post transcriptional regulatory events underpinning the above observations, I next performed screening for miRNA targeting Collagen I/III , IL-33 and ST2 genes. To do this I merged the results of several computational prediction algorithms provided at the miRGen database. Bio computational predication screening tools are well established screening tools for miRNA profiling. Among other miRNA, such as the let-7 family, miR-26, and miR-155, the miR-29 family members were identified as potential post-transcriptional regulators of type I collagen, type III collagen and ST2.

Given that most miRNA act as post-transcriptional repressors and that in tendinopathy the expression of collagen genes are up regulated I sought to explore a role for the microRNA 29 family in tendon biopsies.

### 6.8.1) Down regulation of miR 29 expression in tendinopathy

We therefore analysed the expression of the miR29 family in torn supraspinatus, matched subscapularis and control tendon samples having previously carried out collagen analysis on the same samples which are shown in Figure 4-4A. We found that all members of the miR-29 family were expressed in control, torn and matched tendon. Control tendon showed the highest level of miR29 family expression with miR-29a showed the highest level of expression (mean  $\pm$ SEM  $\Delta$ Ct, control  $17\pm0.5$ , supraspinatus,  $20\pm1.9$ , subscapularis  $25\pm2.4$ ), followed by 29b (control  $23\pm1.9$ , supraspinatus,  $26\pm2.4$ , subscapularis  $28\pm2.8$ ), and 29c (control  $21\pm0.6$ , supraspinatus,  $24\pm1.3$ , subscapularis  $21\pm0.9$ ). Both torn and matched subscapularis showed significantly decreased levels of miR29 a and b compared to control tendon. No significant difference was noted in miR 29c levels compared to control tendon.

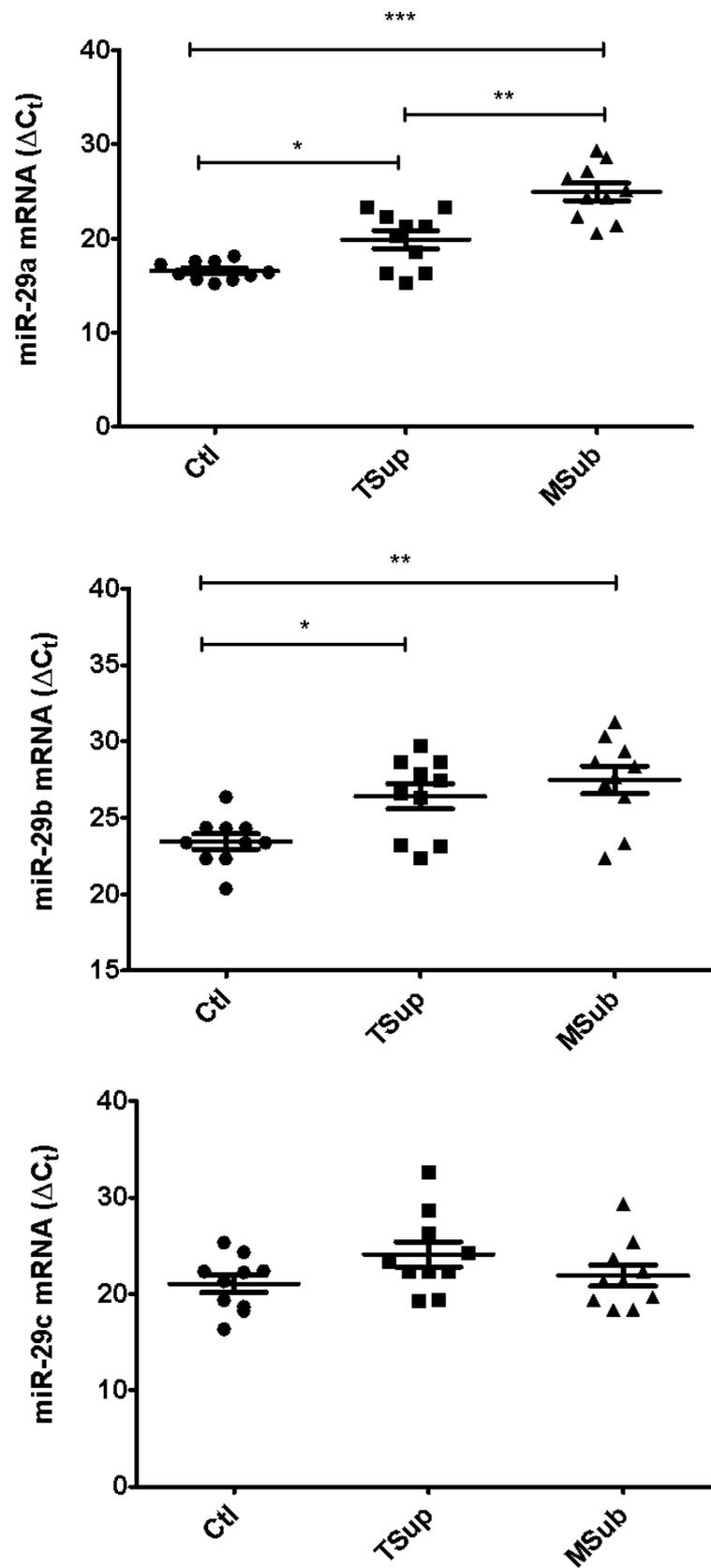
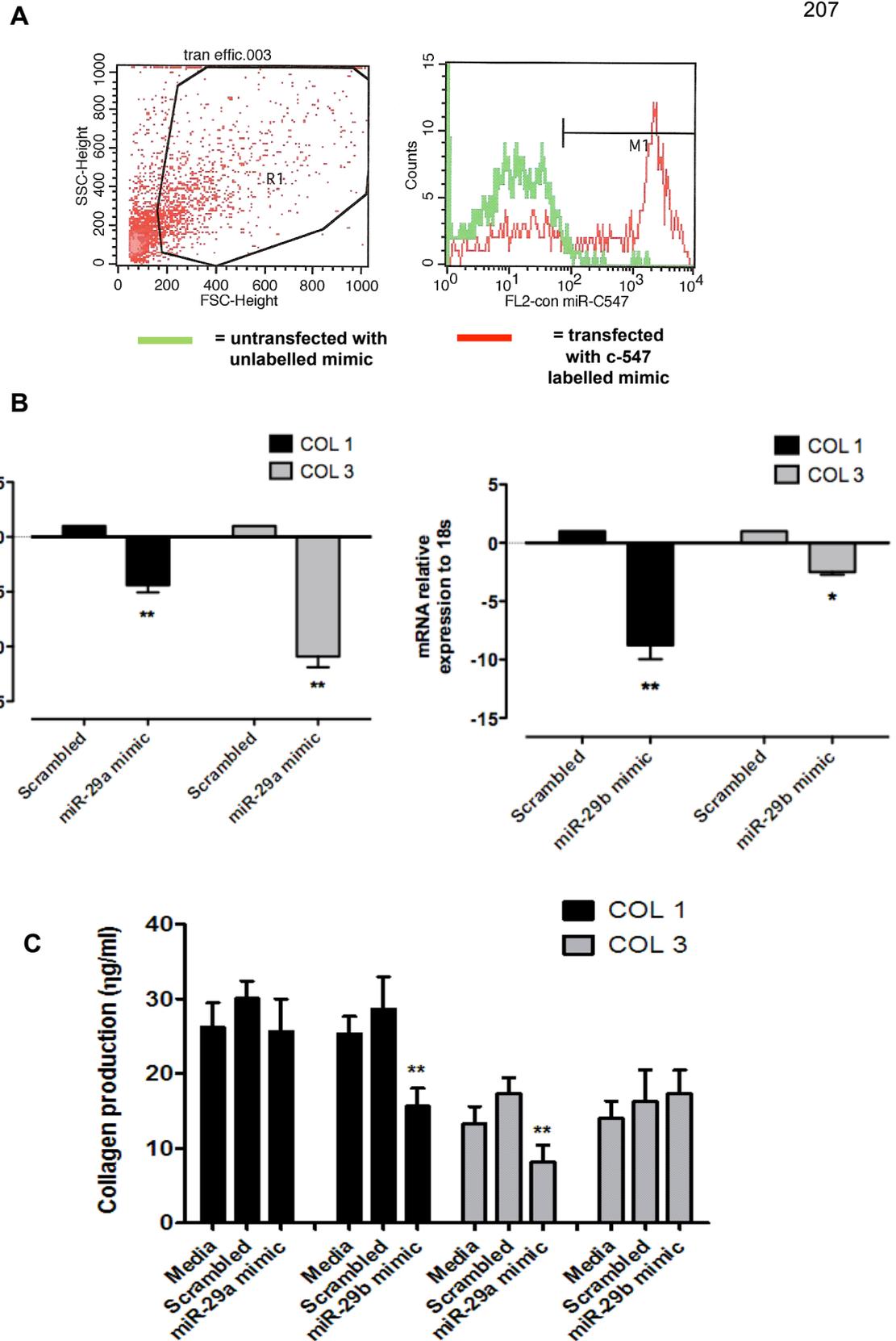


Figure 6-17 Micro RNA 29 family expression in tendon biopsies  
 miR 29 family gene expression in Control (Ctl), torn supraspinatus (TSUp) and matched subscapularis tendon ( MSub). Data shown as the mean ± SD of duplicate samples and represent experiments on ten patient samples. \*p<0.05, \*\*p<0.01

### 6.8.2) Altered collagen expression following over expression of miR29 a and b

I next aimed to evaluate the functional effects of altered miR-29 a & b expression on Collagen matrix synthesis on tenocytes in vitro. Evidence exists showing that mir29 a and b can act by directly targeting Collagen III and I respectively[324]. Thus to test whether miR-29 a & b indeed regulate the levels of the candidate target mRNAs, we transfected miR-29a & b mimics into tenocytes. Tenocytes were also transfected in parallel with a control mimic miRNA. Transfection efficiency was calibrated by FACS analysis of the labelled control mimic. The mean transfection rate of all experiments was  $58 \pm 10$  %. Transfection with pre-miR-29a/29b increased the level of the respective miRNAs by a mean  $\pm$  SEM of  $80.2 \pm 23.2$  fold and  $57.8 \pm 10.6$  fold.

Collagen I and III mRNA were reduced significantly by miR29 a & b mimic transfection respectively. miR 29 mimic transfection reduced collagen III mRNA almost 6 fold compared to scrambled control whereas collagen III protein was reduced by 50% at 24 hours post transfection. miR 29 b mimic reduced Collagen I mRNA 2 fold with a 43% reduction in Collagen I production at the protein level. Thus collagen changes in vitro may possibly reflect 'cross talk' between the miR 29 family with subsequent post transcriptional effects on collagen matrix synthesis.

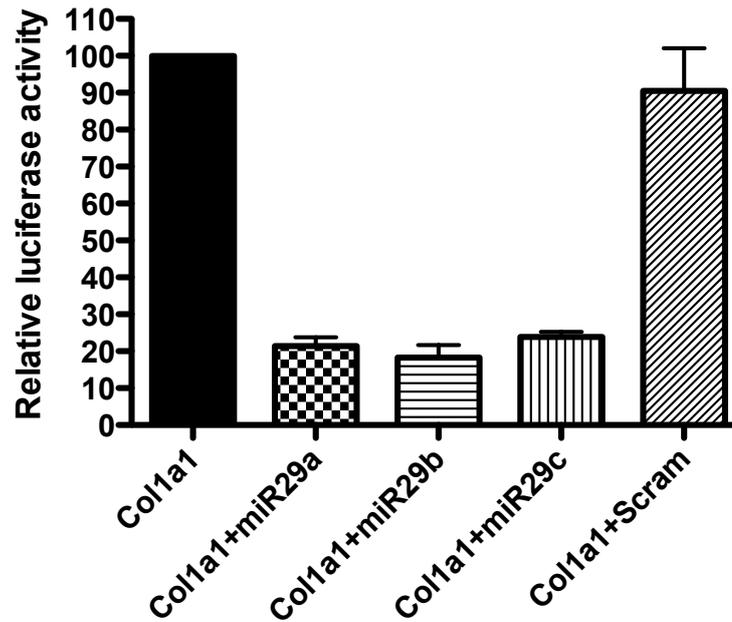


**Figure 6-18 Collagen expression following transfection with miR 29a & b**  
 (A) FACS of labelled c547 mimic control showing shift of curve to right indicating transfection of tenocytes with miR29a mimic. Image show represents 65% transfect ion efficiency in tenocyte culture.(B) Collagen I and III gene expression following transfection with scrambled mimic or mimic for miR29 a and b. (C) Collagen I and III protein levels in tenocytes transfected with mimic miR 29a or b. miR29 a significantly ( $p < 0.01$ ) reduces Collagen III message and protein while miR29b significantly ( $p < 0.01$ ) reduces Collagen I message and protein.

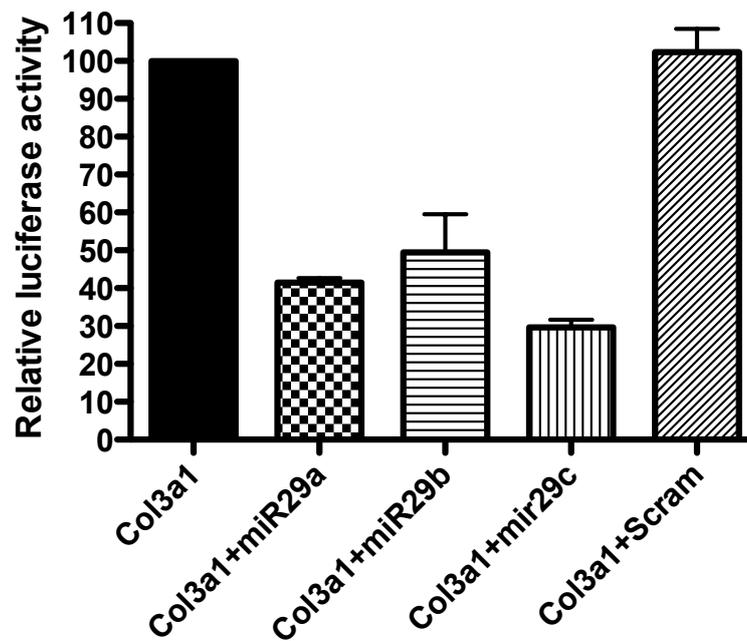
### **6.8.3) Collagen I and III are directly targeted by miR29a and b**

To confirm whether there was direct regulation of collagen genes, we created a luciferase reporter gene system by cloning a part of the 3'UTR of Col 3A1 and Col1A1 with respective binding sites for miR-29a and miR-29b. In the reporter gene assay, co-transfection of cells with pre-miR-29a or b and the luciferase reporter plasmid containing the 3'UTR of COL3A1 or COL1A1 decreased the relative luciferase assay

A



B



**Figure 6-19 Collagen III is a direct target of miR29a**

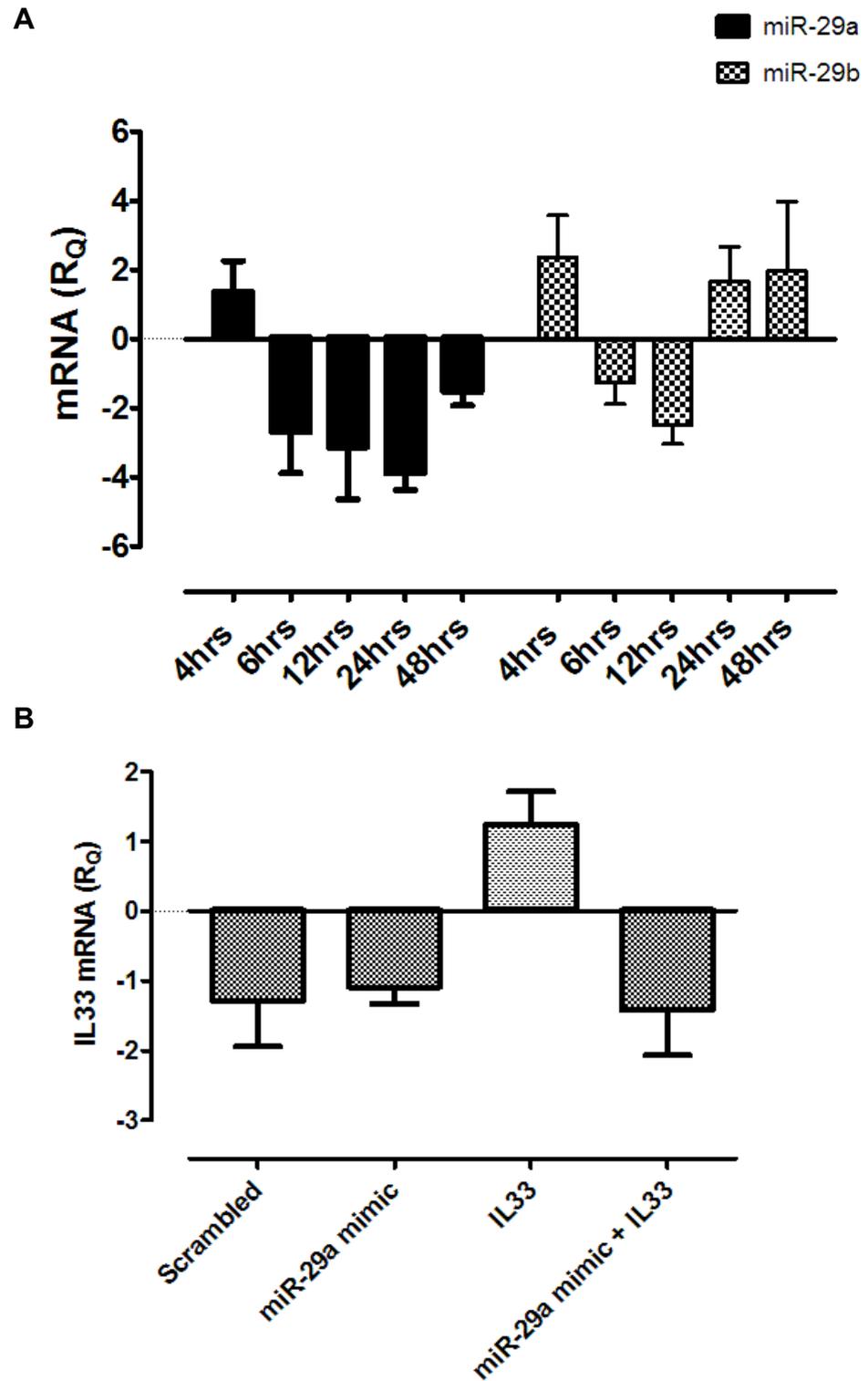
The human miRNA target site was generated by annealing the oligos: of COL I/COLIII 3'UTR which were cloned in both sense and anti-sense orientations downstream of the luciferase gene in pMIR-REPORT luciferase vector (Ambion). These constructs were sequenced to confirm inserts and named pMIR-COL I/COL III-miR29a/b/c and pMIR(A/S)-COL I/III miR29a/b/c, and used for transfection of HEK293 cells. HEK293 cells were cultured in 96-well plates and transfected with 0.1  $\mu$ g of either pMIR-COL I/III miR29a/b/c, pMIR(A/S)-COL I/III-miR29a or pMIR-REPORT, together with 0.01  $\mu$ g of pRL-TK vector (Promega) containing Renilla luciferase and 40 nM of miR-29 or scrambled miRNA (Thermo Scientific Dharmacon®). This was repeated in 3 independent experiments.

#### 6.8.4) IL-33 treated tenocytes show altered miR 29a expression

As I had previously discovered that the addition of rhIL-33 significantly increased the production of Collagen III compared to collagen I, I sought to investigate whether there was a role for the microRNA family 29 in IL-33 induced matrix changes in vitro.

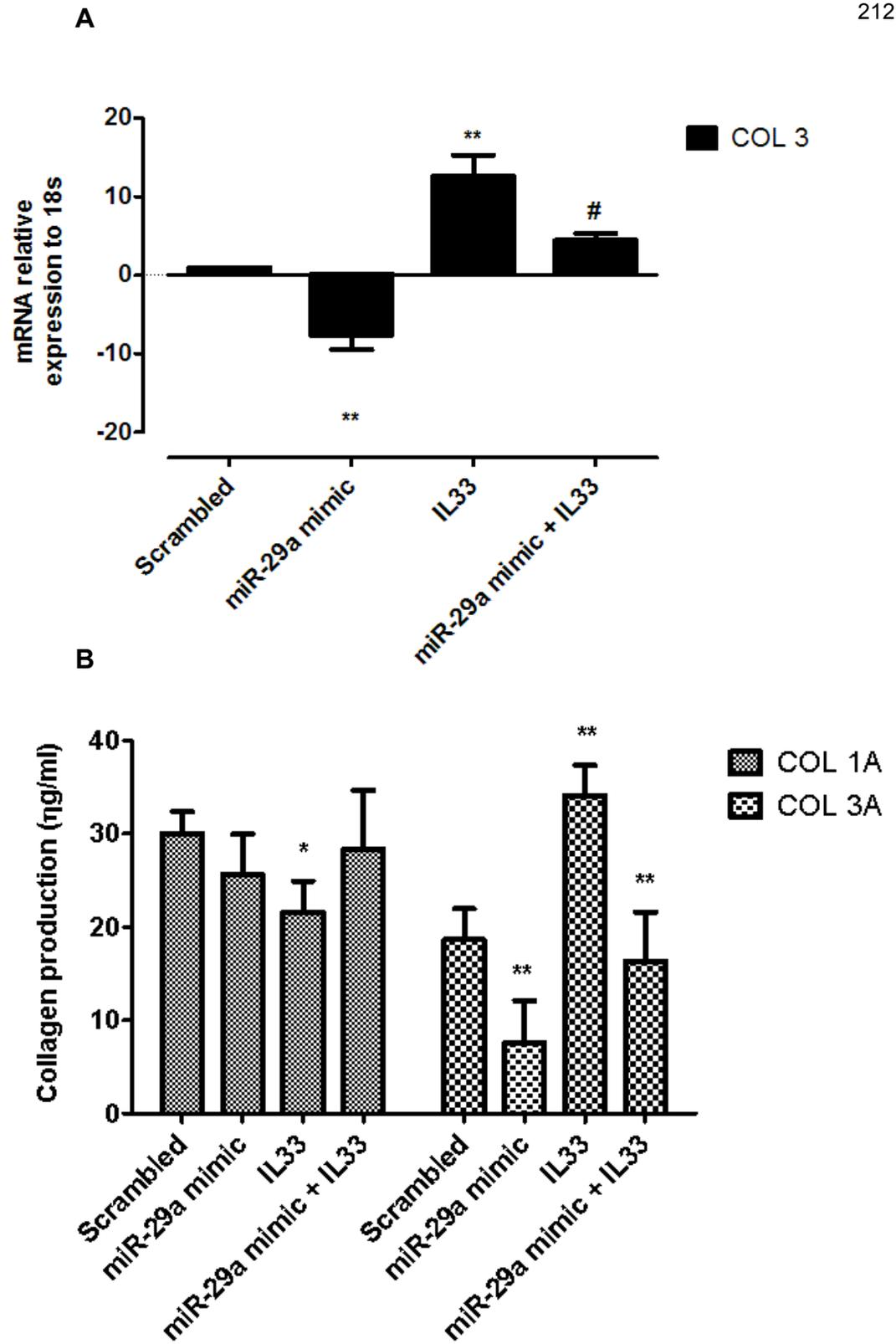
Following incubation with 100ng rhIL-33 the levels of miR 29 a,b &c were analysed in tenocytes cultures at 4,6,12,24 and 48 hours post incubation (Figure 6-20A). IL-33 significantly reduced the expression of miR 29a at 6 ( $\Delta\Delta\text{CT} -2.7$ ), 12 ( $\Delta\Delta\text{CT} -3.1$ ) and 24 hours ( $\Delta\Delta\text{CT} -3.9$ ) post incubation. IL-33 mediated significant effects on mir 29b expression only at 12 hours ( $\Delta\Delta\text{CT} -2.5$ ) post incubation but no other time points. This set of experiments suggested that IL-33 mediated collagen III changes may be in part regulated by miR 29a.

To assess the validity of the observed effects of exogenous IL-33 on miR 29a I then introduced a miR 29a mimic to the culture system. Tenocytes were transfected with miR 29 mimic as previously described. At 24 hours post transfection 100ng of rhIL-33 was added to the culture system and the levels of Collagen I and III were assessed. miR 29a mimic was able to significantly abrogate both collagen III message (+IL-33  $\Delta\Delta\text{CT} 4.9$ , mimic + IL-33  $\Delta\Delta\text{CT} 1.7$ ) and protein (+IL-33  $34\pm 3$  ng/ml, mimic +IL33  $16\pm 5$  ng/ml). miR 29a had no significant effect on Collagen I protein or message levels. The addition of miR 29a mimic  $\pm$  IL-33 had no significant effect on IL-33 gene expression.



**Figure 6-20** The effects of IL-33 on miR 29a expression

(A) The addition of 100ng/ml of rh IL-33 resulted in a significant and persistent reduction in miR 29a expression whilst there was no obvious pattern to miR 29b expression. (B) The addition of mimic± rhIL-33 had no significant effect on IL-33 gene expression.

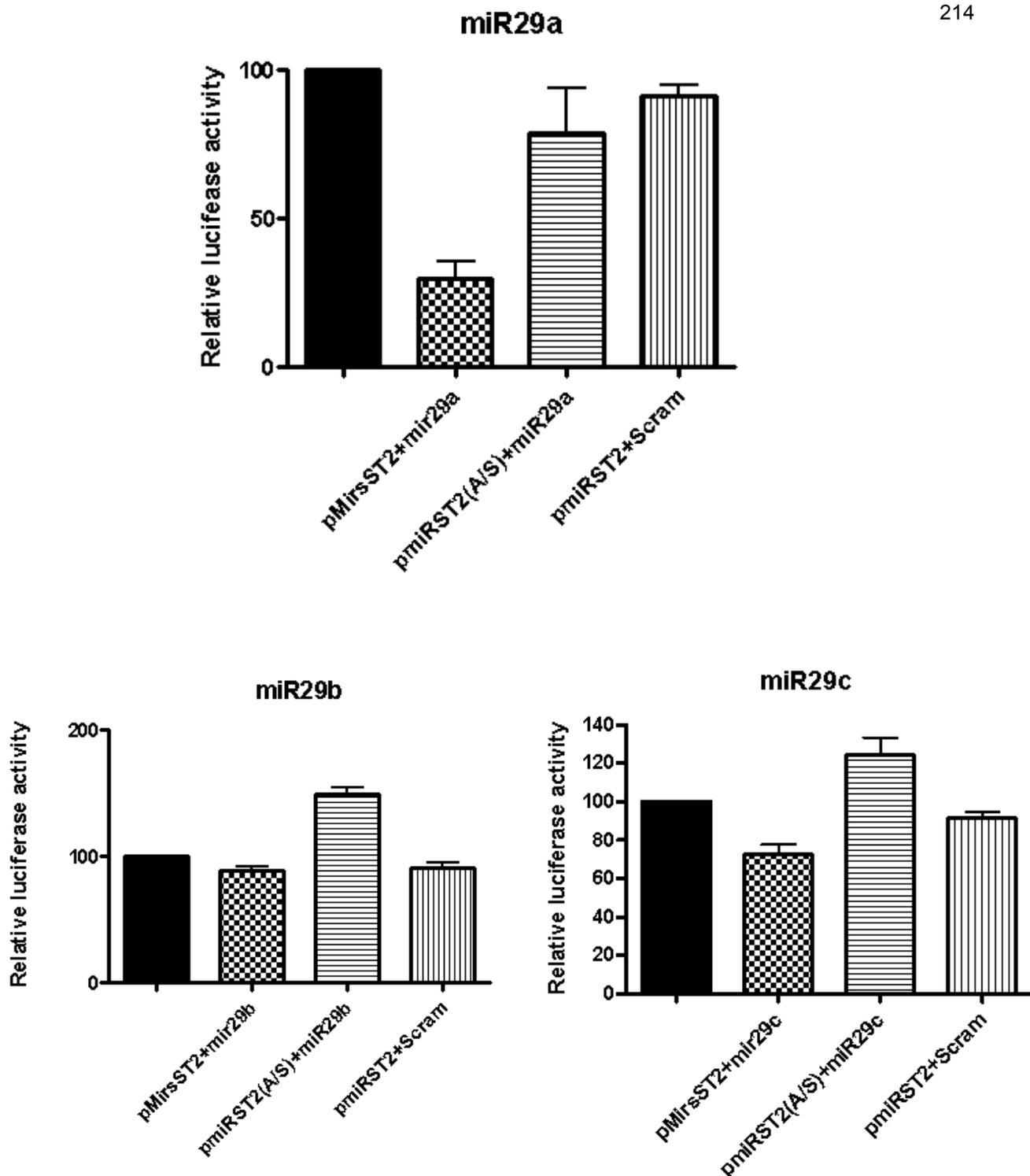


**Figure 6-21** The effects of mir 29a mimic ad IL-33 on Collagen expression  
 The addition of 100ng/ml of rhIL-33 significantly reduced the gene and protein expression of type III collagen in vitro highlighting a possible interaction between microRNA 29a and IL-33 mediated collagen matrix changes.

### 6.8.5) Soluble ST2 is a direct target of miR29a

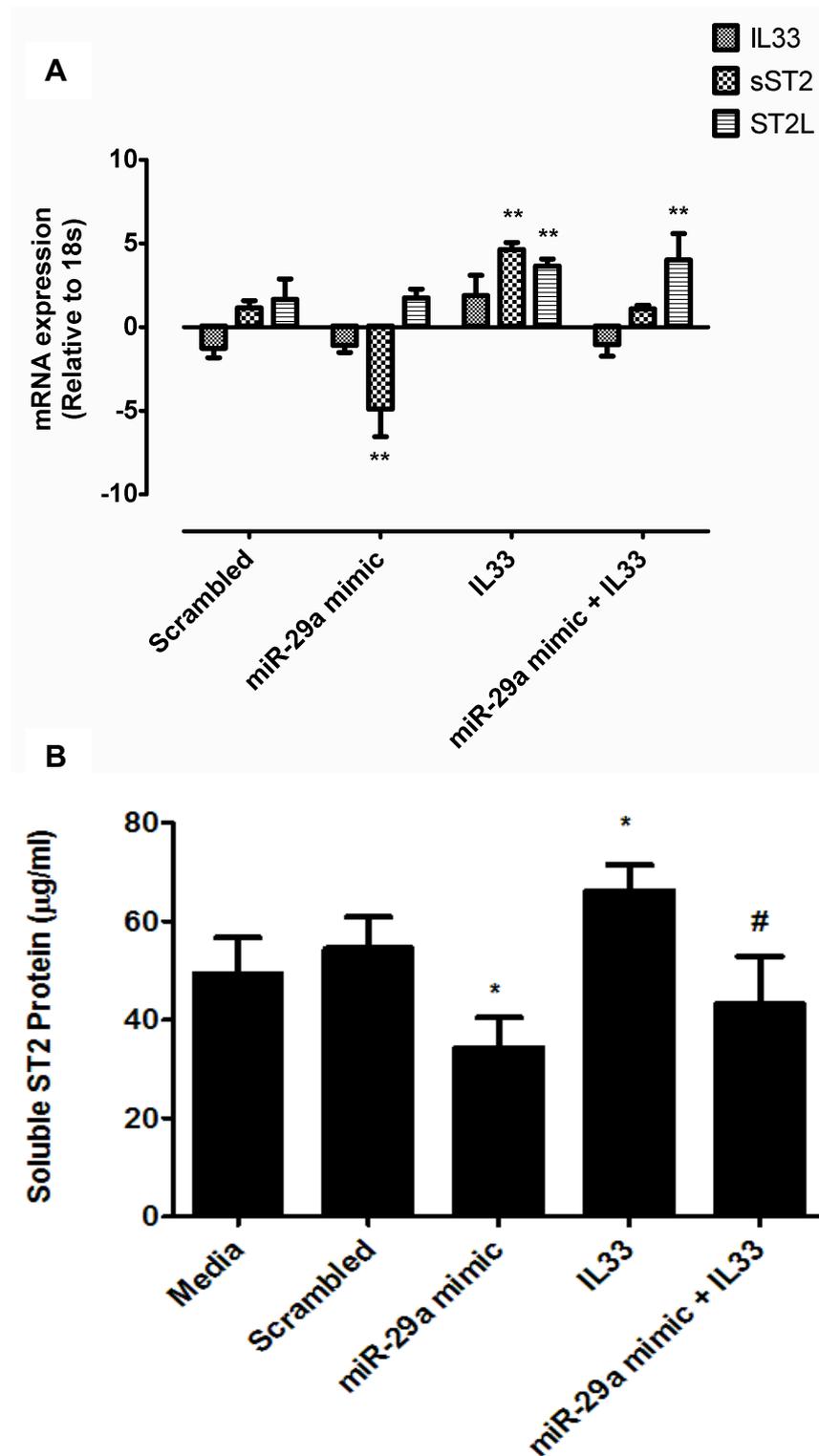
Computational analysis had already suggested miR 29a as a possible target for soluble ST2. In an attempt to link IL-33 mediated collagen changes and miR 29a ability to directly target the 3'UTR of collagen III I next investigated whether the miR29 family was a direct target of sST2. A luciferase reporter gene system was generated by cloning a part of the 3'UTR of soluble ST2 with respective binding sites for miR-29a and miR-29b. In the reporter gene assay, co-transfection of cells with pre-miR-29a and the luciferase reporter plasmid containing the 3'UTR of sST2 decreased the relative luciferase assay (Figure 6-22).

To confirm whether miR-29a did indeed regulate the levels of the candidate target mRNAs, we again transfected miR-29a mimic into tenocytes. Tenocytes were also transfected in parallel with a control mimic miRNA. Transfection efficiency was ensured by FACS analysis of the labelled control mimic as previously described. Soluble ST2 message was significantly ( $p < 0.01$ ) down regulated by transfection with miR 29a mimic by approximately 5 fold with a corresponding modest, yet significant ( $p < 0.05$ ), reduction in soluble ST2 protein (mean sST2 protein  $\mu\text{g/ml} \pm \text{SEM}$ , scrambled  $55 \mu\text{g/ml} \pm 6$ , miR 29a mimic  $34 \mu\text{g/ml} \pm 7$ ) confirming mi29a as a target for soluble ST2. Indeed, miR29a was able to significantly ( $p < 0.05$ ) reduce the IL-33 induced increase in soluble ST2 protein (IL-33  $66 \mu\text{g/ml} \pm 5$ , miR 29a mimic + IL-33  $42 \mu\text{g/ml} \pm 10$ ).



**Figure 6-22 Soluble ST2 is a direct target of miR29a**

The human miRNA target site was generated by annealing the oligos: of sST2 3'UTR which were cloned in both sense and anti-sense orientations downstream of the luciferase gene in pMIR-REPORT luciferase vector (Ambion). These constructs were sequenced to confirm inserts and named pMIR-sST2-miR29a/b/c and pMIR(A/S)-sST2-miR29a/b/c, and used for transfection of HEK293 cells. HEK293 cells were cultured in 96-well plates and transfected with 0.1  $\mu$ g of either pMIR-sST2-miR29a/b/c, pMIR(A/S)-sST2-miR29a or pMIR-REPORT, together with 0.01  $\mu$ g of pRL-TK vector (Promega) containing Renilla luciferase and 40 nM of miR-155 or scrambled miRNA (Thermo Scientific Dharmacon®). This was repeated in 3 independent experiments.



**Figure 6-23 miR29a targets soluble ST2 message and protein in tenocytes**

Confirmation of direct targeting of sST2 by miR29a was analysed using quantitative PCR (A) showing a significant negative fold change in sST2 and a human sST2 ELISA which show a modest but significant reduction of soluble ST2 with miR29a mimic (B). miR-29a was also able to significantly reduce sST2 message and protein following stimulation with rhIL-33

#### 6.8.6) miRNA 29 a & b altered in tendon healing in vivo

Based on the foregoing experiments I next sought to explore the functional relevance of this in an in vivo model of tendon healing. Levels of miR 29 a, b and c were analysed at all-time points post injury in the patellar tendon healing mouse model.

The most profound effect was seen with miR 29a (Figure 6-24). Tendon injury in WT mice resulted in a 22 fold decrease in mir29a on day 1 which was reduced to a 6 fold decrease on day 3. By days 7 and 21 no significant difference was noted in miR29a expression. This effect was significantly abrogated in ST2<sup>-/-</sup> mice with a 7 fold decrease in miR29a on day 1 and a 2 fold decrease on day 3. There was a significant up regulation of miR29a on day 7 ( $\Delta\Delta\text{CT}$  2.3) and day 21 ( $\Delta\Delta\text{CT}$  2.6) in ST2<sup>-/-</sup> mice. Thus in WT injured mice at early time points there is concurrent downregulation of miR29a and up regulation of soluble ST2 message and Collagen III production in keeping with our in vitro findings. ST2<sup>-/-</sup> injured mice have a significantly reduced capacity to down regulate mir29a partially explaining their reduced early collagen matrix response.

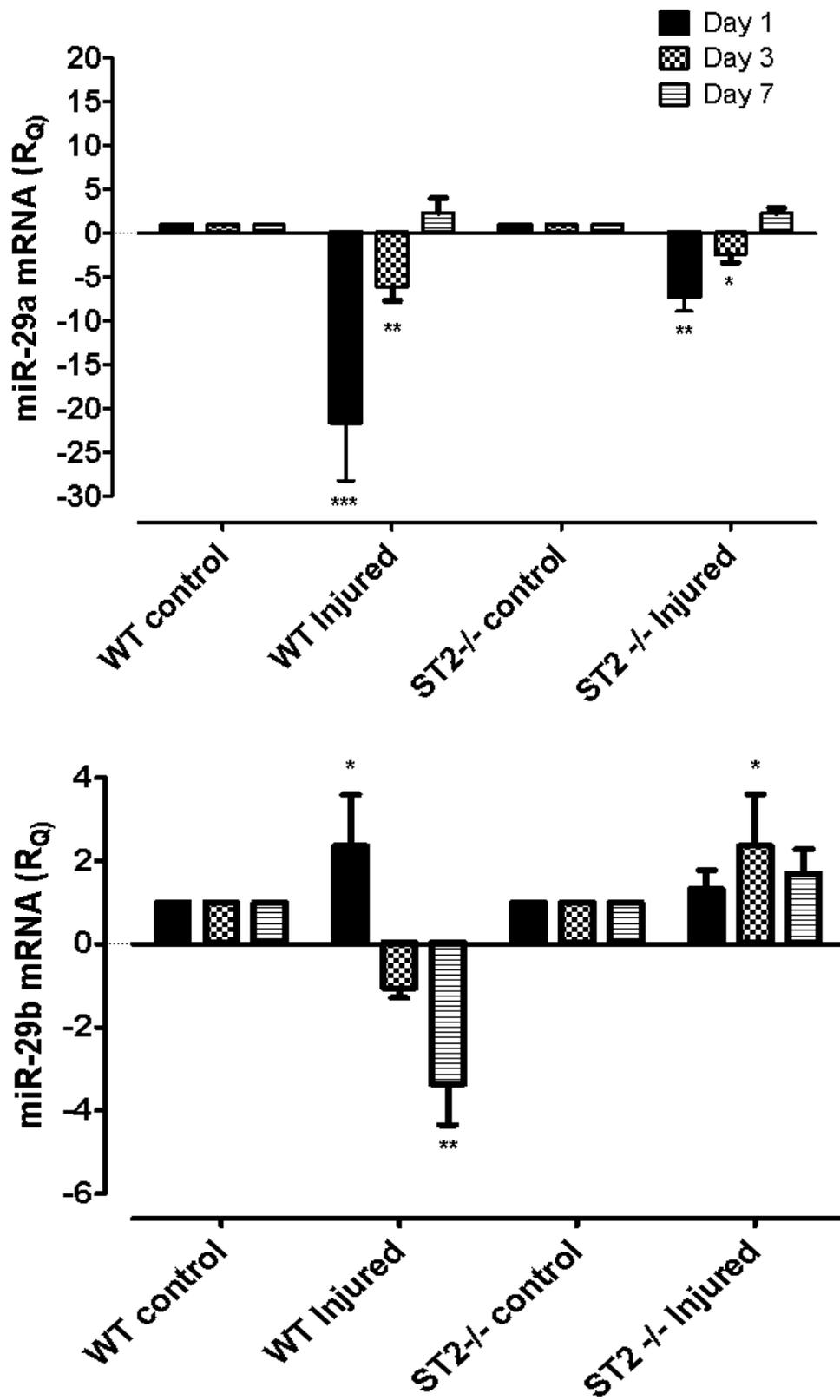
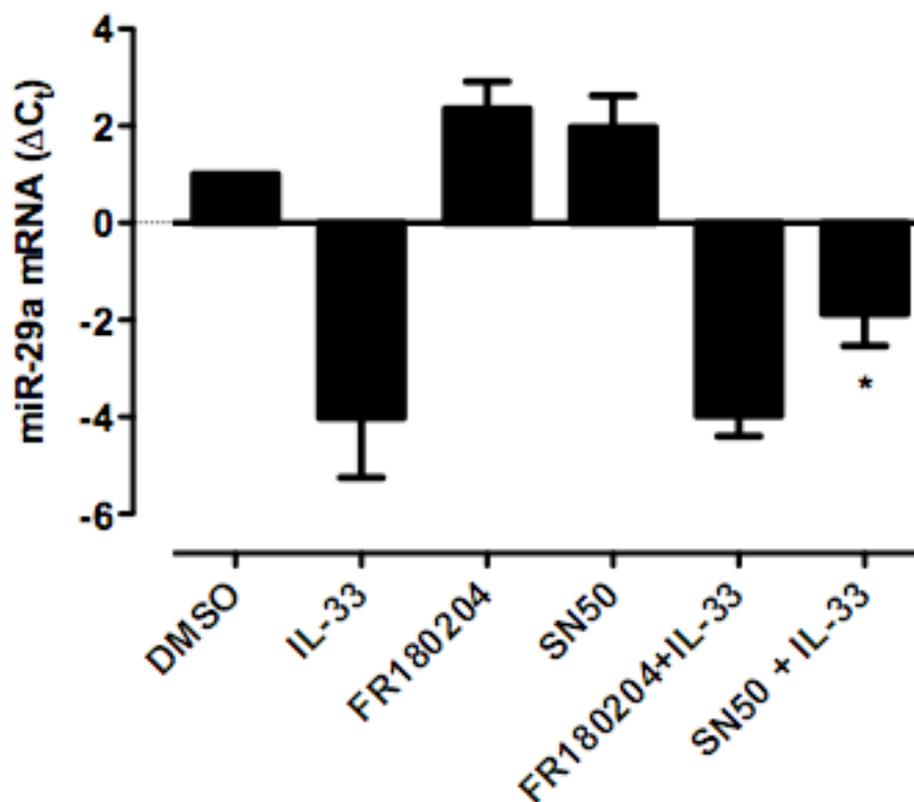


Figure 6-24 miRNA 29 a&b in tendon healing in vivo

Quantitative PCR showing mean fold change  $\pm$  SEM in miRNA 29 a (A) and b (B) in WT and ST2-/- mice in injured versus uninjured animals. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

#### **6.8.7) NFκB signalling modulates miR29a activity in IL-33 treated tenocytes**

Having previously identified NFκB as potential downstream regulators of IL-33 induced collagen III changes and recent evidence that NFκB mediates the suppression of miR29 by directly binding to the promoter of the miR29 gene[402] or activating the transcription repressor YY1[403] we investigated whether inhibition of NFκB had an effect on miR29a expression. SN050 treatment alone had no significant effect on endogenous miR29a expression in tenocyte culture (Figure 6-25). However SN050 attenuated miR29a expression following IL-33 stimulation promoting NFκB as a repressor of miR29a particularly as ERK inhibition appeared to have no direct effect on miR29a expression.



**Figure 6-25 ERK and NFκB inhibitors effects on miR29a expression**

The levels of miRNA 29a were determined by real time PCR. NFκB inhibition significantly reduced the IL-33 induced down regulation of miRNA 29a while ERK inhibition had no direct effect. Data shown as the mean ± SD of triplicate samples and represent experiments on three individual patient samples. \*p<0.05, \*\*p<0.01 compared to control samples

#### **6.8.8) Addition of rhIL-33 alters miR 29 a and b expression in vivo**

The effect of rhIL-33 was next analysed on micro RNA 29a as the most profound effect had been noted in this miR in vivo. The addition of rh-IL33 significantly reduced miR29a expression in uninjured tendons at all-time points compared to PBS injected controls (Figure 6-26) suggesting that IL-33/ST2 signalling reduces mir29a expression with a subsequent change in the collagen matrix.

The affect was most profound in injured WT mice with a further 10 fold reduction in miR 29a with the addition of rhIL-33. Addition of rhIL-33 in ST2<sup>-/-</sup> mice had no significant effect on miR 29a expression in injured or uninjured tendons again suggesting that miR29a down regulation is in part directly affected by IL-33/ST2 signalling.

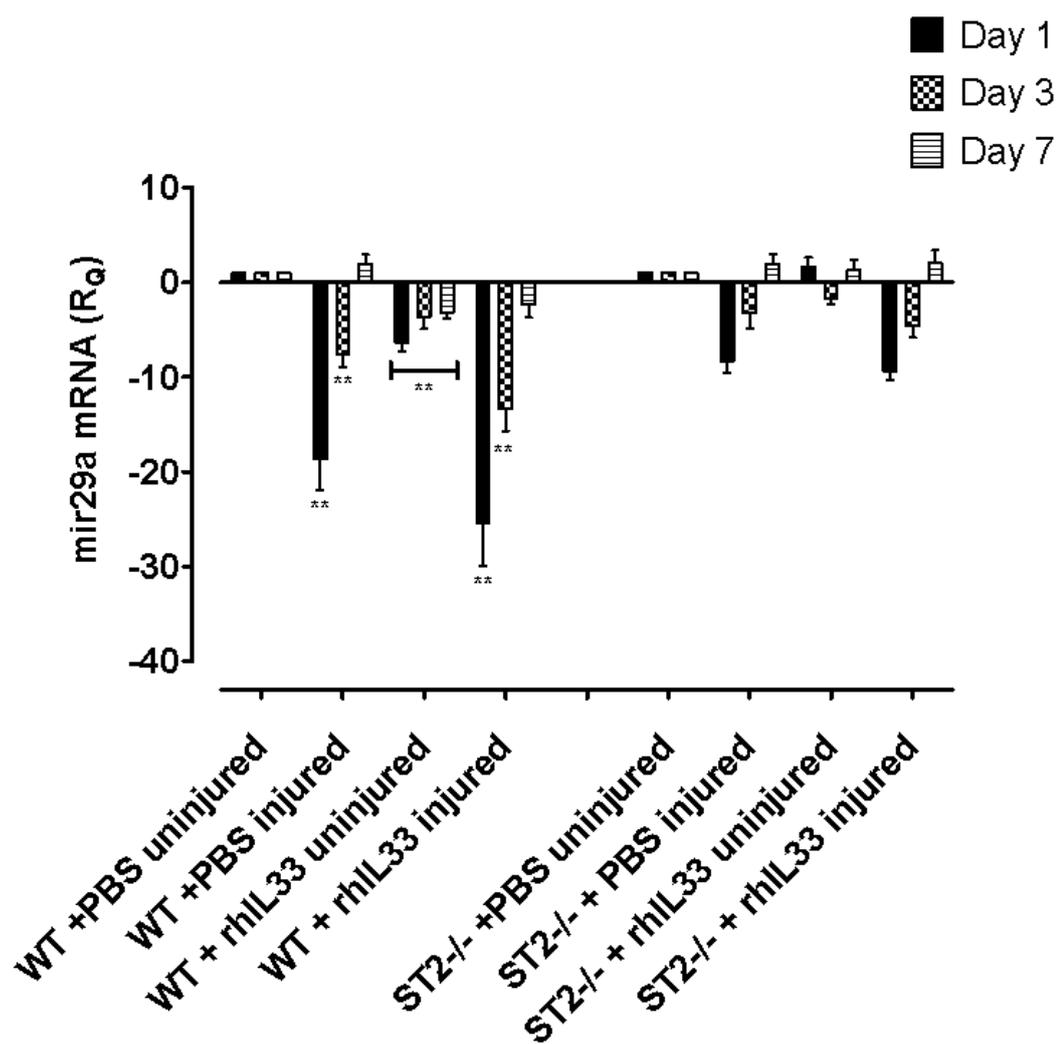


Figure 6-26 miRNA 29a following IL-33 treatment in vivo.

Quantitative PCR showing mean fold change  $\pm$  SEM in miRNA 29a in WT and ST2<sup>-/-</sup> mice in injured versus uninjured animals following treatment with rhIL-33 or PBS. rh-IL33 significantly reduced mi29a expression in uninjured tendons at all-time points compared to PBS injected controls. Data are shown as the mean fold change  $\pm$  SD of duplicate samples and are representative of experiments using four mice per group.

## 6.9) Discussion and Conclusions

These experiments clearly demonstrate a role for IL-33 as a possible *alarmin* in early tendon injury and indeed human tendinopathy. The novel finding of a possible role for microRNA 29a in the regulation of IL-33 mediated effects provides further detailed and entirely novel mechanistic insight and thus targets in tendinopathy.

The previous chapters of this thesis have illustrated a key role for inflammation and the subsequent production of cytokines in human tendon disease. IL-33 has become increasingly associated with musculoskeletal pathologies over the past few years [233]. The experiments in this section show IL-33 to be present in human tendon biopsies at the early stage of disease and localisation studies appear to suggest that the majority is expressed in endothelial cells but also a proportion in fibroblast like tenocytes. This is in keeping with the current immunohistological evaluations in the published literature. End stage biopsies have significantly less IL-33 expression at the message and protein level promoting the concept of IL-33 as an early tissue mediator in tendon injury and subsequent tissue remodelling. Upon cell injury endogenous danger signals, so called damage associated molecular patterns, are released by necrotic cells including heat shock proteins [404], HMGB1 [405], uric acid [406] and IL-1 family members [407, 408] including IL-33 [199, 401]. These danger signals are subsequently recognised by various immune cells that initiate inflammatory responses. These sentinel cells are prepositioned in tissue allowing a rapid response and are capable of producing and secreting selective mediators required for the induction of an inflammatory response.

Mast cells possess these important characteristics [365] and have their activation has been strongly associated with IL-33 [210]. Recent work has demonstrated a key role for mast cells in responding to cell injury by recognising IL-33 released from necrotic cells [409] while IgE activated mast cells produce IL-33 [410] thus initiating proinflammatory responses. Importantly IL-33 has also been shown to be released from endothelial cells following mechanical injury [199] and may be relevant to IL-33's egress in tendon injury/damage. The studies in this thesis have shown a significant proportion of mast cells in human tendinopathic biopsies and tendon healing in vivo. Therefore the IL-33/mast cell axis may provide a mechanism whereby early tendon injury results in IL-33 release/up

regulation, in turn activating mast cells located in close proximity to the injury/degeneration thus perpetuating an inflammatory or healing process. Indeed IL-33 induces mast cell production of IL-1, IL-6, IL-13, and a range of chemokines [155] all of which have previously been shown to effect matrix metalloproteinases production in fibroblasts [330] may also be important in matrix remodelling. Based on these findings I propose IL-33 as an important DAMP in early tendon damage.

Interestingly the biomechanical data suggests a pathogenic role for IL-33 in tendon injury/healing. The addition of rhIL-33 significantly reduced the load to failure of WT mice by approximately 30% at early time points and could be as a result of the concomitant collagen III matrix changes, which are known to result in mechanically inferior tendon. Although the patellar tendon injury model is not a model of tendinopathy my data may give an insight into the repetitive tendon micro trauma and mechanical stress that tendinopathic tendon suffers. Thus one plausible mechanism may be that upon repeated micro injury IL-33 is up regulated with its subsequent release through apoptosis/necrosis, which in turn drives the matrix degeneration and proinflammatory cytokine production propelling the tendon toward a pathological state such as that seen in early tendinopathy biopsies. Further work is now required with neutralising antibodies to IL-33 to ascertain whether the pathogenic role for IL-33 in tendinopathy may have any translational benefits.

In vitro experiments suggest IL-33 switches collagen matrix production significantly toward a Collagen III phenotype. Tendon injury in vivo results in a large increase in IL-33 message immediately post injury with the appearance of nuclear and cytosolic protein expression. The subsequent change in Collagen III message and protein levels strongly promotes a mechanistic role for IL-33 in collagen matrix changes in tendon healing/injury. Others have implicated IL-33 in collagen synthesis. In an elegant study soluble collagen levels were increased 4-fold in skin punch biopsies with the majority of infiltrating leukocytes found to be eosinophils in mice administered with IL-33 s.c [247]. Administration of IL-33 resulted in significant modulation of collagen III and VI and increased expression of IL-13 mRNA. They established that IL-33 induced fibrosis required IL-13 using IL-13 KO mice and eosinophils using  $\Delta$ dbiGATA mice. Increasing evidence points toward IL-33 release from necrotic cells [200] which is a plausible mechanism for release in post injury tendons. IL-33 activates ERK1/2 and NF $\kappa$ B in tenocytes both of which are known to modulate both collagen I gene expression through activated

of NF $\kappa$ B [411, 412] and collagen III which is a target for ERK 1&2 [413, 414]. Indeed we were able to marginally reduce the amount of collagen type III through ERK inhibition. The substantial collagen change in vivo with IL-33 administration may provide novel therapeutic approaches through selective inhibition of downstream targets such as ERK1/2 and warrants further investigation

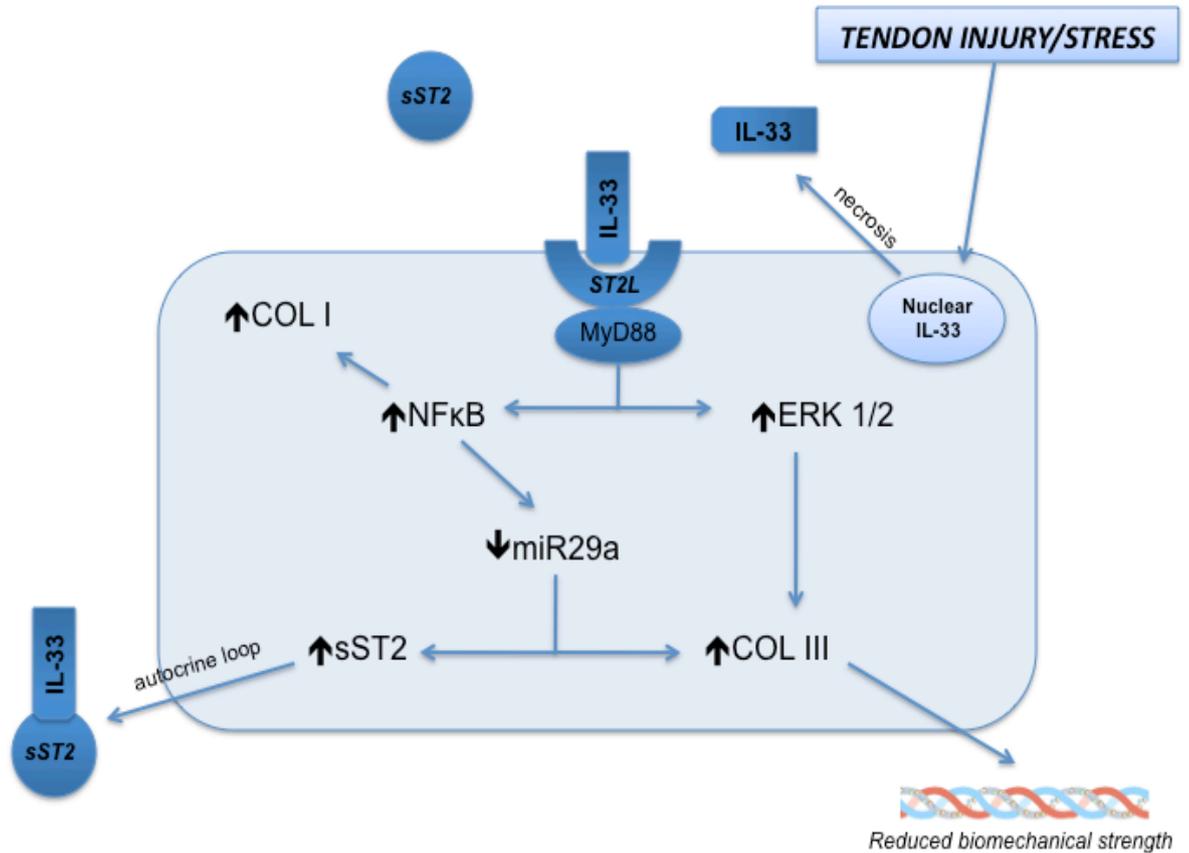
The current set of experiments provide convincing evidence for a functional role for miR-29 in murine and human tendon injury. The direct posttranscriptional regulation of collagens by miR-29 adds a novel aspect to the complex network of factors that regulate the expression of collagen genes in tendinopathy/tendon healing at the level of transcription which include TGF- $\beta$  [415], Smad-7 [416], angiotension receptor [417], TGF-1 [418] and mechanical stress [419]. The regulation of collagen by the mir 29 family has been highlighted in studies of human liver fibrosis, prostate cancer and systemic sclerosis. miR29 mediates the regulation of collagen matrix genes through TGF- $\beta$  and NF $\kappa$ B downregulation of miR 29 family members in hepatic stellate cells(HSCs) [420]. Indeed in another study on HSCs miR 29b was the most effective suppressor of type I collagen at the mRNA and protein level via its direct binding to the Col1A1 3'UTR [399]. Elegant studies in human systemic sclerosis reveals miR 29a was strongly down regulated in SSc fibroblast compared to controls [324]. Functional studies suggested a direct regulation of collagen by miR29a whilst TGF $\beta$ , PDGF-B and IL-4 reduced miR 29a levels in fibroblasts. This was confirmed in a bleomycin model of skin fibrosis where inhibition of PDGF-B and TGF $\beta$  pathways by imatinib resorted the levels of miR-29a. Our results suggest that miR-29a acts as a repressor to fine-tune collagen expression in tendon healing. The decreased expression in human biopsies also permissively implicates it in tendinopathy.

The precise mechanisms that lead to downregulation of miR29, especially miR 29a in tendon injury/tendinopathy remain to be determined but these experiments show that IL-33, decreased the expression of miR 29a in tenocytes and in vivo. Moreover the stimulatory effect of IL-33 on collagen synthesis could be partially rescued with miR29a, suggesting that the reduced levels of miR 29a occur downstream of IL-33 and at least partially regulates its collagen effects. Indeed the discovery that soluble ST2 is a direct target of mi29a promotes the hypothesis that IL-33 is a central early mediator of tendon matrix changes driving a collagen 'switch' than may ultimately change the phenotypical tendon structure with fundamental clinical implications. Indeed the targeting of miR-29 family

members as posttranscriptional regulators could be a potent modulator of collagen changes in tendinopathy. This needs to be tested in future studies. Specifically the effects of modification of miR-29a levels on the synthesis of extracellular matrix proteins should be analysed in an animal tendinopathy model. Optimally miR-29-KO mice could be created and tested in the patellar tendon healing model. So far miRNA based therapeutic approaches in animal studies of other diseases have been promising since toxic effects have not been observed and a recent human trial targeting a liver specific miRNA involved in hepatitis C has yielded promising results. Thus the development of strategies to maintain expression or to prevent repression of miR-29a in tendinopathy may be a promising future therapeutic.

Further investigation in this area are now required. To tie up the role of endogenous IL-33 on mi29a expression and matrix changes I intend to administer neutralising antibody to IL-33 in WT and ST2<sup>-/-</sup> mice and carry out biomechanical studies to appreciate any benefit to IL-33 targeted therapy in tendinopathy.

Based on the experiments in this chapter I propose IL-33 as an important and influential *alarmin* in early tendon injury and tendinopathy which may be influential in the balance between reparation and degeneration in tendon disease (Figure 6-27). A novel role for miR29a as a posttranscriptional regulator of matrix genes in tendon healing and tendinopathy has been discovered and may provide a targeted path for matrix remodelling in tendinopathy.



**Figure 6-27 Schematic diagram illustrating the role of the IL33/ST2 axis in tendinopathy. An tendon injury or repetitive micro tears causing stress that a tendon cell experiences results in the release interleukin 33 and the downstream phosphorylation of NFκB which in turn represses miR29a causing an increase in collagen type III and soluble ST2 production. An increase in collagen III reduces the tendons ultimate tensile strength lending it to early failure while soluble ST2 acts in an autocrine fashion which may ultimately be a protective mechanism whereby excess IL-33 is removed from the system.**

## Chapter 7 Discussion and Future work

As always, experiments leave more questions than answers. However this thesis has I believe opened a debate on the key role of inflammation and its downstream molecular events in the role of tendon disease. For many years inflammation has been regarded throughout the tendinopathy field as misnomer and to have no influence in the pathogenesis of the disease mainly based on examination of end stage surgical specimens lacking an inflammatory infiltrate. I always believed this was a rather 'missed the boat' situation where something that had already happened was being checked for and was thus unlikely to be there. Inflammation is a key mechanism whereby injured tissue and cells recover and protect themselves against further insult with the attraction of leukocytes and sentinel immune cells. Many musculoskeletal pathologies including osteoarthritis, tendon rupture, seronegative/ positive spondyloarthropathies and muscle damage have a key inflammatory component. My findings of inflammation in an early human model of tendinopathy were chance findings when I hypothesised that subscapularis tissue would be normal. Nonetheless the fact that this showed early tendinopathy changes and was reproducible has finally allowed cellular and mechanistic studies into the early disease where clearly inflammation is a key molecular process.

Many studies on tendinopathy have lacked mechanistic investigation. My studies have shown a key role for the cytokines IL-17 and IL-33 in tendinopathy. Rigorous mechanistic investigation has yielded exciting results around the modulation of extracellular matrix genes and proteins by these cytokines and their by-products. Firstly the deciphering of a molecular role for hypoxia in tendinopathy adds credence to hypoxia as an important regulator of tendinopathy. Since Jozsa's classic manuscript on this subject in 1982 many textbooks and review articles have hypothesised that hypoxia is a key factor in tendon disease. While recent *in vivo* evidence from Professor Carr's group in Oxford have shown altered O<sub>2</sub> tensions in human rotator cuff, the investigations in this thesis add substance to the theory of hypoxic tendon injury. We show that hypoxia alters collagen matrix production at the message and protein level but also show how tenocytes may regulate this process through MAPK activation and cytokine production with a possible drive toward tenocytes apoptosis.

Secondly through parallel studies in RA with a colleague I investigated the presence and subsequent mechanisms behind IL-17 in tendinopathy. Whilst surprising that a tissue process that has relatively few T cells, which are still considered in the literature to be the main source of IL-17A, has IL-17 present at the message and protein level on single and double immunofluorescent staining it does support the growing body of evidence that mast cells are important immune *sentinel* cells and are capable of producing a significantly potent cytokine such as IL-17. IL-17 in particular was able to drive a significant proportion of tenocytes toward apoptosis, result in a significant increase in type III collagen and increase the production of proinflammatory cytokines such as TNF $\alpha$  and MIP-1 $\alpha$ . This in addition to recent microarray analysis of torn supraspinatus samples from the US which has highlighted IL-17F in near end stage biopsy tissue may well suggest that targeting of IL-17 and its downstream events could be important in tendinopathy

Thirdly is the significant role that IL-33 may play as an alarmin in tendinopathy/tendon injury. Whilst at the start of these studies I hypothesised that IL-33 may be a novel discovery in tendinopathy and play a role in inflammation my studies have revealed it as an important regulator early tendon injury. In particular the role of IL-33 as early warning system to the tendon with subsequent matrix changes has for the first time given us a true molecular regulator whose downstream effects translate to biomechanical weakness. The US microarray that I mentioned has also shown a significant increase in IL-13RA2 compared to controls. Rankin and colleagues found IL-13 to be a key component of IL33 mediated collagen changes in a skin healing model and although not specifically investigated in this thesis further work to explore a possible role for IL-13/IL-33 mediated collagen changes in tendinopathy would be appropriate. The elusive nuclear function of IL-33 and its probable role as a repressor are currently being investigated at our laboratory. The information yielded from microarray analysis of nuclear regulated IL-33 genes will hopefully give us an insight into its role in the nuclear compartment.

Finally again due to parallel studies in the laboratory this thesis has identified a role for microRNA in the post transcriptional regulation of collagen in tendon injury and likely tendinopathy. Collagen regulation and subsequent matrix changes particularly the replacement of Collagen I with III have long been identified as the key pathophysiological mechanisms in reducing biomechanical

strength with subsequent damage and possible clinical rupture of tendons. The miRNA 29 family is slowly becoming a key molecule in collagen matrix regulation in numerous diseases including liver fibrosis, prostate cancer and systemic sclerosis. Indeed Maurer and colleagues work in systemic sclerosis has led to a patent request for mir29a therapy in systemic sclerosis which is extremely promising and exciting. Thus the identification that miRNA 29a is significantly altered in vivo along with in vitro work highlighting it as direct target of Collagen III and indeed soluble ST2 it may well translate into a possible target to alter the collagen III *switch* in tendinopathy particularly if IL-33/ST2 signalling is an upstream modulator of collagen genes. These experiments have brought up to date cutting edge science to the field and hopefully identified a key target for future work.

In summary whilst previous investigations have painted the tenocyte as a mere bystander in the pathological process I would argue based on these results that the biology and mechanistic investigation of up and downstream regulators of molecular processes in tenocytes including collagen synthesis, apoptosis and cytokine production should be studied as a means to unravel the pathophysiology of tendon disease for true translational benefits to patients.

## Chapter 8 References

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