***Persistent stromal fibroblast activation is present in chronic tendinopathy***

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**ABSTRACT**

**Background:** Growing evidence supports a key role for inflammation in the onset and progression of tendinopathy. However, the effects of the inflammatory infiltrate on tendon cells is poorly understood.

**Methods:** We investigated stromal fibroblast activation signatures in tissues and cells from patients with tendinopathy. Diseased tendons were collected from well phenotyped patient cohorts with supraspinatus tendinopathy before and after sub-acromial decompression treatment. Healthy tendons were collected from patients undergoing shoulder stabilisation or Anterior Cruciate Ligament repair. Stromal fibroblast activation markers including podoplanin (PDPN), CD106 (VCAM-1) and CD248 were investigated by immunostaining, flow cytometry and RT-qPCR.

**Results:** PDPN, CD248 and CD106 were increased in diseased compared to healthy tendon tissues. This stromal fibroblast activation signature persisted in tendon biopsies in patients 2-4 year’s post-treatment. PDPN, CD248 and CD106 were increased in diseased compared to healthy tendon cells. IL-1β treatment induced PDPN and CD106 but not CD248. IL-1β treatment induced NF-κB target genesin healthy cells which gradually declined following replacement with cytokine-free media, whilst *PDPN* and *CD106* remained above pre-stimulated levels. IL-1β treated diseased cells showed a more profound induction of *PDPN* and *CD106* and sustained expression of *IL6* and *IL8* mRNAcompared to IL-1β treated healthy cells.

**Conclusions:** We conclude stromal fibroblast activation markers are increased and persist in diseased compared to healthy tendon tissues and cells. Diseased tendon cells show distinct stromal fibroblast populations. IL-1β treatment induced persistent stromal fibroblast activation which was more profound in diseased cells. Persistent stromal fibroblast activation may be implicated in the development of chronic inflammation and recurrent tendinopathy. Targeting this stromal fibroblast activation signature is a potential therapeutic strategy.

**Key words:** tendon, tendinopathy, inflammation, stromal fibroblast

**BACKGROUND**

Musculoskeletal diseases account for 5 of the top 15 causes of years lived with disease in well-resourced health systems [1]. Soft tissue pathologies such as tendinopathy are a common cause of pain and loss of function and an important and increasing component of health expenditure in ageing societies [2]. Diseased tendons heal by forming a repair scar; however, the normal architecture, composition, and tissue function are not fully restored, increasing susceptibility to chronic injury. The aetiology of tendinopathy is complex and multifactorial, encompassing the effects of repetitive damage, daily exercise, ageing and genetic factors [3, 4]. Growing evidence supports the contribution of inflammation to the onset and progression of tendinopathy [5-8], however the mechanisms underpinning the development of chronic tendon inflammation are poorly understood.

Recent work highlights the complex activation states of immune cells including macrophages populating diseased human shoulder tendons [8]. Investigation of inflammation activation pathways in cultured stromal cells from diseased human tendons revealed that diseased stromal cells may be primed for inflammation [8]. However, the mechanism for this priming and the relative contribution of tendon stromal cells to sustaining chronic inflammation are unknown.

Recent studies support the importance of tissue microenvironments and the innate immune response in perpetuating the inflammatory process. Non-myeloid/non-lymphoid populations such as resident stromal fibroblasts are known to play a prominent role in the generation and maintenance of chronic synovial inflammation [9, 10]. Stromal fibroblast activation is reported in Rheumatoid Arthritis (RA) in which resident stromal cells fail to switch off their inflammatory programme. Phenotypic alterations in RA synovial fibroblasts play an important role in the switch from resolving to persistent disease [11, 12]. The process by which fibroblasts produce cytokines, chemokines, prostanoids and extracellular matrix proteins is termed ‘fibroblast activation’. Activated fibroblasts are found in damaged, inflamed or healing tissues and promote the retention of immune cells and regulate their behaviour [12]. Previously identified stromal fibroblast activation markers in RA synovium include podoplanin (PDPN), CD106 (VCAM-1) and CD248 (tumour endothelial marker-1/endosialin). PDPN is a transmembrane glycoprotein implicated in invasiveness of cancer metastasis, CD106 functions as a cell adhesion molecule [13]. CD248 is a transmembrane receptor whose ligands include collagen 1 and fibronectin [14]. CD248 expression is up-regulated by inflammation, fibrosis, angiogenesis and malignancy [15, 16]. These stromal fibroblast activation markers have been identified in different locations of RA synovium. PDPN and CD106 are located in the synovial lining and CD248 in the sub-lining layer and are thought to represent distinct fibroblast subsets [12, 13].

In this study, we identify the potential role of tendon stromal fibroblasts (resident fibroblasts populating tendons) as an important tissue resident population implicated in the development of chronic tendon inflammation. We studied supraspinatus tendon tissues from a well-phenotyped longitudinal cohort of symptomatic patients pre and post-treatment. We characterised distinct stromal fibroblast activation signatures in healthy, diseased and post-treatment tendon tissues. As IL-1β induces NF-κB target genes known to be highly expressed in early stage tendinopathy [8], we further investigated if IL-1β treatment of cultured tendon cells induced persistent stromal fibroblast activation, and if this response differed between healthy and diseased cells. We hypothesized that diseased tendons would express stromal fibroblast activation markers and that IL-1β treated diseased tendon cells would show profound induction of PDPN, CD106 and NF-κB target genes.

**METHODS**

**Collection of tendon tissues**

Patients were recruited from shoulder referral clinics where the structural integrity of the rotator cuff was determined ultrasonographically. Patients presenting to the referral shoulder clinic had failed non-operative treatment, including a course of physical therapy and glucocorticoid injections into the sub-acromial space and had experienced pain for a minimum of 3 months. Patients completed the Oxford Shoulder Score (OSS), a validated and widely used clinical outcome measure scoring from 0 (severe disease) to 48 (normal function) [17]. Samples of healthy supraspinatus tendons (n=5) were collected intra-operatively from patients undergoing shoulder surgery for post-traumatic instability. These biopsies were collected from male and female patients (ages 20-30 years; mean, 23±3.8 years) that had intact supraspinatus tendons on ultrasound, which was confirmed at surgery. Healthy subscapularis tendons (n=4) were collected from male or female patients undergoing shoulder surgery for post-traumatic instability (ages 61-77 years; mean, 66±8 years). BMI from the healthy shoulder tendon patient group was 24.5 (±1.5).

Diseased supraspinatus tendons were collected from male and female patients undergoing sub-acromial decompression surgery (biopsies collected from n=6) or surgical debridement of a supraspinatus tendon tear (n=9). Tendon tear sizes were classified as small (≤1 cm), medium (>1 and ≤3 cm), large (>3 and ≤5 cm) and massive (>5 cm in anterior-posterior length) [18]. Patients with diseased supraspinatus tendons were aged between 44 and 75 years (mean, 55±18.3 years). BMI for the diseased tendon patient group was not significantly different to that of the healthy group (27.8 ±1.2). Torn tendons were collected under research ethics from the Oxford Musculoskeletal Biobank (09/H0606/11). Biopsies were also taken from patients between 2-4 years after undergoing sub-acromial decompression surgery, in whom pain had resolved completely (n=6) or pain persisted (n=5). Post-treatment biopsies were collected via percutaneous ultrasound-guided biopsy under local anesthesia. The biopsy specimen was taken using a trucut needle 5-10 mm posterior to the anterior edge of the supraspinatus tendon. This validated biopsy technique is described in detail elsewhere [19]. Exclusion criteria for all patients in this study included previous shoulder surgery, other shoulder pathology or acute trauma, rheumatoid arthritis and systemic inflammatory disease.

For cell experiments, healthy hamstring tendons were collected from 10 male and female patients undergoing surgical reconstruction of their anterior cruciate ligament. All patients were aged between 18 and 48 years (mean, 25.5 ± 11 years). BMI from the healthy hamstring tendon patient group was 24.9 (± 2.1) and was not significantly different to that of the diseased patient group. Hamstring tendons were collected under research ethics from the Oxford Musculoskeletal Biobank (09/H0606/11). Hamstring tendons were immediately placed in Dulbecco’s minimum essential medium (DMEM)/F12 (Lonza) and processed in tissue culture to isolate tendon derived stromal cells.

**Processing of tendon samples**

***Immunohistochemistry and immunofluorescence.*** Healthy and diseased supraspinatus tendons were immersed in 10% buffered formalin for 0.5 mm/hour. After fixation, tendons were processed using a Leica ASP300S tissue processor and embedded in paraffin wax. Tissues were sectioned at 4 μm using a rotary RM2135 microtome (Leica Microsystems Ltd.) onto adhesive glass slides and baked at 60°C for 30 min and 37°C for 60 min.

***Histological assessment of healthy and diseased supraspinatus tendons***

Histological assessment of tendons collected from the study cohort was performed on hematoxylin and eosin–stained sections using the Bonar scoring system (0 to 12) that evaluates tissue structure [20]. Healthy supraspinatus tendons exhibited a more normal tissue architecture (median, 2; interquartile range, 1 to 2) compared to tendinopathic (median, 7; interquartile range, 6 to 8) and torn supraspinatus tendons (median, 10; interquartile range, 8.25 to 10).

***Gene expression.*** Samples of healthy subscapularis and diseased supraspinatus tendons were immediately snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**Immunohistochemistry and immunofluorescence for stromal fibroblast activation markers**

For antigen retrieval, slides were baked at 60°C for 60 min, and tissue sections subjected to deparaffinization and target retrieval steps (heat-mediated antigen retrieval at high pH) using an automated PT Link (Dako). For single staining immunohistochemistry, antibody staining was performed using the EnVision FLEX visualization system with an Autostainer Link 48 (Dako). Antibody binding was visualized using FLEX 3,3′-diaminobenzidine (DAB) substrate working solution and hematoxylin counterstain (Dako) using the recommended manufacturer protocols. After staining, slides were taken through graded alcohol and xylene and mounted in Pertex mounting medium (Histolab). For multiple antibody immunofluorescence staining and image acquisition, protocols were adapted from Dakin et al 2015 [8] using primary antibodies listed in Table 1. Sections of diseased Rheumatoid synovium were used as positive controls to confirm immunostaining for PDPN and CD248. Isotype control antibodies were a cocktail of mouse immunoglobulin G (IgG1), IgG2a, IgG2b, IgG3, and IgM (Dako) and rabbit immunoglobulin fraction of serum from non-immunized rabbits, solid-phase absorbed (Dako).

**Isolation of tendon derived stromal cells**

Tendon cells were isolated from healthy hamstring and diseased supraspinatus tendons. Diseased tendon cells were isolated from patients with small to medium tendon tears (< 3cm in length). Small to medium tendon tears are known to express genes and proteins induced by Interferon and NFκB inflammation activation pathways [8]. Tendons were cut into 2-mm3 explants and incubated in DMEM/F12 (Lonza) containing 50% fetal calf serum (FCS; Labtech) and 1% penicillin-streptomycin (Lonza). Fresh media were replaced every 4 days, and cells were allowed to grow out from explants over time in a tissue culture incubator at 37°C and 5% CO2. Once cells were confluent, explants were removed and media replaced with DMEM/F12 containing 10% FCS and 1% penicillin-streptomycin. Cells between passages 1 and 3 were used for all experiments.

**Treatment of tendon-derived stromal cells with IL-1β**

Tendon-derived stromal cells isolated from healthy hamstring and diseased supraspinatus were seeded at a density of 30,000 cells per well in a 12 well plate (mRNA) or 60,000 cells in a 6 well plate (flow cytometry). Cells were allowed to reach 80% confluence prior to stimulation with IL-1β (10ngml-1). Tendon cells were incubated in DMEM F12 medium (Lonza) containing 1% heat-inactivated human serum (Sigma). Media containing sterile filtered 0.1% endotoxin free BSA (Sigma) diluted in PBS was used for vehicle only controls. After IL-1β or vehicle treatment, cells were incubated for 24 hours at 37°C and 5% CO2 until harvest of the lysate for mRNA or flow cytometry.

**Extraction of RNA from tendons**

Protocols for RNA extraction from healthy and diseased tendon tissues and cells, complementary DNA synthesis and quantitative polymerase chain reaction are described elsewhere [8]. 2 μL cDNA was used in a 10 μL qPCR volume with Fast SYBR Green Master Mix (Applied Biosystems) and diluted Qiagen validated human primers including *PDPN* (QT01015084), *CD248* (QT00216356), *CD106* (QT00018347) *β-actin* (QT00095431) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (QT00079247). Duplicate reactions for each gene were run on a ViiA7 qPCR machine (Applied Biosystems) and results were calculated using the DDCt method using reference genes for human β-actin and GAPDH. Results were consistent using these reference genes and data are shown normalized to β-actin.

**Flow cytometry**

After harvest, tendon cells were washed twice in Cell Staining Buffer (CSB) (BioLegend) and blocked for 15 mins at room temperature (RT) in 20% human FcR blocking reagent (Miltenyi Biotech) diluted in CSB. All dead cells were excluded from analysis using fixable viability dye ef780(1:1000 dilution) (eBioscience). Cells were stained in a buffer containing 20% FcR blocking reagent diluted in CSB at RT for 30 mins. Antibody and isotype cocktails were prepared as indicated in Table 2. After washing, cells were fixed using Cytofix fixation buffer (BD Biosciences) for 20 mins at RT. Flow cytometry was performed on a BD LSR Fortessa instrument calibrated daily with BD cytometer setup and tracking beads. Analysis of data was carried out using FlowJo software (Treestar).

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Normality was tested using the Shapiro-Wilk normality test. Kruskal-Wallis tests followed by pairwise post hoc Mann-Whitney U tests were used to compare *PDPN*, *CD248* and *CD106* mRNA expression in healthy, diseased and post-treatment tendons. Pairwise Mann-Whitney U tests were used to test for differences in gene and protein expression of PDPN, CD106 and CD248 between vehicle and IL-1β treated healthy and diseased tendon cells. Pairwise Mann-Whitney U tests were used to test for differences between mRNA expression of *PDPN*, *CD106* and NF-κB target genes in cytokine-treated healthy and diseased tendon cells. P<0.05 was considered statistically significant.

**RESULTS**

***Diseased tendon tissues express stromal fibroblast activation markers***

Markers of stromal fibroblast activation including PDPN, CD248 and CD106 have not been investigated in healthy and diseased tendon tissues. Post-treatment supraspinatus tendon biopsy samples were collected from patients 2-4 years after surgical sub-acromial decompression (SAD) treatment. This post-treatment patient group consisted of 6 patients who were asymptomatic after treatment and 5 who remained symptomatic.

Diseased supraspinatus tendons showed significantly increased *PDPN,* *CD248* and *CD106* mRNA compared to healthy subscapularis tendons (Figure 1A) (p=0.001, p=0.003 and p=0.0007 respectively). This stromal activation signature was also present in supraspinatus tendon biopsies from post-treatment patients (p=0.0015, p=0.0015 and p=0.006 respectively). Diseased and post-treatment supraspinatus tendons showed increased immunopositive staining for PDPN, CD248 and CD106 compared to healthy supraspinatus tendons (Figure 1B). Co-staining revealed co-localization of PDPN, CD106 and Toll-like receptor 4 (TLR4) in diseased tendons (Figure 1C). CD248+ cells were closely associated with clusters of PDPN+ cells. However only very few cells expressed both PDPN and CD248 (Figure 1D).

***Markers of stromal fibroblast activation are increased in diseased compared to healthy tendon-derived stromal cells***

Markers of stromal fibroblast activation identified in diseased human tendons were further studied in healthy and diseased tendon-derived stromal cells to investigate the effects of cytokine treatment on stromal fibroblast activation *in vitro*.

Diseased cells showed increased *PDPN* mRNA compared to healthy cells under baseline unstimulated conditions (media containing vehicle only) (p=0.008) (Figure 2A). IL-1β treatment of healthy and diseased cells for 24 hrs further induced *PDPN* (p=0.008 respectively). Induction of *PDPN* was more profound in IL-1β treated diseased compared to IL-1β treated healthy cells (p=0.03). PDPN protein was increased in diseased compared to healthy cells under baseline unstimulated conditions (p=0.008) (Figure 2B and C). IL-1β treatment of healthy and diseased cells further induced PDPN (p=0.008 respectively). Induction of PDPN was more profound in IL-1β treated diseased compared to IL-1β treated healthy cells (p=0.008).

Diseased cells showed increased *CD106* mRNA compared to healthy cells under baseline unstimulated conditions (p=0.03) (Figure 3A). IL-1β treatment of diseased cells further induced *CD106* (p=0.008). Induction of *CD106* was more profound in IL-1β treated diseased compared to IL-1β treated healthy cells (p=0.008). CD106 protein was increased in diseased compared to healthy cells under baseline unstimulated conditions (p=0.03) (Figure 3B and C). IL-1β treatment of healthy and diseased cells further induced CD106 (p=0.008 respectively).

Diseased cells also showed increased *CD248* mRNA compared to healthy cells under baseline unstimulated conditions (p=0.03) (Figure 4A). IL-1β treatment did not significantly attenuate *CD248* mRNA. CD248 protein was increased in diseased compared to healthy cells under baseline unstimulated conditions (p=0.008) (Figure 4B and C). IL-1β treatment of diseased cells reduced CD248 (p=0.008).

***Inflammation induces persistent stromal activation in tendon-derived stromal cells***

Having identified that stromal fibroblast activation markers were present in diseased tendon tissues 2-4 years after treatment, we sought to investigate if IL-1β treatment induced stromal fibroblast “memory” in healthy and diseased tendon cells *in vitro*. Diseased cells showed increased *PDPN* and *CD106* mRNA compared to healthy cells under baseline unstimulated conditions (p=0.03 respectively). IL-1β treatment for 24 hours induced *PDPN* and *CD106*, induction was more profound in diseased compared to healthy cells (p=0.03 respectively) (Figure 5 A & B). In healthy cells *PDPN* and *CD106* mRNA remained elevated beyond pre-stimulated levels following replacement with cytokine-free media for 4 days (p=0.03 respectively). In diseased cells, PDPN returned to pre-stimulated levels, however CD106 remained elevated beyond pre-stimulated levels following replacement with cytokine-free media for 4 days (p=0.03).

IL-1β treatment of healthy and diseased tendon cells also induced NF-κB target genesincluding *IL6, IL8, STAT-1* and *IFIT1* (Figures 5C-F)*.* Healthy cells showed a gradual decline of these pro-inflammatory genes following replacement with cytokine-free media. IL-1β treated diseased cells showed a more sustained induction of *IL6* and *IL8* compared to IL-1β treated healthy cells between 2 and 4 days following replacement with cytokine-free media (p=0.03 for each respective time point) (Figures 5C and D).

**Positive and isotype controls for immunostaining**

Sections of diseased Rheumatoid synovium were used as positive controls to confirm immunostaining for PDPN and CD248 (Figure 6). Isotype control staining was performed using sections of diseased supraspinatus tendons (Figure 7A and B).

**DISCUSSION**

The effects of the inflammatory process on resident tendon stromal cells is poorly understood. This study provides new insights into the pathogenesis of diseases affecting these musculoskeletal soft tissues. We identify a mechanism whereby diseased tendon tissues and cells become activated after exposure to an inflammatory stimulus, which induces a sustained change their phenotype whereby cells express markers of stromal fibroblast activation including PDPN, CD106 and CD248. This longitudinal cohort study of phenotyped human tendon tissues collected pre and post-treatment revealed that this stromal fibroblast activation signature persisted in tendon biopsies from patients up to 4 year’s post-treatment. Stromal activation and its persistence *in vivo* were further confirmed by IL-1β treatment of cultured tendon stromal cells *in vitro*.

Stromal fibroblast activation is a feature of RA synovium, where markers including PDPN, CD106 and CD248 have been identified [11, 12]. To our knowledge this is the first study to identify these stromal fibroblast activation markers in non-immune mediated musculoskeletal disease. IL-1β treatment of cultured tendon stromal cells induced markers of fibroblast activation including PDPN and CD106 but not CD248, suggesting distinct stromal responses in diseased tendons. In support of this, immunostaining of diseased tendons revealed co-localization of PDPN, CD106 and TLR4, suggestive of a pro-inflammatory tendon cell phenotype. CD248+ cells were closely associated with clusters of PDPN+ cells, but only a small number of cells expressed both PDPN and CD248 in diseased tendons. In RA, PDPN and CD106 are located in the synovial lining and CD248 in the sub-lining layer and are thought to represent distinct fibroblast subsets [12, 13]. PDPN expressing fibroblasts possess a pro-inflammatory phenotype in RA and malignancy [21, 22]. CD248 expressing fibroblasts have also been identified in hepatic and renal fibrosis, representing a reparative population [23].

Cancer associated fibroblasts are known to maintain a state of permanent activation after the effects of the initiating stimulus subsides. This activated phenotype persists until cell senescence [24]. Cells populating diseased tendons are known to be apoptotic and senescent [5]. Furthermore, turnover of tendon collagenous matrix components is known to be low [25, 26]. This may account for the sustained expression of stromal fibroblast activation markers in tendon tissues from patients up to 4 years after treatment in both symptomatic and asymptomatic patients.

In our previous work investigating inflammation in cultured stromal cells derived from diseased human tendons, we proposed that diseased cells may be primed for inflammation [8]. In the current study, we identified IL-1β treated diseased cells showed a more profound induction of *PDPN* and *CD106* and sustained expression of *IL6* and *IL8 mRNA* compared to IL-1β treated healthy cells. Our findings support the concept that diseased tendon cells previously exposed to inflammation are primed and become hyper-responsive on subsequent exposure; possessing ‘stromal memory’. In support of this, studies of cancer associated fibroblasts [27, 28] and of RA synovial fibroblasts [9, 10] emphasize the important contribution of resident stromal cell populations to the persistence of chronic inflammation. Epigenetic changes in stromal cell populations are thought to be implicated in fibroblast activation. Changes in the epigenome can modulate the inflammatory response and promote the development of chronic disease [29].

Diseased tendon tissues investigated in the current study were collected from patients undergoing sub-acromial decompression surgery or surgical debridement of a supraspinatus tendon tear ranging from small (<1cm) to massive (>5cm) in size. Patients with large to massive tendon tears likely represent the end stage of the disease spectrum. Stromal fibroblast activation markers were highly expressed in all tendinopathic and torn tissues investigated in this study. We recently identified the plasticity and complexity of inflammation activation signatures in diseased human shoulder tendons [8]. The current study using tissues from these same patient cohorts identified that markers of stromal fibroblast activation are highly expressed in diseased tendons, and that disease stage does not influence the degree of expression of PDPN, CD106 and CD248. These findings further support the concept of stromal fibroblast ‘memory’ and that exposure of tendon cells to an inflammatory stimulus induces a sustained change in their phenotype.

Repetitive damage through cumulative loading is a known contributor to the development of tendon disease. Mechanical cues can also induce tendon cells and tissues to release pro-inflammatory mediators including prostaglandins and pro-inflammatory cytokines [30, 31]. The effects of mechanical loading on stromal activation in tendinopathy have not been directly investigated. However, it is conceivable that in a disease setting, both mechanical and chemical cues may potentially induce stromal fibroblast activation as both stimuli possess the capacity to induce inflammation.

We acknowledge there are potential limitations with the use of hamstring tendon as a comparator to diseased tendons including tendon type and age differences. However, hamstring tendon was taken from live healthy donors without history of tendinopathy. We believe this is a more suitable comparator than cadaveric shoulder tendon tissues where little is known about whether the tendons were healthy or diseased and tendons were not affected by post mortem changes.

**CONCLUSIONS**

The findings from this study support the important and sustained contribution of diseased tendon stromal cells to the development and persistence of non-resolving tendon inflammation. We show for the first time that stromal fibroblast activation markers are increased in diseased compared to healthy human tendon tissues and cells and identify distinct stromal responses in diseased tendons. We demonstrate that inflammation induces stromal fibroblast activation and stromal ‘memory’ which is more profound in diseased compared to healthy tendon cells. We propose persistent stromal fibroblast activation is an important mechanism for the development of chronic inflammation and recurrent tendinopathy. Targeting activated tendon stromal cells is a potential therapeutic strategy for curative intervention.

**ABBREVIATIONS**

CSB Cell Staining Buffer

DAB 3,3′-diaminobenzidine

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GCR Glucocorticoid receptor

IFIT1 Interferon Induced Protein with Tetratricopeptide Repeats 1

IFN Interferon

IHC Immunohistochemistry

IL Interleukin

NF-κB Nuclear Factor Kappa Beta

OSS Oxford Shoulder Score

PBS Phosphate buffered saline

PBST Phosphate buffered saline with Tween

PDPN Podoplanin

RA Rheumatoid Arthritis

RT-qPCR Real Time quantitative polymerase chain reaction

SAD Sub-acromial decompression

STAT Signal transducer and activator of transcription

TLR4 Toll-like receptor 4

**Ethics approval and consent to participate:**

Ethical approval for this study was granted by the local research ethics committee, Oxfordshire REC B references 10/H0402/24, 09/H0605/111, and 10/H0606/60 and South Central Oxford B reference 14/SC/0222. Full informed consent according to the Declaration of Helsinki was obtained from all patients.

**Consent for publication:**

Not applicable

**Availability of data and material:**

All data generated or analysed during this study are included in this published article.

**Competing interests:**

There are no potential conflicts of interest relevant to this article.

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**Authors' contributions**

**Study concept and design**: SGD, AJC, CDB

**Acquisition of data**: SGD, RH, MHA, FM

**Analysis and interpretation of data**: SGD, RH, MHA

**Drafting of the manuscript**: All authors

**Critical revision of the manuscript and approval of final version**: All authors

**Statistical analysis**: SGD

**Obtained funding**: SGD, AJC

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**Figure Legends**

**Figure 1. Diseased tendon tissues express markers of stromal fibroblast activation**. **(A)** Graphs show mRNA expression of stromal fibroblast activation markers *PDPN*, *CD248* and *CD106* in healthy subscapularis tendons and diseased and post-treatment supraspinatus tendons. Bar shows median values. Statistically significant differences were calculated using Kruskal-Wallis tests with pairwise post hoc Mann-Whitney U tests. \*\* p<0.01, \*\*\* p<0.001. **(B)** Panels show representative images of 3,3’-diaminobenzidine immunostaining (brown) for PDPN, CD248 and CD106 in healthy, diseased and post-treatment supraspinatus tendons. Nuclear counterstain is haematoxylin. Scale bar, 50μm. **(C and D)** Representative immunofluorescence images of sections of diseased supraspinatus tendons stained for markers of stromal activation (PDPN, green; CD248 and CD106, purple) and TLR4 (red). Cyan represents POPO-1 nuclear counterstain. Scale bar, 20μm.

**Figure 2.** **Expression of PDPN mRNA and protein in healthy and diseased tendon cells after IL-1β treatment.** Tendon cells were derived from healthy hamstring (n=5 donors) and diseased supraspinatus tendons (n=5 donors). Tendon cells were treated with media containing vehicle only or IL-1β (10ngml-1) for 24 hours. *PDPN* mRNA expression **(A)** and **(B)** PDPN protein are shown in healthy and diseased tendon cells after vehicle and IL-1β treatments. **(C)** Representative FACS contour plots for PDPN from healthy and diseased tendon cells after vehicle and IL-1β treatments gated on CD45-CD34- cells. Bar shows median values. Statistically significant differences were calculated using Kruskal-Wallis tests with pairwise post hoc Mann-Whitney U tests. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

**Figure 3.** **Expression of CD106 mRNA and protein in healthy and diseased tendon cells after IL-1β treatment.** Tendon cells were derived from healthy hamstring (n=5 donors) and diseased supraspinatus tendons (n=5 donors). Tendon cells were treated with media containing vehicle only or IL-1β (10ngml-1) for 24 hours. **(A)** *CD106* mRNA expression and **(B)** CD106 protein are shown in healthy and diseased tendon cells after vehicle and IL-1β treatments. **(C)** Representative FACS contour plots for CD106 from healthy and diseased tendon cells after vehicle and IL-1β treatments gated on CD45-CD34- cells. Bar shows median values. Statistically significant differences were calculated using Kruskal-Wallis tests with pairwise post hoc Mann-Whitney U tests. \* p<0.05, \*\* p<0.01.

**Figure 4.** **Expression of CD248 mRNA and protein in healthy and diseased tendon cells after IL-1β treatment.** Tendon cells were derived from healthy hamstring (n=5 donors) and diseased supraspinatus tendons (n=5 donors). Tendon cells were treated with media containing vehicle only or IL-1β (10ngml-1) for 24 hours. **(A)** *CD248* mRNA and **(B)** CD248 protein are shown in healthy and diseased tendon cells after vehicle and IL-1β treatments. **(C)** Representative FACS contour plots for CD106 from healthy and diseased tendon cells after vehicle and IL-1β treatments gated on CD45-CD34- cells. Bar shows median values. Statistically significant differences were calculated using Kruskal-Wallis tests with pairwise post hoc Mann-Whitney U tests. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001

**Figure 5. Effects of IL-1β on *PDPN*, *CD106* and NF-κB genes in diseased tendon cells over time.** Tendon cells were derived from healthy hamstring (n=4 donors) and diseased supraspinatus tendons (n=4 donors). Cell lysates were sequentially harvested at day 0 (pre-stimulation), day 1 (24 hrs after IL-1β treatment, 10ngml-1) and days 2, 3, 4 and 5 when cells were incubated in cytokine free media containing vehicle only. mRNA expression is shown for **(A)** *PDPN*, **(B)** *CD106*, **(C)** *IL6*, **(D)** *IL8*, **(E)** *STAT-1* and **(F)** *IFIT1*.Data show mean ± SEM. Statistically significant differences were calculated using pairwise Mann-Whitney U tests for healthy and diseased cells at each time point. \* p<0.05.

**Figure 6. Positive control staining of Rheumatoid synovium for markers of stromal fibroblast activation.** Representative confocal immunofluorescence images show PDPN (green), CD248 (purple) and TLR4 (red). Cyan represents POPO-1 nuclear counterstain. Scale bar, 20μm.

**Figure 7.** **Isotype control staining of diseased human supraspinatus tendons. (A, B)** Panel shows representative bright field images of diseased tendon sections stained with isotype control antibodies for mouse IgG1, IgG2a, IgG2b and rabbit IgG fractions. Nuclear counterstain is haematoxylin. Scale bar, 50μm. **(C, D)** Representative confocal immunofluorescence images showing merged image of diseased tendon sections stained with isotype control antibodies for mouse IgG1, IgG2a, IgG2b and rabbit IgG fractions. Cyan represents POPO-1 nuclear counterstain. Scale bar, 20μm.

**Table 1.** Primary antibodies used for immunohistochemistry and immunofluorescence

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibody** | **Clone** | **Isotype** | **Species** | **Dilution** |
| Podoplanin (PDPN)  Abcam  Ab10288 | 18H5 | IgG1 | mouse | 1:100 |
| CD248 (TEM1)  Abcam  Ab204914 | EPR17081 | IgG | Rabbit monoclonal | 1:1000 |
| CD106 (VCAM-1)  LS-Biosciences  LS\_C313019 |  | IgG | rabbit  polyclonal | 1:100 |
| TLR4  Abcam  Ab22048 | 76B357.1 | IgG2b | mouse | 1:200 |

**Table 2.** Antibodies used for flow cytometry. All antibodies were diluted 1:50 for staining.

|  |  |  |
| --- | --- | --- |
| **Antibody** | **Catalog #** | **Clone** |
| PDPN Alexa Fluor 488 anti-human | 337005 BioLegend | NC08 |
| Alexa Fluor 488 Rat IgG2aκ isotype | 400525  BioLegend | RTK2758 |
| CD248 Alexa Fluor 647 anti-human | 564994  BD Biosciences | B1/35 |
| Alexa Fluor 647 mouse IgG1κ isotype | 557714  BD Biosciences | MOPC-21 |
| CD106 PE anti-human | 305806  BioLegend | STA |
| PE mouse IgG1κ isotype | 400112  BioLegend | MOPC-21 |
| CD34 PerCP/Cy5.5 anti-human | 343521  BioLegend | 581 |
| PerCP/Cy5.5 mouse IgG1κ isotype | 400149  BioLegend | MOPC-21 |
| CD45 BV605 anti-human | 304041  BioLegend | H130 |
| BV605 mouse IgG1κ isotype | 400161  BioLegend | MOPC-21 |