Modulating Inflammation in an Engineered Ligament Model

By

ALEC AVEY
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Declaration

I declare that this dissertation is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of California, Davis.

It has not been submitted for any degree or examination at any another University.

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To everyone who has come through the Baar Lab during my time here—post-docs, graduate students, undergraduates, and the many visitors—thank you for making our lab an incredible place to work.

To Dr. Clare Yellowley and Dr. Lucas Smith, thank you for the opportunity to rotate in your labs, conducting my qualifying exam, and serving on my dissertation committee. I am greatly appreciative of the time and effort you have devoted towards my graduate school progress.

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Abstract

Musculoskeletal injuries account for the highest percent of time away from work and affect over 100 million people in the United States [1,2]. Ligament and tendon injuries account for approximately 50% of all musculoskeletal injuries [3]. Despite the prevalence of these injuries, current tendon and ligament research is lacking, with little advancement in the treatment of tendinopathies for decades. Tendinopathy describes an injured or diseased tendon/ligament. Underneath this umbrella term, tendinitis is used to describe an injured tendon/ligament that has signs of inflammation. There are many molecular pathways which have been explored in great lengths in muscle and bone; however, the same signals in tendon/ligament have significantly less research devoted to them – including the pro-inflammatory pathways upregulated in tendinitis. In this dissertation, we characterize an engineered ligament model, determine the effects of pro-inflammatory cytokines on engineered ligaments, and explore possible interventions to treat tendinitis such as anti-inflammatories and isometric loading.

The *in vitro* engineered ligament model used throughout this dissertation was first developed by the Baar lab group [4]. In order to further validate the use of this model, we characterized the effect of passage number on cell gene expression and ligament function, as well as the ligament development over time. Despite previous studies suggesting multiple passages of fibroblasts led to decreased gene expression of typical tenoblast markers, our results found that there was no significant change in gene expression across multiple passages. Furthermore, from passage 4 to passage 11 there was no observed change in mechanical function or matrix composition of engineered ligaments. These results validate the use of this *in vitro* engineered ligament model using both early and later passage cells. We then characterized the development of engineered

ligaments over the course of 5 weeks. The results showed that from Day 7 to Day 14 there was an exponential increase in both collagen content and percent collagen, while these measures began to plateau after Day 14. There also was a linear increase in mechanical function from Day 7 to Day 21. Taken together, this data suggests that Day 7 to Day 14 best represents a developing or regenerating tissue, while after Day 14 the plateau of collagen content best represents a more mature tissue. In total, the work in Chapter 2 demonstrates the efficacy of the model used throughout this dissertation and provides multiple timepoints for interventions based on desired modeling of a regenerating vs mature tissue.

To screen for potential therapeutics for tendinitis, we first sought to develop a model for tendinitis using engineered ligaments. Treating ligaments with pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 individually led to decreased mechanical function and reduced collagen content. Using a Box-Behnken design of experiments, we developed an optimal combination of all three cytokines to decrease ligament mechanics by 50%. Treatment with this cytokine cocktail led to impaired mechanical function and decreased collagen, resulting in the first multi-cytokine *in vitro* model for tendinitis. Utilizing this reproducible model, we targeted the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway since it had been identified as a potential target for treatment of chronic tendinopathies [5]. Surprisingly, treatment with a known NF-κB inhibitor alone actually decreased the mechanical function of ligaments, while treatment alongside the cytokine cocktail had little to no positive effects. We then targeted another proinflammatory pathway – Janus kinase 1 (JAK1) and signal transducer and activator of transcription (STAT)3. Inhibition of JAK1, either alone and in the presences of the cytokine cocktail, resulted in increased mechanical function and increased collagen content in engineered

ligaments. Furthermore, the quality of the tissue was improved by JAK1 inhibition, as demonstrated by an increase in enthalpy when undergoing differential scanning calorimetry. In total, the work presented in Chapter 3 resulted in the first multi-cytokine model for tendinitis in an *in vitro* engineered ligament. Additionally, inhibition of JAK1 has been identified as a possible new treatment for tendinitis.

Isometric loading has previously been reported to improve tendon function, reduce pain, and even reverse the appearance of a central core patellar tendinopathy on MRI [6–8]. Given the positive effect of isometric loading, we tested the combined effects of isometric loading and JAK1 inhibition on engineered ligaments in Chapter 4. Isometric load by itself tended to increase mechanical functional with no significant effect on collagen, resulting in the first successful reproduction of isometric loading effects in this model. Additionally, isometric load alone increased enthalpy which suggests that load improved mechanical function of the tissue through improved matrix organization. Inhibition of JAK1 again improved mechanical function and collagen content by itself. However, there was no interaction effect between load and JAK1 inhibition, instead there were independent positive effects that together led to an additive increase. Interestingly, the combination of isometric loading and JAK1 inhibition did not increase collagen content relative to load alone, suggesting that isometric load may actually prevent an increase in collagen while simultaneously improving collagen fibril organization, diameter, and/or cross-linking. Overall, the combined intervention of isometric load and JAK1 inhibition improve engineered ligament mechanical and material properties. This further supports the possibility of JAK1 inhibition as a novel therapeutic for tendinopathy and may improve upon the previously reported effective isometric loading protocols.

In sum, this dissertation better characterizes and validates the use of engineered human ligaments to make fundamental discoveries concerning tendon biology. Using this model, I developed a novel multi-cytokine treatment to reliably reproduce the negative effects of pro-inflammatory cytokines on ligaments and screen for therapeutics. This resulted in the identification of a new potential treatment for tendinitis – inhibition of JAK1 using a class of drugs that have FDA approval. Ultimately, this work furthers understanding within the field of tendon/ligament physiology and sets the foundation for future *in vivo* studies to improve treatment of tendinitis.

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Chapter 1: Muscle-Tendon Crosstalk: A Lack of Emphasis on Tendon Research

This chapter was adapted from a previously published review.

A.M. Avey, K. Baar, Muscle-tendon cross talk during muscle wasting, Am J Physiol Cell Physiol. 321 (2021) C559–C568.

Abstract

In organisms from flies to mammals, the initial formation of a functional tendon is completely dependent on chemical signals from muscle (myokines). However, how myokines affect the maturation, maintenance, and regeneration of tendons as a function of age is completely unstudied. Here we discuss the role of three myokines – fibroblast growth factors (FGF), myostatin, and the secreted protein acidic and rich in cysteine (SPARC) – in tendon development and hypothesize a role for these factors in the progressive changes in tendon structure and function as a result of muscle wasting (disuse, aging and disease). Furthermore, we highlight the inflammatory response to mechanical load and injury in muscle and discuss how these same signals could alter tendon structure and function. Because of the close relationship between mechanical loading and muscle and tendon regulation, disentangling muscle-tendon crosstalk from simple mechanical loading is experimentally quite difficult. Therefore, I propose an experimental framework that will be useful in demonstrating muscle-tendon crosstalk *in vivo*. Though understudied, the promise of a better understanding of muscle-tendon crosstalk is the development of new interventions that will improve tendon development, regeneration, and function throughout the lifespan.

Introduction

Muscle wasting occurs as a result of disuse, aging and disease. Muscle disuse can result from traumatic injury (such as nerve crush or spinal cord injury), forced inactivity (bed rest, immobilization, or pandemic-induced inactivity), or voluntary changes in activity (such as athletes during the offseason or a change in lifestyle or job). The loss of muscle mass resulting from disuse, aging, and disease is associated with decreased muscle function [9–11]. Interestingly though, similar amounts of muscle wasting can result in large differences in muscle function, for example in young and old individuals [12]. Specifically, adult and old animals lose approximately the same amount (~30%) of muscle over a two week period of hindlimb unloading; however, the old animals lose 50% of force production, whereas the adult animals only lose only 10% of their strength [12]. Further, during normal aging muscle strength is lost at a rate 3-times faster than muscle mass [13]. These data suggest that strength loss is dependent on more than just a loss of contractile proteins from muscle and suggest that force transfer through the matrix and tendon to the bone may play a role in strength loss.

A significant amount of research has been devoted to elucidating the cellular mechanisms underlying muscle atrophy. Mechanical loading prevents the cell signaling associated with muscle atrophy [14,15] and more importantly short bouts of neuromuscular activity are sufficient to prevent muscle wasting [16]. Interestingly though, whereas neuromuscular electrical stimulation prevents muscle atrophy, it is not enough to prevent the loss of strength that occurs with immobilization [16]. Like muscle, strength training increases and detraining decreases tendon size and mechanical properties [17]. However, whether the change in tendon size and function are strictly the result of the altered mechanical environment, or whether there is

molecular crosstalk between muscle and tendon has yet to be determined. Muscle-tendon crosstalk has been elegantly demonstrated during development of the of the muscle-tendon unit [18–20] and we will use this excellent work to describe how muscle-tendon crosstalk could function within the adult. However, few studies have determined whether the developmental signals between muscle and tendon are modulated during muscle wasting in the adult.

We found only a handful of studies where muscle derived or circulating factors were shown to increase tendon-specific markers or tendon strength. Because of the complexities of isolating mechanical and chemical signals *in vivo*, both studies used cell culture for the initial experiments. In the first, Ghebes and colleagues used a transwell system to show that co-culturing human hamstring tendon cells with muscle cells increased the expression of tendon specific markers like scleraxis and tenomodulin, whereas osteoblast or chondrocyte co-culture had no such effect [21]. Further, longer term co-culture of tendon with muscle cells increased the amount of collagen the tendon cells produced, suggesting that a factor secreted from muscle had an anabolic effect on tendon. In the second study, young men provided a blood sample before and 15 minutes after a heavy strength training session, the serum was isolated, and the effect of the different sera was determined using an engineered human ligament model [22]. Engineered ligaments grown in the post-exercise serum contained more collagen and were mechanically stronger than those grown in the resting serum, again suggesting that a circulating factor released from active muscle could improve tendon size and strength. Given that both studies suggest that a circulating factor can influence tendon size and strength, the next question is what is that factor and how does it work? In principle, the muscle-derived factor could be a protein that is produced in muscle cells under load that diffuses or circulates to the associated tendon cells. As we will

see below, this is what happens during development. Alternatively, the muscle-derived factor could be exosomal, potentially a microRNA (miR) or long non-coding RNA that alters mRNA quantity or translation in the tendon cell. Either way, the protein or exosomal factor would help coordinate the functional status of both the muscle and tendon.

In the current sections below we will discuss muscle-tendon crosstalk in development, potential mediators of muscle-tendon crosstalk in the adult, how inflammation is a classic example of crosstalk, and what would be required experimentally to identify the factors beyond mechanical load that drive tissue crosstalk.

Muscle Tendon Crosstalk in Development

Much of the early work in the development of the muscle-tendon unit was performed in *Drosophila*. This work was done in *Drosophila* since each muscle connects to a specialized epidermal attachment cell called a tendon cell. Before the discovery of scleraxis (*Scx)*, a molecular marker of tendon precursor cells in mammals [23], there was no way to determine whether changes in muscle altered tendon formation in rodent models. In *Drosophila,* the fate of the tendon and muscle cells could more easily be tracked, leading to early progress in the molecular understanding of the development of muscle-tendon unit.

***Stripe* in Muscle/Tendon Development**

The earliest example of muscle-tendon crosstalk during development involves the *stripe* genes In *Drosophila, stripe* is a tissue-specific gene found in tendon precursor cells that is required for tendon cell differentiation. The *stripe* gene is necessary to initiate the differentiation program of

epidermal muscle attachment (tendon) cells and in the absence of *stripe* these cells do not differentiate [24]. Expression of *stripe* transforms epidermal cells into tendon cells, and these cells initiate myotube adhesion to their target cells through the *stripe*-regulated production of thrombospondin, a tendon cell produced extracellular matrix (ECM) protein [25]. The *stripe* gene has been shown to produce two splice variants, *stripeA* and *stripeB* [26]. *StripeB* defines the tendon precursor cells, and its levels are held constant throughout development. By contrast, *stripeA* expression increases in the muscle-bound tendon cell following adhesion of myotubes to a tendon precursor cell. The increase in *stripeA* leads to tendon cell differentiation [27]. The upregulation of *stripeA* in tendon cells is established by the expression and secretion of the paracrine myokine *vein* by muscle cells. *Vein* produced in the muscle cell is secreted and binds to the epidermal growth factor receptor (EGFR) on the surface of the attached tendon cell. Binding of *vein* to the EGFR activates ERK1/2 signaling [28] and this promotes the high level and sustained expression of *stripeA* in the differentiated tendon cell [29]. Thus, autonomous *stripeB* expression in early development defines the tendon precursor cell and initiates myotube attachment. Once contact is made, *vein* is released from the muscle cell and drives the expression of *stripeA* that is necessary for differentiation and maturation of the interface.

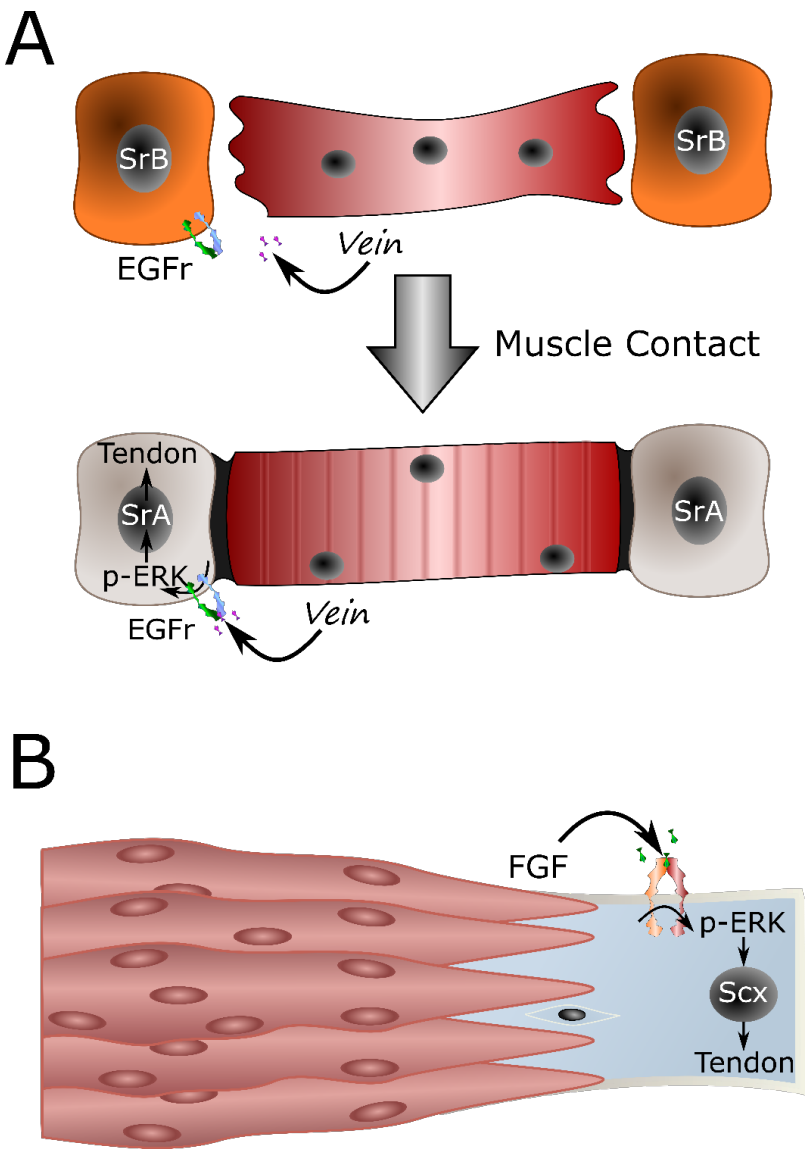


Figure 1. Cartoon comparing muscle-tendon crosstalk in the development of the myotendinous junction in (A) *Drosophila* and (B) mammals. In *Drosophila*, prior to muscle contact epidermal cells express stripe (SrB) and muscle produced vein does not reach the epidermal cell. As muscle comes into contact with the epidermal cell, *vein* binds to the EGF receptor activating signaling through ERK1/2 resulting in the expression of stripe A (SrA) and the differentiation of the epidermal cell into a tendon cell. In mammals (B), developing myotubes secrete FGFs, which bind to FGF receptors on tendon cells once again activating ERK1/2 and this time increasing the production of scleraxis (Scx) and driving tendon cell differentiation.

FGF Signaling in Muscle/Tendon Development

Whereas in Drosophila *vein* is released from muscle and drives tendon development, in mammals the functional homologs are the fibroblasts growth factors (FGFs). During development, FGF4 and/or 8 are secreted from the myotome and are necessary for the formation of *Scx* expressing tendon progenitors. When FGF4 or 8 are secreted from the myotome they act in a paracrine fashion on cells within the sclerotome where they bind to FGF receptors. As with *vein* in *Drosophila*, FGF receptor activation leads to the phosphorylation and activation of the mitogen activated protein kinases (ERK1/2) and subsequent activation of the ETS-domain transcription factors *Pea3* and *Erm*, resulting in *Scx* expression [30]. Using FGF coated beads, Brent and Tabin demonstrated that they could establish ectopic expression of Scx in a *Pea3*-dependent manner. Consistent with the role of muscle-derived FGF in tendon formation, the expression of *Pea3* and *Sprouty*, another FGF target gene, is diminished in tendons of *muscleless* mice, demonstrating that *Pea3 and Sprouty* gene expression is dependent on the presence of muscle [31]. The FGF signal coming from muscle is also proportional to the contractility of the muscle. In the absence of muscle contraction, the expression of tendon related FGF signaling components are decreased [32]. In support of the role of contraction on FGF secretion from muscle and the development of tendon, in the absence of muscle contraction, the addition of mFgf4-expressing retroviruses to immobile limbs can rescue ERK1/2 signaling and *Scx* expression [32]. These data suggest that during development muscle contraction drives FGF secretion, and that FGF derived from contracting muscles binds to FGF receptors on tendon precursor cells resulting in the phosphorylation and activation of ERK1/2. The activation of ERK1/2 increases the expression of *Pes3, Erm,* and *Sprouty*, which drive the scleraxis expression required for the development of the associated tendon.

As with the *Drosophila stripe* genes, their mammalian homologues, Egr1 and Egr2, are also important in the development and maintenance of tendons *in vivo*. Forced expression of Egr1 or 2 is sufficient to drive *Scx* and *ColIa1* expression in both mesodermal and ectodermal tissues [33]. Further, ablation of Egr1 decreases the expression of *Scx* and other tendon-associated genes by ~50%, suggesting that Egr1 and 2 are necessary and sufficient for tendon cell differentiation in mammals. In support of this hypothesis, Egr1 knockout mice produce tendons that have a quarter the tensile strength of WT tendons and fail to repair after injury [34]. Similar to their *Drosophila* homologue *stripeA*, which is upregulated by vein, Egr1 and 2 are upregulated during tendon development by FGFs. Implanting a bead coated in FGF4 into a developing limb results in the rapid (<4 hrs) upregulation of Egr1 and 2 ahead of increased expression of tendon-enriched genes like *Scx* (6 hrs) and *ColIa1* (24 hrs) [33]. Interestingly, the regulation of Egr1 by muscle contraction is maintained in adult animals. Injection of botox to inactivate the gastrocnemius muscle of adult mice results in a rapid 60% decrease in Egr1 expression and a concomitant decrease in *Scx* and *Col1a2* mRNA [35]. Transient overexpression of Egr1 in the tendon before botox injection was able to increase the expression of *Scx, Col1a1*, and tenomodulin. These data suggest that Egr1 remains important in the regulation of tendon function in adult animals; however, whether Egr1 is activated directly by the mechanical load, or a secreted factor such as FGF remains to be determined.

Taken together, these data suggest that, in mammals, actively contracting muscle cells secrete FGF proteins that bind to FGF receptors on tendon cells, activate ERK1/2 signaling, and during development this signal can increase the production of *Pea, Sprouty, Egr1* and *2*. In the adult, the

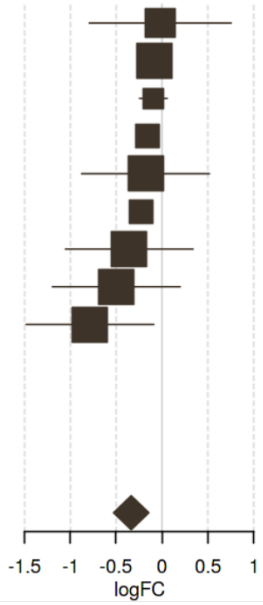
same ERK signal increases *Egr1* and this periodic high level of *Egr1* expression is necessary to maintain functional tendons.

FGF and Muscle Atrophy: Potential Crosstalk

Beyond their role in development, fibroblast growth factors have also been studied in the regulation of muscle mass and regeneration from injury in adults. In model organisms, FGFs play a critical role in the renewal of muscle satellite cells and therefore the maintenance of adult muscle mass [36]. FGF is released following mechanical loading [37], suggesting that decreased activity could decrease secreted FGF levels concomitant with decreased tendon function. In support of this hypothesis, FGFR1 levels were found to be increased in atrophying muscles due to hindlimb suspension, and further overexpression of FGFR1 in immobilized muscle prevented muscle atrophy [38]. In a model of unloading in humans, release of FGF from muscle decreased in proportion to the reduction in muscle fiber cross-sectional area [39]. Further, using the exercise transcriptomic profiling tool MetaMEx [40], we find that the key FGF protein FGF8 is transcriptionally repressed by 80% following inactivity (Figure 2). Consistent with a drop in *FGF8* expression, the FGF8 target protein polyoma enhancer activator 3 (PEA3), also known as ETS translocation variant 4 (ETV4), decreases almost the same amount. These data suggest that, in inactive people there is a loss of FGF8 signaling that may play a role in the loss of tendon function; however, it remains unclear whether decreased FGF8 signaling in adult muscle is sufficient to inhibit *Pea*/*Egr1* expression resulting in a decrease in the size and strength of the tendon.

A

FGF8 in inactivity studies	logFC	FDR	n
GSE24215_BED_VAL_M_YNG_ACT_LEA_HLY_D10	-0.02	9.9e-01	10
GSE148152_BED_VAL_M_YNG_ACT_LEA_HLY_D84	-0.08	1.2e-01	12
GSE21496_LIM_VAL_M_YNG_SED_LEA_HLY_D02	-0.09	9.8e-01	7
GSE14798_BED_VAL_F_YNG_SED_LEA_HLY_D60	-0.16	4.9e-02	8
GSE14901_LIM_VAL_M_YNG_SED_LEA_HLY_D02	-0.18	9.4e-01	12
GSE14798_BED_SOL_F_YNG_SED_LEA_HLY_D60	-0.23	3.1e-03	8
GSE14901_LIM_VAL_M_YNG_SED_LEA_HLY_D14	-0.36	7.4e-01	12
GSE14901_LIM_VAL_F_YNG_SED_LEA_HLY_D02	-0.50	7.5e-01	12
GSE14901_LIM_VAL_F_YNG_SED_LEA_HLY_D14	-0.79	3.5e-01	12
GSE113165_BED_VAL_F_ELD_ACT_OWE_HLY_D05	NA	NA	
GSE113165_BED_VAL_F_YNG_ACT_LEA_HLY_D05	NA	NA	
GSE113165_BED_VAL_M_ELD_ACT_OWE_HLY_D05	NA	NA	
GSE113165_BED_VAL_M_YNG_ACT_LEA_HLY_D05	NA	NA	
Meta-analysis score	**-0.34**	**5.9e-04**	**93**



B

ETV4 in inactivity studies	logFC	FDR	n
GSE33886_LIM_VAL_M_YNG_SED_LEA_HLY_D21	-0.01	1.0e+00	3
GSE14798_BED_SOL_F_YNG_SED_LEA_HLY_D60	-0.03	8.8e-01	8
GSE148152_BED_VAL_M_YNG_ACT_LEA_HLY_D84	-0.12	7.9e-02	12
GSE104999_BED_VAL_M_YNG_ACT_LEA_HLY_D21	-0.17	7.1e-01	12
GSE14798_BED_VAL_F_YNG_SED_LEA_HLY_D60	-0.17	3.1e-01	8
GSE14901_LIM_VAL_M_YNG_SED_LEA_HLY_D14	-0.20	8.0e-01	12
GSE24215_BED_VAL_M_YNG_ACT_LEA_HLY_D10	-0.29	6.5e-01	10
GSE14901_LIM_VAL_F_YNG_SED_LEA_HLY_D14	-0.78	1.0e-01	12
GSE113165_BED_VAL_F_ELD_ACT_OWE_HLY_D05	NA	NA	
GSE113165_BED_VAL_F_YNG_ACT_LEA_HLY_D05	NA	NA	
GSE113165_BED_VAL_M_ELD_ACT_OWE_HLY_D05	NA	NA	
GSE113165_BED_VAL_M_YNG_ACT_LEA_HLY_D05	NA	NA	
Meta-analysis score	**-0.30**	**2.3e-05**	**77**

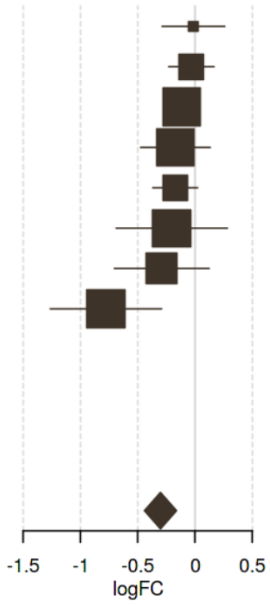


Figure 2. Analysis of the expression of (A) FGF8 and (B) Pea3 (ETV4) in human inactivity studies using the MetaMEx expression tool [40]. Both *FGF8* and *Pea3* are decreased as a result of inactivity or unloading by a log10 fold change (logFC) of ~0.3, equating to a percent change of ~80%.

Myostatin

Another myokine that is associated with muscle size and tendon function is myostatin. During development, inhibition of myostatin activity leads to increased muscle fiber size and total fiber number [41] and increases bone mass [42]. In muscle, myostatin signals through the binding and activation of the ActRIIB and ALK4/ALK5 receptor complex. The activated receptor complex phosphorylates Smad2 and/or Smad3 [43]. Phosphorylated Smad2 or 3 then binds with Smad4 and translocates into the nucleus to alter gene expression [44] resulting in decreased muscle mass [45–47]. Physiologically, myostatin activity can be reduced through decreased expression of myostatin [48,49], increased c-ski protein [50–52], or increased Notch activity [53]. All of these are associated with an increase in muscle mass. For example, resistance exercise results in the cleavage of Notch protein at the membrane resulting in the release of the notch intracellular domain, and a decrease in myostatin activity that leads to increased expression of Akirin1 (mighty) [54]. Since mighty is a direct target of myostatin [55], this gene is an important indicator of myostatin activity. In support of the role of myostatin activity and mighty in muscle growth, the production of mighty 6 hours after resistance exercise is directly proportional to the increase in muscle mass as a result of 6 weeks of training. Together, these data suggest that increased loading results in reduced myostatin activity in the loaded skeletal muscle, which is associated with muscle hypertrophy.

In contrast, following three days of unloading, myostatin mRNA and protein levels increase significantly in humans [56]. Consistent with the increase in myostatin following a short period of inactivity, MetaMEx analysis of mighty mRNA shows that akirin1 goes down ~40% following 2-5 days of inactivity (Figure 3; [40]). Similarly, in hind limb suspension models,

myostatin increases with unloading [57] and decreases with subsequent reloading [58]. Taken in total, myostatin activity is inversely related to muscle mass such that myostatin, and its downstream targets, is upregulated during muscle wasting.

Given the fact that myostatin activity increases during muscle atrophy and that myostatin is a paracrine factor whose protein has local/global affects, it is an important factor to consider when addressing potential muscle-tendon crosstalk. Myostatin is a member of the transforming growth factor (TGF) β family. This family of more than 30 cytokines includes the growth and differentiating factors (GDF), activins, and bone morphogenetic proteins (BMP). These proteins regulate many bodily functions including fibrosis, making them likely regulators of tendon function. In support of this hypothesis, tendons are smaller, stiffer, and hypocellular in myostatin deficient mice when compared to control mice [59]. Further, treatment of fibroblasts *in vitro* with myostatin induces proliferation [59] and can drive tenogenic differentiation of C2C12 myoblasts and rat bone marrow-derived mesenchymal stem cells [60,61]. These data suggest that myostatin regulates progenitor cell differentiation into fibroblasts and their subsequent proliferation, which could be important in tendon maintenance and regeneration following injury. Further support for the role of muscle derived myostatin comes from the fact that the expression of the myostatin inhibitor follistatin in tendon decreases following injury and is further decreased with loading even though myostatin expression itself is unchanged in the tendon [62]. In support of these results, Heinemeier and colleagues have shown that myostatin expression decreases with 4 days of loading in muscle, whereas tendon expression is unaffected by loading or unloading [48,63]. Moreover, myostatin is able to stimulate the differentiation of bone marrow-derived stem cells into tenocytes in a regenerating tissue [64]. Using myostatin coated sutures to repair an injured

rabbit Achilles tendon did not improve tendon mechanics 4 or 7 days following the repair [65]. However, Muraoka and colleagues did find that the myostatin coated suture increased *ColIa1* and *ColIa2* mRNA more than 10-fold at both 4 and 7 days [65], suggesting that the early increase in collagen production has yet to be translated into mechanical properties. Together, these data suggest that myostatin effects tendon maintenance and/or regeneration and that myostatin represents a potential mechanism of muscle-tendon crosstalk during muscle wasting. In such a model, myostatin production would increase with muscle wasting, move to the tendon, and increase collagen production and tendon stiffness. However, whether this occurs or not has yet to be demonstrated experimentally.

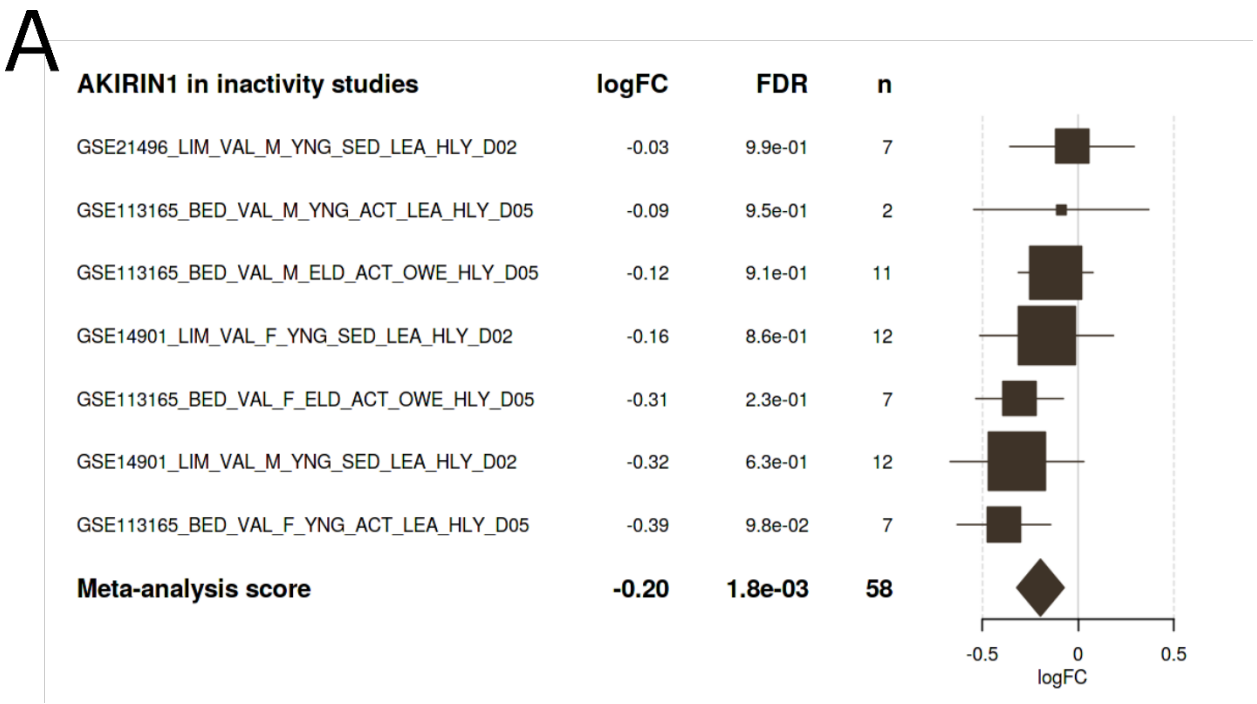


Figure 3. Analysis of the expression of (A) akirin1 (*mighty*) in short-term (2 to 5 days) human inactivity studies using the MetaMEx expression tool [40]. Mighty decreased as a result of short-term inactivity or unloading by a log10 fold change (logFC) of 0.2, equating to a percent change of 40%.

SPARC

The secreted protein acidic and rich in cysteine (SPARC) is a multifunctional extracellular matrix (ECM) protein that is associated with development, tissue remodeling, and the response to injury [66]. SPARC is expressed during muscle development and regeneration, as well as in satellite cells and myoblasts suggesting a potentially important role in muscle [67]. SPARC enhances myotube formation in differentiating C2C12 cells and increases ECM protein production during proliferation and after myotube formation [68]. By contrast, inhibition of SPARC decreases ECM production throughout the myogenic process [68]. Important for its potential as a myokine, muscle-derived SPARC is released during exercise [69]. Given that SPARC is present in muscle, and plays a role in muscle development and regeneration, SPARC may be important in the regulation of muscle mass. In support of this hypothesis, SPARC knockdown mice have smaller myofiber diameters than WT mice [70]. This suggests SPARC is required to maintain muscle mass even when muscle is being loaded normally. Additionally, SPARC expression is significantly decreased following 14 days of unloading and increases following either 1 or 7 days of reloading, suggesting that SPARC is regulated by loading [71]. In contrast, SPARC protein levels were unchanged in muscle following 12 weeks of ladder climbing and then increased within the muscle following 7 days of immobilization [72]. Interestingly, even though SPARC protein increased in muscle, the circulating level of SPARC was lower in the immobilized animals, suggesting that other tissues may be receiving less SPARC when muscle is wasting. More research is necessary to determine the precise role of SPARC with regards to muscle mass regulation; however, current work suggests that SPARC is important for the maintenance of muscle mass and function [73].

As in muscle, lower SPARC is associated with smaller, less functional tendons. In mice, SPARC protein decreases with age and this is associated with a decrease in the expression of tendon-specific genes [74]. To better determine the cause-and-effect relationship between SPARC and tendon function, Gehwolf and colleagues generated a whole body SPARC knockout [74]. In the resulting animals, Achilles tendon diameter was smaller and cellularity was increased compared to WT controls [74]. The knockout mice also had smaller collagen fibril diameters, increased tendon stiffness and a decreased maximum tensile force [74], suggesting that in the absence of SPARC tendons became small and brittle. A follow-up study on the SPARC knockout mice showed that SPARC was essential for the response to mechanical loading in tendon. Instead of tendons becoming thicker and stronger following exercise training, Achilles tendons in the SPARC knockout mice spontaneously ruptured, suggesting that in the absence of SPARC tendons were more prone to load-induced injury. By contrast, inhibiting muscle contraction using Botox resulted in an increase in tendon-specific gene expression, and patellar tendon thickness and a decrease in cell number. Interestingly, gene expression and structure of the SPARC knockout and WT tendons looked similar with the addition of Botox, suggesting that the phenotype of a tendon attached to an inactive muscle resembles that of a SPARC knockout [75]. Wang and colleagues also identified a heterozygous missense mutation in SPARC that results in impaired collagen I production and may increase the risk of tendon injury in humans [75]. Even though these data are suggestive of muscle-tendon crosstalk through SPARC, to determine whether SPARC functions as a myokine that regulates tendon function, the structure/function of tendons from mice with a muscle-specific knockout or overexpression of SPARC needs to be determined.

Inflammatory Response to Load and Injury

The inflammatory response is another form of tissue crosstalk. Following mechanical load or injury whole body, as well as muscle, inflammation can increase. Inflammation typically has a negative connotation and is often associated with disease or injury. However, a normal response to muscle damage includes an acute inflammatory response that is important for healthy recovery [76]. Following muscle damage, cytokines are released to recruit immune cells to the damaged site. Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are two crucial pro-inflammatory cytokines [77] that activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [78] and signal transducer and activator of transcription (STAT3) [79], respectively. In TNF-α receptor knockout mice, regeneration of injured soleus muscle was significantly impaired through the disruption of normal myogenic differentiation [80]. Furthermore, *TNF-α* expression is elevated immediately after resistance exercise and remains high through 24 hours [81], while another study showed an immediate increase but a return to baseline by 24 hours [82]. These results indicate a critical role for TNF-α in muscle regeneration following loading. However, elevated TNF-α has also been implicated in muscle wasting [83] and chronic low levels of inflammation result in lower muscle mass and strength [84]. IL-6 has a similarly conflicting role in muscle health. High levels of IL-6 have been reported to lower muscle mass and strength in elderly population [85] while chronic overexpression of IL-6 causes muscle wasting in rats [86]. These findings alongside similar outcomes with chronic increases in TNF-α highlight this idea that unresolved inflammation leads to muscle atrophy. However, just like TNF-α, IL-6 has been found to be critical in a healthy response to muscle damage [87]. In IL-6 knockout mice, myoblasts had an impaired inflammatory response to injury resulting in reduced myoblast proliferation as well as less myotube formation [87]. Therefore, IL-6 is crucial

in the early stages of regeneration characterized by increased cell proliferation as well as necessary for differentiation typically associated with anti-inflammatory stage of an injury response. An explanation for these different roles proposed by Howard et al. is that both the duration and concentration of these cytokines determines whether there is a corresponding increase in proliferation or differentiation [88]. Altogether, TNF-α and IL-6 clearly play a crucial yet complex role in muscle regeneration and atrophy that requires more research to further clarify the timing and regulation of the signals involved.

When considering potential crosstalk between muscle and tendon, it is important to consider the role of these same cytokines in tendon. Inflammation in tendon/ligament has long been controversial when considering tendinopathy – the term describing an injured or diseased tendon. It has long been thought that inflammation was an acute response to tendon injury that resolved within days, but did not play a role in chronic tendinopathies [89–91]. However, more recent work has identified elevated markers of inflammation in chronic tendinopathies [92–94], similar to the detrimental effects observed in muscle with chronic inflammation discussed above. The NF-κB pathway, which can be activated by TNF-α, has been indicated as a pro-inflammatory pathway that is elevated in early, intermediate, and chronic tendon injury [5,95]. TNF-α signaling leads to decreased collagen synthesis and increased matrix metalloproteinase activity [96], while early inhibition of TNF-α actually improves rotator cuff healing [97]. Although inhibition of TNF-α appears to improve tendon healing, it also plays a critical role in early debris clearance and increased cellularity necessary to repair damaged tissue [98]. Like the TNF-α/NF-κB pathway, the IL-6/JAK/STAT signaling pathway has been a target for anti-inflammatory interventions [99]. IL-6 has been shown to be elevated in patients with rheumatoid arthritis [100]

and in transected rat patellar tendon [101], again demonstrating a role in both chronic and acute inflammatory responses. However, complicating the role of JAK/STAT signaling in tendon degeneration are recent studies suggesting that STAT3 activity may reduce inflammation and scar formation after tendon injury [102]. Also, cyclic loading of tendon fascicles resulted in increased IL-6 expression and collagen, once again showing similar conflicting roles to TNF-α/NF-κB. Ultimately, the complex inflammatory response in both muscle and tendon suggest that there may be crosstalk between tissues as a function of infection and injury. However, more work on the role of inflammatory signals within tendon is needed.

Muscle Exosomes and Regulation of Matrix Production

Even though protein signals can drive muscle adaptations, the work by West and colleagues that showed that engineered ligaments grown in post-exercise serum made more collagen and were mechanically stronger than those grown in serum from resting individuals were unable to find proteins within the serum that could explain the difference [22]. This suggests that the muscle-tendon crosstalk could be mediated by a different molecular mechanism. One possible mechanism is through exosomal transmission of non-protein factors. Exercise increases the production of exosomes by muscle [103] and exosomes can alter the production of matrix [104]. Fry and colleagues have shown that satellite cell-derived exosomes can decrease the production of collagen by fibro/adipogenic precursor (FAP) cells. Removing dicer reversed the effect of the satellite cell-derived exosomes, suggesting that the inhibition of collagen production by FAPs was the result of a microRNA. The authors went on to identify the miRNA as miR-206 and showed that removal of miR-206 resulted in an increase in the production of collagen by FAPs. Lastly, combining load and removal of satellite cells (to remove miR-206) resulted in an increase

in collagen within the matrix of the muscle [104]. Together, these data suggest that muscle-derived exosomes can modify collagen production within the matrix; however, whether these exosomes can affect tendon cell collagen production and mechanics has yet to be demonstrated.

If exosomal transmission of miRNAs underlies muscle-tendon crosstalk, the next question is which miRNA(s) could mediate the effect on muscle wasting of tendon physiology? A 2018 systematic review of the effect of miRNAs on tenocytes and tendon-related gene expression identified 12 miRNAs that met their criteria [105]. Of these, the miR-29 family (a, b, or b-3p) is the best described. miR-29 was first described by van Rooij and colleagues as being down-regulated in response to a myocardial infarction and targeting mRNAs that encode proteins involved in fibrosis, including multiple collagens, fibrillins, and elastin [106]. Using an anti-miR to decrease miR-29b induced the expression of collagen, whereas over expression of miR-29b resulted in a dose-dependent decrease in collagens Ia1, Ia2, and IIIa1. Interestingly, Hu and colleagues found a 5- to 13-fold increase in members of the miR-29 family in sarcopenic muscle [107]. If this increase in miR-29 in old muscle resulted in a similar effect on exosomal levels of miR-29, and these exosomes reached the tendon, the expected result would be a decrease in collagen mRNA within the tendon with age, as has been described [108].

The understanding of how miRNAs could signal tissue crosstalk through exosomes is in its infancy. The understanding of how miRNAs affect tendon collagen expression and mechanical function is also poorly understood. Both areas need far more research moving forward so that we can advance our understanding of muscle-tendon crosstalk.

Demonstrating Muscle-Tendon Crosstalk

Only a small number of studies have actively looked at both tendon and muscle during muscle growth or wasting [63,109–111]. Furthermore, we are not aware of any studies that have specifically explored muscle-tendon crosstalk via cellular signals originating from adult muscle in response to anabolic/catabolic stimuli. This is a particularly important area of research since the identification of chemical signals originating in muscle that can modulate tendon structure/function would have a dramatic effect on recovery following injury or the maintenance of strength in the face of muscle wasting.

With this in mind, it is important to lay out how one could experimentally demonstrate muscle-tendon crosstalk to investigate the roles of the previously mentioned signals on tendon health. Experimentally, this could take several forms.

1. To test individual cytokines in a controlled environment, use of an *in vitro* engineered tendon/ligament model would avoid the potential genetic compensation that occurs when using an *in vivo* knockout model [112]. The engineered ligament model, originally developed by the Baar lab group [4], utilizes fibroblasts isolated from the human anterior cruciate ligament (ACL) which both respond to and secrete cytokines [113,114]. Furthermore, this model has increased cell mass and protein turnover compared to the slow turnover and relatively low cell mass (10%) of *in vivo* tissues [115], which allows for easier biochemical analysis and higher throughput. Treatment with TNF-α or IL-6 at different doses and durations would allow for specific testing of the role of TNF-α/NF-κB or IL-6/STAT3 signaling within tendon/ligament. Although use of muscle-derived TNF-α or IL-6 would best represent muscle-tendon crosstalk, use of exogenous cytokines

would likely improve repeatability between experiments which would be crucial in working to clarify such a complex signaling event.

2. The most effective way to study crosstalk as a function of loading is to combine circulating factors from *in vivo* tissues following loading with an *in vitro* system that has not been loaded. For these experiments, proteins or exosomes isolated from muscles that are undergoing a growth stimulus (after resistance exercise or overload hypertrophy) would be expected to increase collagen synthesis and improve mechanics of cultured tendon cells or engineered tendons/ligaments, respectively. By contrast, proteins or exosomes isolated from muscles that are undergoing an atrophy stimulus (unloading, immobilization, aging) should have the opposite effect. The constituents of exosomes could then be sequenced, and the individual components tested in the same model. Here, a specific miRNA sponge could be transfected into the tendon cells *in vitro* and the ability of the exosomes to improve collagen synthesis and mechanics measured directly.
3. To test a positive factor (such as FGF or SPARC) *in vivo*, overexpression in the gastrocnemius muscle of mice using viral transduction [116] or inducible overexpression [117] should increase collagen synthesis and improve tendon mechanics even in the absence of load. For example, overexpressing a positive factor during hind limb unloading would still result in more collagen and a stronger tissue than a control limb that has been unloaded.
4. Similarly, to test negative regulators (such as myostatin or inflammatory cytokines), that increase in muscle as the muscle gets smaller, overexpression of inhibitors (follistatin to inhibit myostatin; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) to inhibit NF-κB) in the gastrocnemius muscle should decrease collagen

and mechanics of the Achilles as has been described for myostatin [118]. Because over expressing follistatin would increase muscle mass, performing this experiment when the animal was unloaded would highlight the effect of muscle mass in determining tendon mass. In this example, if muscle mass drove tendon mass, unloading an animal where follistatin was over expressed would result in a bigger muscle and tendon. If load and crosstalk were more important, the muscle would be bigger, but the tendon would have lower collagen content and mechanics.

Using these techniques, the obvious questions raised by the discussion above are:

1. Do pro-inflammatory cytokines alone alter tendon function in the absence of immune cells?
2. Can the effects of pro-inflammatory cytokines on tendon be manipulated by both the dose and duration of exposure?
3. Is increased expression of FGF in muscle in response to load necessary for increased *Pea3/Egr1* expression and mechanics in tendon?
4. Can FGF release from muscle overcome the negative effect of unloading on tendon gene expression and mechanics?
5. Can blocking myostatin activity during unloading decrease tendon function even while increasing muscle mass?
6. Is SPARC expression in muscle sufficient to improve tendon structure/function even in the absence of loading?
7. Does ablation of SPARC specifically from muscle prevent load-induced improvements in tendon gene expression and mechanical function?

8. What miRNAs are found within exosomes of loaded and unloaded muscles that might affect tendon function?
9. Does miR-29 released from muscle alter collagen expression in tendons?

The focus of this dissertation was crosstalk mediated by inflammatory cytokines. I targeted the first two questions raised above. I then went on to determine how loading and inflammatory cytokines combined to alter ligament function. My hope is that a better understanding of this communication will lead to improved interventions to maintain tendon health. To begin this work, I identified a drug that could reverse the negative effects of inflammation on ligament structure and function. In the long term, such an intervention could reduce the occurrence of tendon injury and improve clinical outcomes following periods of illness, forced inactivity, or immobilization.

Chapter 2: Characterization of an *In Vitro* Engineered Ligament Model

This chapter is in press at *Matrix Biology Plus*.

A. Avey, O. Valdez, K. Baar, Characterization of an *In Vitro* Engineered Ligament Model. *Matrix Biology Plus*. (2023).

Abstract

In vivo tendon and ligament research can be limited by the difficulty of obtaining tissue samples that can be biochemically analyzed. In this study, we characterize the most widely used *in vitro* engineered ligament model. Despite previous works suggesting multiple passages change gene expression in 2D primary tenocytes, we found no relationship between passage number and expression of classical tendon fibroblast markers across different biological donors. When engineered into 3D ligaments, there was an increase in maximal tensile load between 7 and 14 days in culture, that corresponded with an increase in collagen content. By contrast, percent collagen increased logarithmically from Day 7 to Day 14, and this was similar to the increase in the modulus of the tissue. Importantly, there was no relationship between passage number and mechanical function or collagen content in the two independent donors tested. These results suggest that the model develops quickly and is reliable across differing passage numbers. This provides the field with the ability to 1) consistently determine functional changes of interventions out to passage number 10; and 2) to time interventions to the appropriate developmental stage: developing/regenerating (Day 7) or mature (Day 14) tissue.

Introduction

Ligaments and tendons are fibrous connective tissues made by specialized fibroblasts that during development express the basic helix-loop-helix transcription factor scleraxis (SCX) [119] and reside within a dense extracellular matrix (ECM). These SCX^+ fibroblasts synthesize and maintain the ECM, which largely consists of aligned type I collagen.

Even though ligaments and tendons are generally grouped together due to similarities in molecular composition, structure, and function [120], there is an important functional difference between the two. Ligaments connect two stiff tissues (bone to bone), whereas tendons connect a stiff tissue to a dynamic compliant tissue (bone to muscle). This functional difference, results in mechanical variation along the length of a tendon that is not present in ligament [121]. Therefore, when materials of the same stiffness are at either end, it is appropriate to describe the tissue as a ligament. Acknowledging this fundamental difference between the tissues, all structures containing aligned collagen as the primary component of the tissue that have been engineered to date should be referred to as ligaments, even though they can likely model the development and adaptation of either tissue.

In vivo, tendons and ligaments are composed of an extensive extracellular matrix and low cell number which can make biochemical analyses such as qPCR or western blotting difficult [4]. Further, protein turnover within adult human tendons may be very low resulting in difficulties quantifying changes in total protein following interventions [5]. Engineered ligaments were developed to overcome these issues and produce an *in vitro* model that could provide cellular,

developmental, and biochemical insights into the response of tendon/ligament cells to stress without compromising the ability to determine the mechanical characteristics of the tissue.

There are several ways in which *in vitro* engineered ligament models may not truly represent the native tissue. In a two-dimensional (2D) cell culture setting, increasing passage number may cause de-differentiation of the primary cells [122,123], resulting in cells that do not reflect the native tissue. In the three-dimensional (3D) setting, the tissues have more cells and less extracellular matrix than adult tendons *in vivo* [124], they express more developmental isoforms of collagen [125], have uniform small collagen fibrils [126], and as a result are much weaker than adult tissues [4]. In many ways, engineered ligaments are therefore more comparable to developing ligaments than their adult counterparts [127]. Even with these limitations, engineered ligaments have similar responses to nutrients [4], growth factors [128], hormones [129], cytokines [130], and exercise [131,132] to those reported for native tendons or ligaments. These data suggest that engineered ligaments may be a good model for understanding the physiology of these complex connective tissues if they were better characterized.

Despite demonstrated effectiveness of engineered ligaments, further characterization of the effect of cell passage number and developmental stage of the organoid is still needed. In this study, we set out to further characterize this model by investigating the effect of increasing passage number on gene expression in a 2D environment as well as mechanical function of 3D engineered ligaments. Furthermore, we characterized the development of human engineered ligament matrix and mechanical properties over time. Ultimately, our objective was to provide insight into the methodology so that engineered ligaments could better model native tissues *in vivo*.

Methods

The human anterior cruciate ligament (ACL) remnants used for cell isolation were obtained following informed consent and all procedures and experiments were approved by the University of California Davis Institutional Review Board (IRB# 779755- A tissue collection study for patients undergoing anterior cruciate ligament (ACL) reconstruction).

Human ACL Fibroblast Isolation

Fibroblast isolation was performed as previously described [4]. Briefly, the remnants of human ACLs were placed into a sterile 50 ml tube and washed 5 times with 25 ml of Dulbecco's phosphate buffered saline (DPBS). The tissue was then transferred to a sterile 50 ml tube and stored in 25 ml 0.5x antibiotic/antimycotic solution at 4 °C for 1 hour. The remnant was then transferred to a sterile 50 ml tube containing 25 ml of a 0.1% Collagenase Type II Solution (Dulbecco's Modified Eagle Medium (DMEM), 20% fetal bovine serum (FBS), 1% penicillin, 0.1% collagenase type II) and incubated at 37 °C overnight. The following day the tube was centrifuged for 5 minutes at 2000 x g, the supernatant aspirated, and washed with 10 ml of growth media (GM; DMEM containing 10% FBS). Washing with GM twice before the cells were plated on 15 cm cell culture treated plates. The freshly isolated cells were considered passage zero (P0).

Passaging Cells

When the cells reached approximately 70% confluence, they were expanded by trypsin-triggered cell dissociation and passaging onto new plates. The growth media was replaced every 3 days until the cells were ready to passage. At each point, cells were passaged 1:5 with one plate at

each passage used for gene expression analysis and four for expansion/engineering ligaments. For engineered ligaments, cells were passaged until sufficient plates (20-25) were produced.

RNA Extraction/qPCR

Human ACL cells were grown in a 2D environment and collected from plates from passage zero until passage seven. Cells were collected in Trizol and vortexed to release RNA. After centrifugation for five minutes at 10,000xg, chloroform extraction of the aqueous solution phase, precipitation with isopropanol, and washing with ethanol, RNA was resuspended and quantified. One microgram of RNA was reverse transcribed (MultiScribe RT, 10x RT buffer, 10x Primers, dNTPs, RNase inhibitor; Applied Biosystems, Foster City, CA) and then diluted to 3 ng/µl prior to qPCR. Samples were amplified in triplicate with 3 µL cDNA, 5 µL SYBR green (BioRad, Hercules, CA; PCRbio.com, Wayne, Pennsylvania), and 2 µL of 10 uM primer (Invitrogen, ThermoFisher, Waltham, MA). qPCR reactions were performed on a CFX384 Touch Real-Time PCR Detection System (BidRad, Hercules, CA). GAPDH was used as housekeeping control. Absolute CT for GAPDH was not different between groups. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Construct Plate Preparation

Minutien pins (0.2mm) were placed into 1.5 mm tear drop anchor molds. The solid phase (β-tricalcium phosphate powder, Plasma Biotal Limited, Derbyshire, UK) was mixed in equal parts (1g:1mL) with ice cold liquid phase (3.5M ortho-phosphoric acid with 100mM citric acid) and then pipetted into molds. Molds were centrifuged for 1 minute at 2250 x g at 4 °C, then left overnight to set. The next day, the anchors were removed from the molds and pinned 12 mm

apart into Sylgard PDMS (Dow, Midland, MI) coated 35 mm plates. Plates were sterilized in 70% ethanol for 1 hour.

Engineering Ligaments

Constructs were engineered as previously described [4]. For each construct, $2.5x10^5$ cells were suspended in 714 µl growth medium containing 5.8 Units of thrombin, 20 µg aprotinin, and 200 µg 6-aminohexanoic acid. This mixture was added to a 35 mm plate with brushite anchors and shaken to make sure the solution covered the plate. To the thrombin/cell mixture, 286 µl of fibrinogen (20 mg/ml) was added and the plate was incubated at 37 °C with 5% CO_2 for 15 minutes to allow fibrin-gel formation. After 15 minutes, 2 ml of feed media (GM supplemented with 200 µM ascorbic acid, 50 µM proline, and 5 ng/ml of transforming growth factor-β1(TGF-β1)) was added to each plate. Feed media is replaced every other day until testing.

Mechanical Testing

On day of testing, constructs were removed from the incubator, and the length measured using digital calipers as the distance between the inside points of the teardrop anchors. The media was aspirated, the plates rinsed with Dulbecco's modified phosphate buffered saline (DPBS) and the width and thickness of the tissue were determined by spectral-domain optical coherence tomography using a OQ Labscope (Lumedica, Durham, NC). After measuring width and depth, 2 ml of DPBS were added back to the plate to keep ligament hydrated until testing. Ligaments still attached to anchors were loaded into 3D printed grips in the test space of a Model 68SC-1 single column tensile tester (Instron, Norwood, MA) containing a 10N load cell. The grips were printed to secure each brushite anchor with an appropriate opening for the tissue to fit through.

At later timepoints when anchor degradation became a limitation, constructs that were mechanically tested were those whose anchors were still able to be loaded into grips without failing. The samples were submerged in 37°C saline within the temperature controlled BioBath, and mechanically tested using 10 cycles of preconditioning to 0.10 N at a rate of 0.25 mm/s prior to loading to failure at a constant rate of 0.25 mm/s. From the test, the maximum tensile load (MTL), ultimate tensile strength (UTS), modulus, and cross-sectional area (CSA) were determined. MTL is the maximal load measured at failure in Newtons. UTS was calculated by normalizing the MTL by CSA (width x depth) and the Young's modulus was calculated as the maximal slope of the stress-strain (displacement divided by the initial length) curve. The position of graft failure was noted, and this data is presented as the % of grafts tested that failed at either the anchor or midsubstance. After testing, ligaments were removed from anchors and dried on a glass plate at 120 °C for 30 min. Dry mass was measured, and samples were left at room temperature until processed for hydroxyproline.

Hydroxyproline Assay

To determine collagen content, a hydroxyproline assay was performed [133]. Briefly, 200 µl of 6 N HCl were added to previously dried samples in 1.7 ml snap-cap tubes. Samples were hydrolyzed on a heat block at 120 °C for two hours (using micro-tube cap locks to prevent cap from opening during boiling). After two hours, the samples were transferred to a heat block in a laminar flow hood for another 90 minutes, this time with the tube lids opened to allow evaporation. After drying the samples, 200 µl of hydroxyproline buffer were added to each sample. Samples were further diluted 1:20 in hydroxyproline buffer and 150 µl of 14.1 mg/ml Chloramine T solution was added to each sample before vortexing and incubating at room

temperature for 20 minutes. Aldehyde-perchloric acid containing 60% 1-propanol, 5.8% perchloric acid and 1 M 4-(dimethylamino)benzaldehyde (150 µL) was added to each sample before vortexing and incubating at 60 °C for 15 minutes. Once removed from heat and allowed to cool at room temperature, 200 µl of each standard and sample were loaded in duplicate on a 96 well plate and absorbance was read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT). Hydroxyproline content of samples was calculated using a standard curve and then converted to total collagen by assuming hydroxyproline makes up 13.7% of the dry mass of collagen. The collagen content was then divided by the dry mass of each sample to determine the percent collagen.

Statistical analysis

For all assays, a technical replicate was a single engineered ligament or 2D tissue culture well within a group at a given time. Biological replicates reflect that the experiments were repeated using a different cell donor or a separate vial of cells from the same donor. Each experiment presented here therefore represents a single biological replicate that was analyzed independently using a one-way ANOVA with time as the primary factor of interest using GraphPad Prism v9. Where statistical differences were detected, a Tukey's honestly significant difference test was used for post-hoc analysis since all groups demonstrated equal variance. Linear regressions were performed separately and both linear and non-linear fit were calculated using Prism. Statistical analyses and the type I error was maintained at $\alpha < 0.05$ for all comparisons.

Results

After obtaining human ACL remnants from two different biological donors, cells were isolated and passaged multiple times (Figure 1A). At each passage, starting at 0, one plate of cells was collected to quantify gene expression of classical markers of tendon differentiation. We found that there was no change in expression of decorin, tenascin-C, collagen I, or collagen III as a function of passage number in either donor (Figure 1).

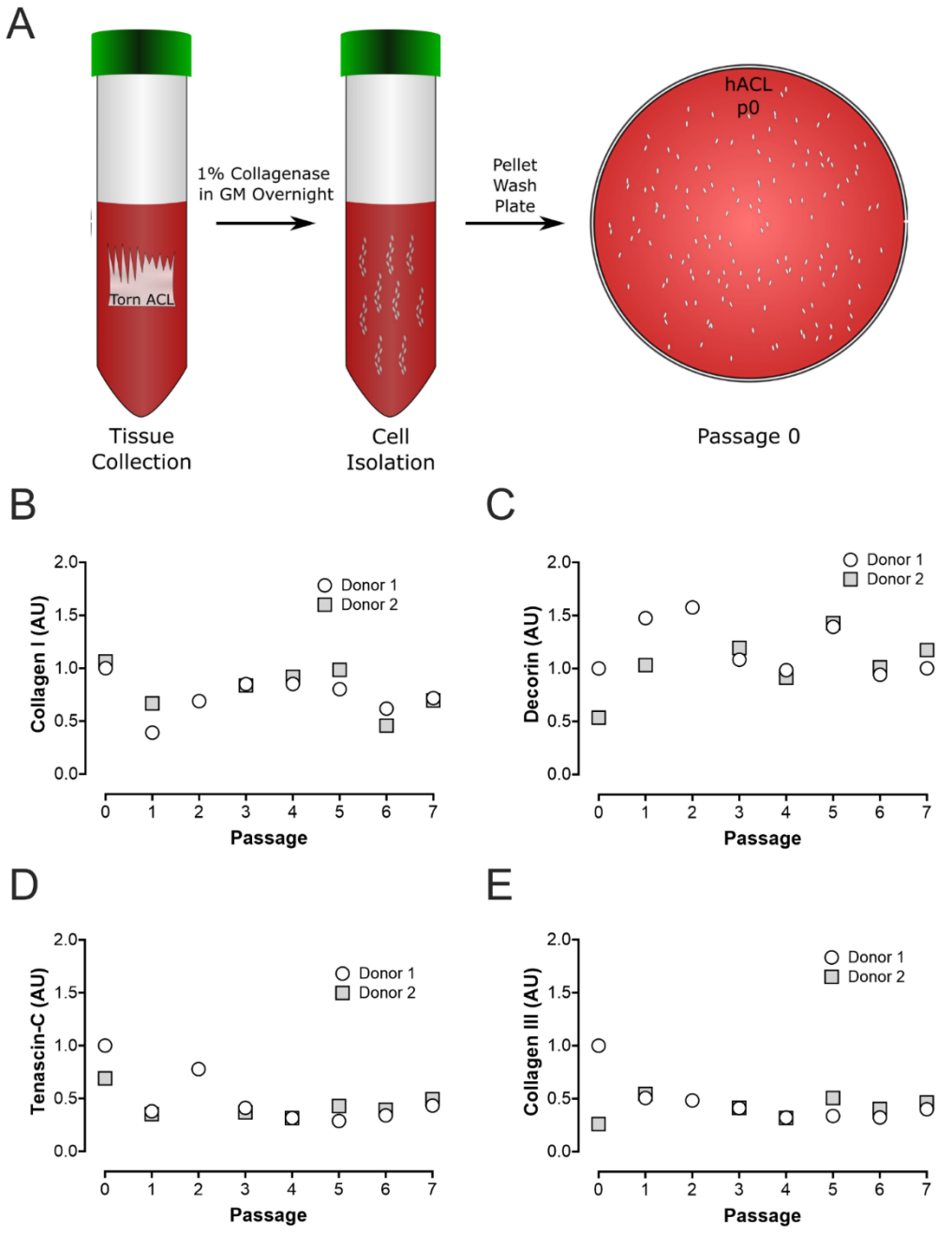


Figure 1: Schematic of the Isolation of Human ACL Cells and Gene Expression as a Function of Passage Number. Relative gene expression did not change as a function of passage number for **A)** Decorin, **B)** Tenascin-C, **C)** Collagen I, **D)** Collagen III. Dotted lines represent 95% confidence interval. n=2 biological replicates with each biological replicate having 3 technical replicates.

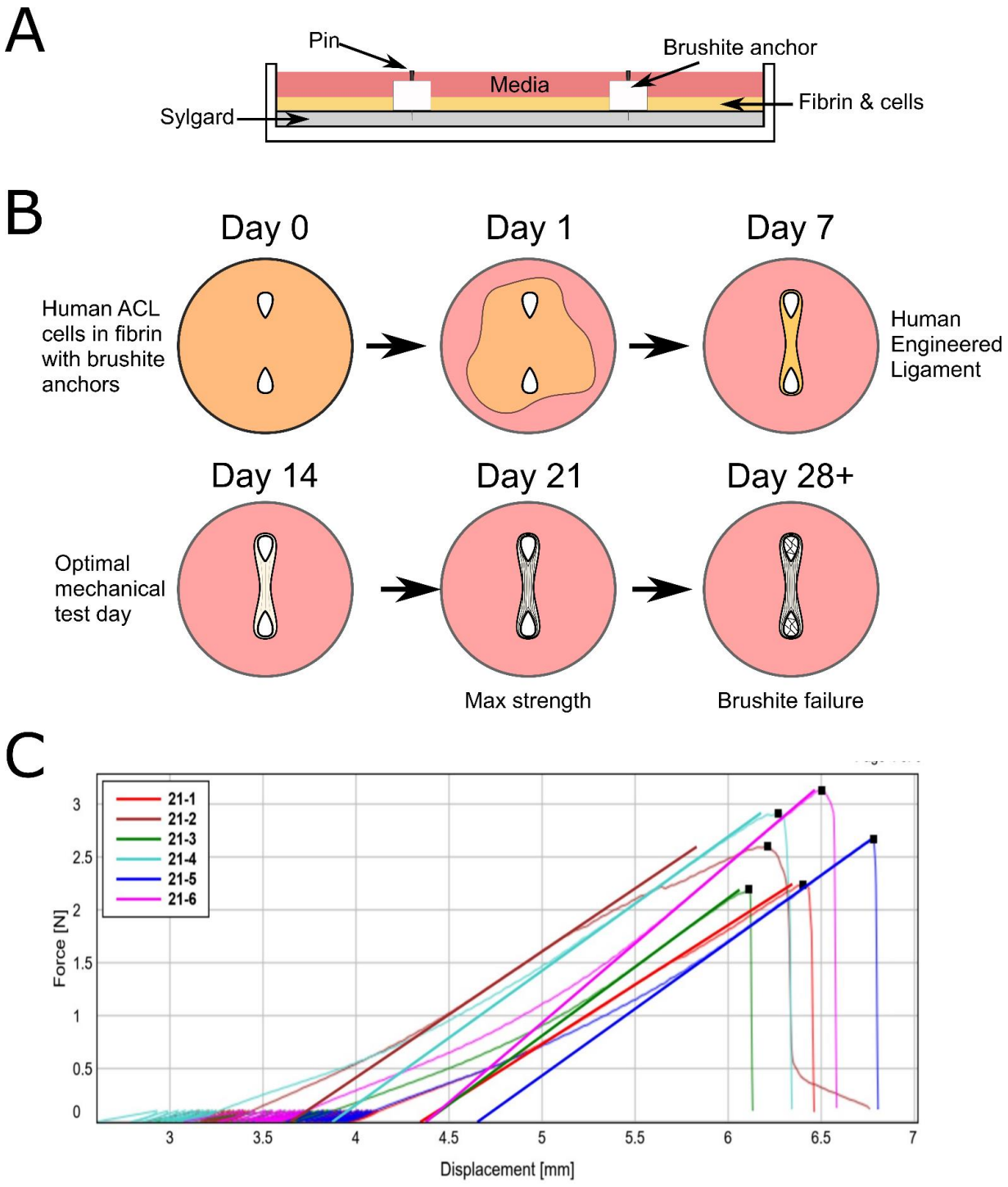


Figure 2: Schematic of the Formation of Engineered Ligaments. A) Ligaments are formed in a 35 mm plate by combining a fibrin/cell mixture and plating this mixture on PDMS substrate

containing two brushite anchors. Once the fibrin gel forms, the grafts are fed 2ml growth media. **B)** The fibrin gel is contracted by the cells around the brushite anchors within the first 4-5 days. Between Day 7 and Day 14 the cells digest the fibrin matrix and replace it with an endogenously produced native collagen matrix (represented by a shift from yellow (fibrin) to white (collagen)). Finally, the resorption of the brushite within the anchor is depicted at Day 28. **C)** The load – elongation curves for the 21-day-old engineered human ligaments reported in Figure 3.

To determine the optimal time to mechanically test engineered ligaments, we characterized the cellular and mechanical development of human engineered ligaments as a function of days in culture. All samples reported at each timepoint were made on the same day with the same batch of fibrin, thrombin, and media. The experiment was repeated on three separate occasions and the results were qualitatively identical. At Day 7, three of the six ligaments in the reported cohort failed during preconditioning and one failed before precondition, indicating that at this early time point the ligaments are extremely weak. Mechanical testing revealed a rapid increase in the maximal tensile load at failure (MTL; $r^2 = 0.917$ for a linear fit and 0.932 for an exponential fit) with over a 10-fold increase in mean MTL from Day 7 to Day 14 and a further 2.5-fold increase in mean from Day 14 to Day 21 (Figure 3A). After day 21, the anchors became mechanically unstable resulting in a drop in MTL due to poor sample gripping. There was a similar rapid increase in ultimate tensile strength (UTS; $r^2 = 0.951$ for a linear fit and 0.932 for a linear fit and 0.939 for an exponential fit) with over a 15-fold increase from Day 7 to Day 14 and a further 2-fold increase from Day 14 to Day 21 (Figure 3B). We did not include Day 28 or Day 35 in statistical analysis of MTL or UTS due to the degradation of the anchors and the resulting lowering of the absolute strength of the tissue. The increase in modulus ($r^2 = 0.904$ for a linear fit

and 0.85 for an exponential fit), a mechanical property that is measured at ~25% maximal load, followed a slightly different pattern increasing 6-fold between Day 7 to Day 14 and a further 1.5-fold increase from Day 14 to Day 21 (Figure 3C). Since the stability of the anchor is less important for modulus, we were able to observe a plateau in modulus between days 21 and 35. With maturation, the location of graft failure changed from at/near the anchor at days 7 and 14 to the midsubstance of the tissue at days 21 and 28 (Figure 3E).

In parallel with the increase in mechanical function with time, there was an increase in collagen content and concentration. Collagen content increased nearly 10-fold from Day 7 to Day 21 (R^2 = 0.958 for a linear fit and 0.947 for an exponential fit) and this increase appeared to be associated with the increase in MTL (R^2 = 0.958). Unlike MTL, collagen content continued to increase from Day 21 to Day 28 but did not change between Day 28 and Day 35 (Figure 3F). The percent of the dry mass of the tissue that was collagen (percent collagen) showed a rapid increase from Day 7 to Day 14 and then plateaued between Day 14 and 35 (Figure 3G). Dry mass of the tissue tended to increase linearly with time (R^2 = .759) increasing 2-fold from Day 7 to Day 35 (Figure 3H).

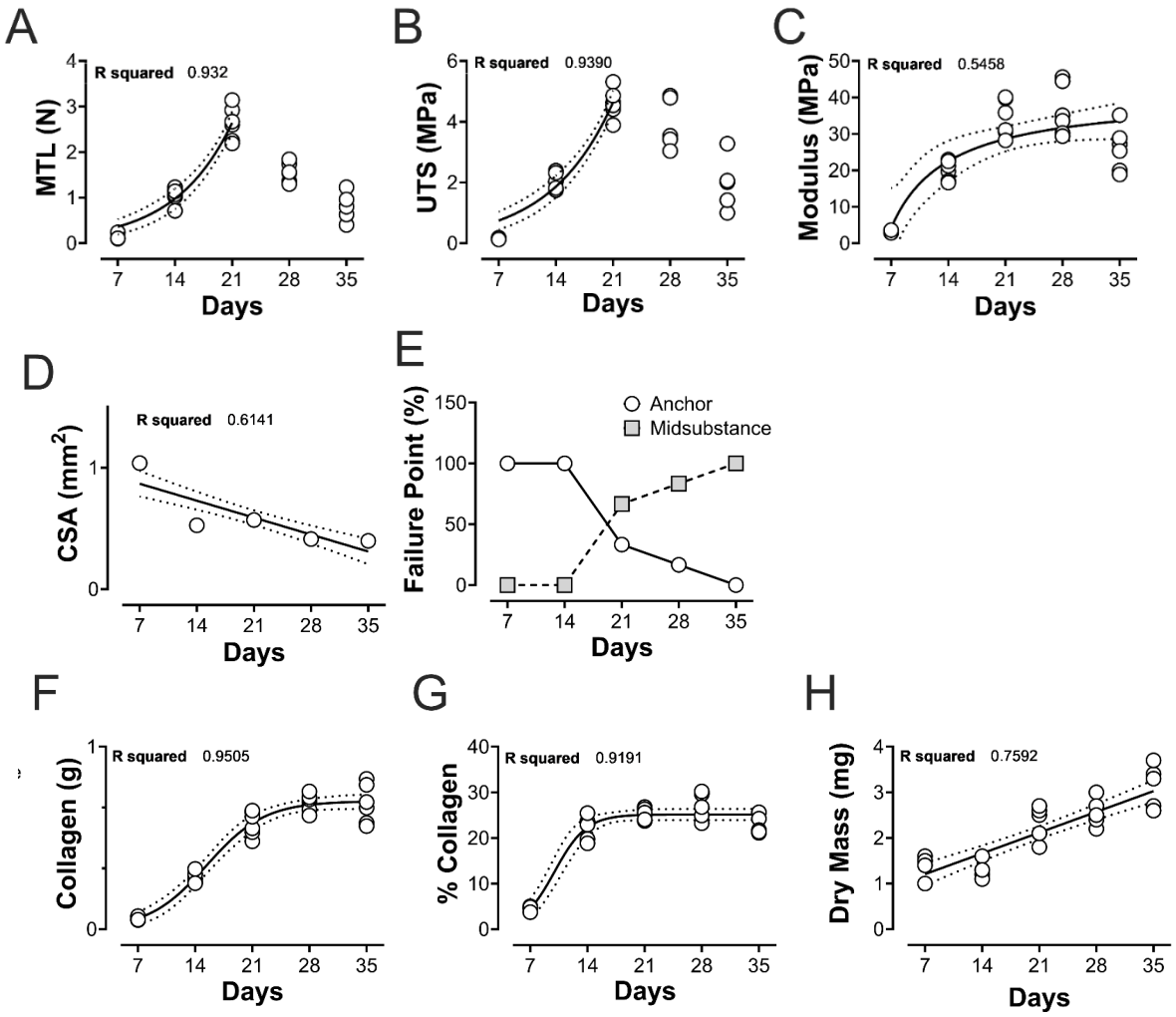


Figure 3: Construct Development Over Time. Mechanical **(A)** maximal tensile load (MTL) and material **(B)** Ultimate tensile strength (UTS) and **(C)** modulus show a rapid increase from Day 7 to Day 21 before regressing or plateauing. **(D)** Cross sectional area (CSA) decreases linearly as a function of time in culture. **(E)** Failure location, the location within the tissue where failure occurred changes with time. Collagen expressed either as **(E)** total content or **(F)** percent of dry mass rapidly increases before plateauing at day 21 and 14, respectively. **(G)** Dry mass increases linearly as a function of time in culture ($R^2 > .759$). Dotted lines represent 95% confidence interval. Dots are the technical replicates (n=6) at each time point. Qualitatively

identical data were seen in three biological replicates of the experiment using at least two different donors.

From the developmental data, we felt that day 14 was the best day to test engineered ligaments since mechanics and collagen were high but had yet to reach maximum tensile strength. We felt this was important since it would allow us to observe interventions that improved and/or impaired function and collagen accumulation. To determine the effect of passage number on human engineered ligament structure/function, ligaments were engineered for six consecutive passages for the two different donors and tested 14 days after plating. There was no significant change in MTL, UTS, and modulus as a function of passage number in either the female (Figure 4A-C) or male (Figure 4G-I) donor. Similarly, there was no change in total collagen, percent collagen, or tissue dry mass as a function of passage number in either the female (Figure 4D-F) or male (Figure 4J-L) donor.

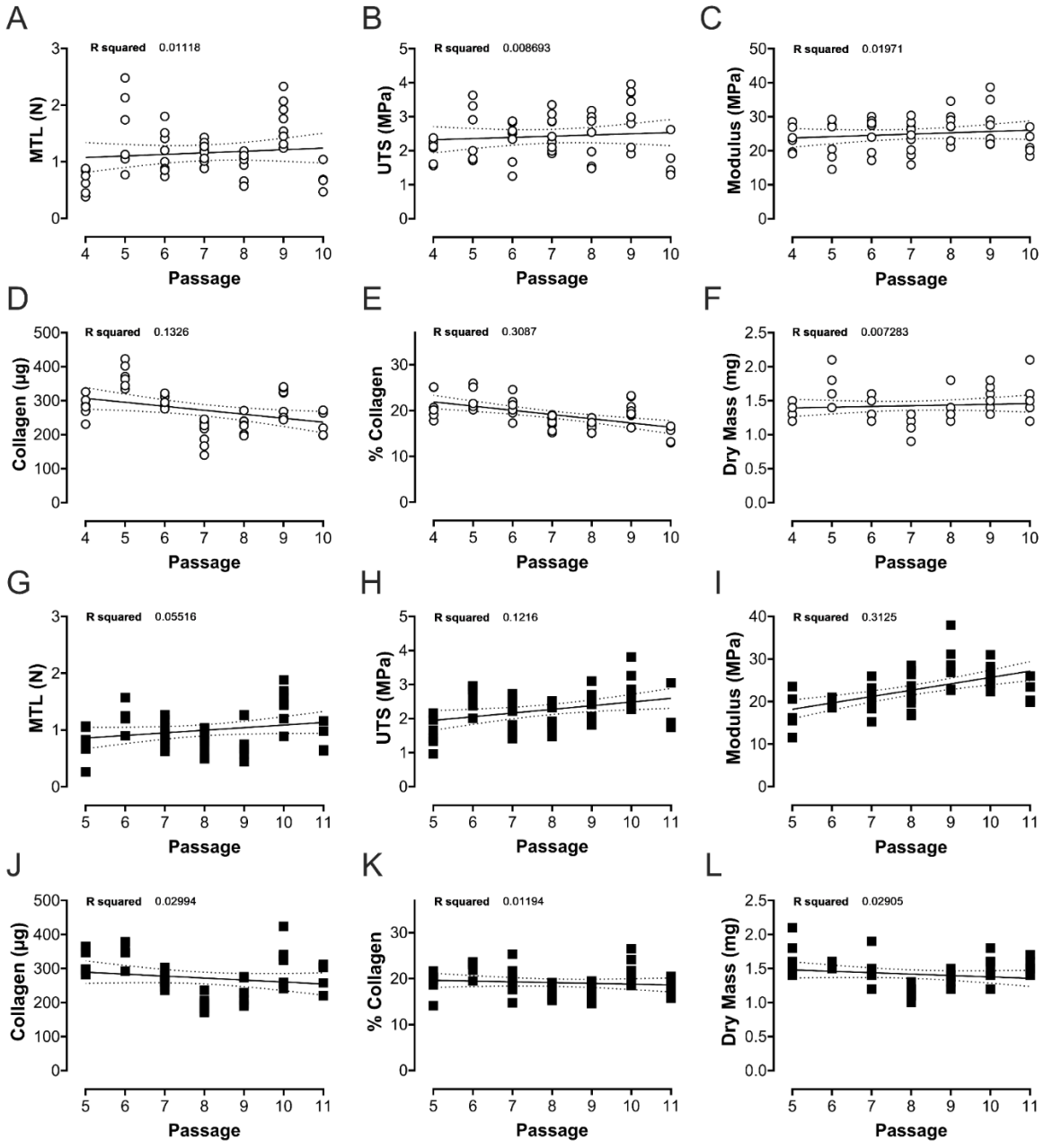


Figure 4: Construct Mechanics and Collagen as a Function of Passage Number. A-F) Are data from a 23-year-old female donor, whereas **G-L)** were collected on constructs made from a 31-year-old male donor. **A-C, G-I)** Mechanical and material properties do not change as a function of passage number. **D-F, J-L)** Total collagen content, percent collagen, and dry mass also do not change as a function of passage number. Dots represent the technical replicates (n=5-

10) at each passage. Solid lines are lines of best fit (R^2 is reported for the line used). Dotted lines represent 95% confidence interval.

Discussion

Despite previous studies demonstrating a shift in gene expression of primary cells *in vitro*[122,123], we found that through multiple passages (0-7) human ACL cells continue to express common tendon cell markers. When engineering ligaments, the tissues develop rapidly from a mechanically weak, collagen poor, tissue at day 7 to strong, collagen dense tissue at day 21. From day 21 to 35, the tissues continue to add collagen; however, the brushite anchors used in the current work begin to dissolve, limiting the strength of the full tissue. When ligaments are tested at day 14 for six contiguous passages there is no loss in either collagen or mechanics, indicating that human ACL cells can be passaged at least 11 times before the function of the ligaments are altered.

Previous studies in the field demonstrated multiple passages of cells isolated from tendons and ligaments and then cultured in a 2D environment showed changes in gene expression[6,7]. When we repeated this work, we did not find that human ACL cells de-differentiated in culture. Not only did the cells not de-differentiate at the level of gene expression, but we also did not detect any noticeable change in proliferation rate; however, this was not actively quantified. There are a few possible explanations for the differences between our findings and those of others. First, our cells were isolated from the remnants of human ACLs following rupture. These tissues are typically collected 2-3 months after injury. The result is that the cells that we are isolating may be different than those taken from an intact ACL or hamstring tendon. We have previously isolated cells from cadaveric human ACLs and hamstring tendons collected during ACL reconstruction and these cells produce qualitatively normal engineered ligaments. Further, Herchenhan and other from the Kjaer group have similarly produced engineered human

ligaments from human hamstring tendons[125,134,135]. Therefore, we do not feel that the cell type is the only issue underlying the difference in cell identity as a function of passage number. Other differences between the current work and previous experiments include the fact that previous studies used 100 mg/ml streptomycin in the culture medium and grew their cells to a higher confluence before passaging. In their manuscript, Mazzocca and colleagues show plates prior to passaging (Fig 1[122,123]) and the cells are completely confluent. A key component of our culturing process is not to let the cells get over 75% confluent. It is possible that passaging at a relatively low confluence may help maintain cellular function.

It is important to understand the development of human engineered ligaments as a function time to better use them to model native tissues. Therefore, we measured collagen content and mechanical properties from Day 7 to Day 35. Interestingly, the shifts in mechanics and collagen that we observed between days 7 and 21 mirrored what was recently shown in the development of rat patellar tendon from Day 7 to Day 28[136]. *In vivo* development of the patellar tendon showed a rapid increase in collagen content and concentration that was coupled with improved mechanical properties. For example, between day 7 and 28 the collagen content and MTL of the rat patellar tendon both increased 9-fold[136]. In the current work, the engineered ligament collagen content similarly increased 9-fold from day 7 to 21. Although the engineered ligaments in this model demonstrated similar mechanical development to what was previously described in rat, these engineered ligaments are still significantly smaller and weaker than an adult human ACL. The average cross-sectional area and mechanical properties of an adult male cadaver ACL are: CSA=72.9 mm; MTL=1818 N; failure stress=26 MPa; Modulus=128 MPa[137]. Compared to the average Day 21 values of engineered ligaments, these measurements are 128-fold, 692-

fold, 5.5-fold, and 4-fold higher, respectively. Interestingly, the material properties indicate that the tissues are within 5-fold of the native ACL. Despite the high material properties in the engineered ligaments, *in vivo* collagen accounts for approximately 80% of the dry mass of a human ACL, whereas the engineered ligaments plateaued at approximately 25%. Potential explanations for this are that the *in vitro* ligaments maintain a higher cell mass than the ~10% value seen *in vivo*; residual fibrin within the matrix may accounts for some of the dry mass; and the density and diameter of the collagen fibrils are likely significantly lower than native tissue. Further research will be needed to investigate matrix organization and composition with maturation of the engineered ligaments. Currently, the engineered ligaments are smaller and weaker with a less developed matrix and greater cellularity. Despite these differences, the data presented demonstrates similarities in development to an *in vivo* tissue even in the absence of the active (movement) and passive (bone growth) forces seen *in vivo*. Additionally, the greater cellularity of engineered ligaments may make interventions and biochemical analysis easier, adding to the usefulness of such a reliable *in vitro* ligament model.

The material properties reported here are some of the highest for engineered tendons/ligaments. For example, Puetzer and colleagues have engineer elegant tendon/ligaments with an integrated compressed interface[138]. These tissues are larger than those reported here. Using advanced imaging, they demonstrate that the collagen fibrils within the tissue are well aligned, and like this model max out at ~35nm[124,126]. However, the material properties of their tissues are an order of magnitude lower (modulus = 1MPa and failure stress = 0.4MPa at 6 weeks) than those reported here. There are a few possible explanations for these differences. Most importantly, Puetzer uses a collagen gel to form ligaments. Using a collagen gel can affect the development of

an engineered ligament since a high-density collagen gel is significantly stiffer than the fibrin used in the current model, and this may decrease the tensional strain on the cells and limit endogenous collagen production. A second issue that arises when using a collagen gel is that the large collagen molecules formed during gelation are likely difficult for cells to modify. By contrast, tendon and ligament cells within a fibrin gel create an endogenous matrix using fibropositors to align the matrix along the line of force[124]. A last possible issue is that within a collagen gel the cells lack developmental cues. Since fibrin is the matrix present when cells begin to form/regenerate a tendon, the stiffness, structure, and biochemistry likely provide proliferative cues. As this developmental matrix is replaced by a stiffer, cell-derived, collagen matrix, new cues for maturation likely help the cells quickly differentiate and drive the production and stabilization of collagen necessary to increase material properties.

The rapid increase in total and relative collagen (day 7-14) followed by a maturation phase (day 14-35) demonstrated by the plateau in both measures suggests the model can be used to study interventions targeting either a developing/regenerating tissue (days 7-14) or a mature (days 14-21) tissue. During development/regeneration *in vivo,* tissues have increased vascularity[139], cellularity[140], lower collagen content[140], and increased collagen synthesis[141]. This is similar to days 7-14 in the human engineered ligament model. On the other hand, interventions designed to target a more mature tissue, identified by reduced collagen turnover[115] and a more developed matrix, would be better modeled between days 14 and 21.

One interesting finding from the timecourse experiment was that as the engineered ligaments matured, the location of graft failure changed. Early in the process, the ligaments would always

fail at the interface between the soft biological tissue and the hard ceramic anchor. The progressive increase in the strength of this interface over time could reflect that as the brushite anchor was beginning to be resorbed the biological matrix of the ligament began to better integrate with the hard calcium phosphate, as we have previously seen by Raman microscopy[4]. As the anchor and the ligament better integrated, the weakness of the tissue shifted to the midsubstance resulting in higher failure rate at the midsubstance, as is often observed *in vivo* where the enthesis is mechanically stronger than the tissue proper[142].

A major limitation to the long-term study (days 28-35) was the degradation of the anchor seen from day 28 onwards. The integrity of the anchor was maintained out to day 21 as evidenced by the high MTLs. However, by day 28 there was noticeable cracking along the anchor surface and the ligaments would pull through the 3D printed grips resulting in lower maximal mechanical properties despite increased total collagen in the construct. The loss of anchor integrity is the result of our use of brushite cement. Originally, when the goal of engineering ligaments was to produce a graft that could be used for ACL reconstruction, we chose a 1:1 (powder:liquid) ratio of βTCP:orthophosphoric acid to make the brushite used as the anchor material for its ability to be rapidly resorbed and replaced by biological tissue *in vivo*[143]. These brushite anchors were never optimized for long-term culture. In fact, FBS is known to accelerate the degradation of 1:1 (P:L) ratio brushite[144]. A simple fix for those looking to keep engineered ligaments for longer than 21 days would be to switch to a 3:1 βTCP:orthophosphoric acid brushite, which loses two thirds less mass in cell culture[143], or calcium deficient hydroxyapatite, which is not passively or actively degraded *in vitro*[145], as the anchor material. This simple change in anchor material would permit much longer studies of *in vitro* tissue development.

We chose day 14 as the best day for determining whether the mechanical properties and collagen content of engineered ligaments changed with increasing passage number. This time point was chosen since it was in the linear range of the increase in both the mechanical and collagen content of the organoids. From this point, we would be able to detect both increases and decreases in function as passage number increased. However, when taken together, there were not differences in either mechanics or collagen as a function of passage number. There were fluctuations in mechanics and collagen from passage to passage; however, we attribute this to the variability inherent in the technique as well as small changes in the biological components of the fibrin gel and media. Importantly, the highest average MTL and total collagen were measured at passages 5 and 9 in one biological donor, and passage 6 and 10 in the other. These data demonstrate that any passage out to at least 11 would all be appropriate in future studies utilizing this engineered ligament model.

Overall, we have characterized the development and progression of a popular *in vitro* human engineered ligament model and have shown that it produces reliable and reproduceable data out to at least passage 11. The protocol is straightforward, requiring only basic sterile technique and common cell culture materials. We suggest that using a media without 100 mg/ml streptomycin and passaging cells at lower confluence may prevent de-differentiation of primary human ACL cells and allow researchers to engineer consistent ligaments (mechanical properties and matrix composition) from passage 4 to 11. We propose that this human engineered ligament model can be used to represent a developmental or injured tissue when interventions are performed between days 7 and 14, as well as a more mature tissue when interventions are performed from day 14-21.

Lastly, when using a 1:1 βTCP:orthophosphoric acid ratio to make brushite anchors, engineered ligaments should be mechanically tested by Day 21 to ensure anchor integrity. If longer interventions are desired, a different anchor material should be used.

Acknowledgements

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Author Contributions

A.A. and K.B. conceptualized the study. A.A. and O.V. contributed to data collection. A.A., O.V., and K.B contributed to data analysis. A.A. and K.B. contributed to manuscript. All authors approved the final manuscript.

Chapter 3: Inhibiting JAK1, not NF-κB, reverses the effect of pro-inflammatory cytokines on engineered ligament function

This chapter is in press at *Matrix Biology*.

A. Avey, F. Devos, A. Roberts, E.S. El Essawy, K. Baar, Inhibiting JAK1, not NF-kB, reverses the effect of pro-inflammatory cytokines on engineered ligament function. *Matrix Biology*. (2023).

Abstract

The role of inflammation in chronic tendon/ligament injury is hotly debated. There is less debate about inflammation following acute injury. To better understand the effect of acute inflammation, in this study we developed a multi-cytokine model of inflammatory tendinitis. The combined treatment with TNF-α, IL-1β, and IL-6, at dosages well below what are routinely used *in vitro*, decreased the mechanical properties and collagen content of engineered human ligaments. Treatment with this cytokine mixture resulted in an increase in phospho-NF-κB and MMP-1, did not affect procollagen production, and decreased STAT3 phosphorylation relative to controls. Using this more physiologically relevant model of acute inflammation, we inhibited NF-κB or JAK1 signaling in an attempt to reverse the negative effects of the cytokine mixture. Surprisingly, NF-κB inhibition led to even greater decrease in mechanical function and collagen content. By contrast, inhibiting JAK1 led to an increase in mechanical properties and collagen content and thermal stability concomitant with a decrease in MMP-1. Our results suggest that inhibition of JAK1, not NF-κB, reverses the negative effects of pro-inflammatory cytokines on collagen content and mechanics in engineered human ligaments.

Introduction

Tendinopathies are the most common musculoskeletal injury in athletes and other active people. In fact, by 80 years of age, 80% of the population are believed to suffer from at least one tendinopathy[146]. Tendinopathy is a general term that includes tendinitis and tendinosis[147]. Tendinitis describes the degeneration of a tendon with visible signs of inflammation (swelling, redness, and warm to the touch). By contrast, tendinosis is the degeneration of a tendon without signs of inflammation.

While the role of inflammation in chronic tendinopathy is still quite controversial, recent work points to a central role of inflammatory mediators in predisposing tendons to subsequent degeneration[148,149]. Following acute injury, tendons can enter an inflammatory state where the cells within the tendon are 'primed' to respond to later pro-inflammatory signals[93]. This inflammatory state results in increased activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which has been shown to be activated in early and intermediate stages of tendinopathy[95]. The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) is an activator of NF-κB[78], and inhibition of TNF-α can improve early-stage healing in a rat rotator cuff injury model[97]. Along with TNF-α, the pro-inflammatory cytokine interleukin 1-beta (IL-1β) can also activate NF-κB and increase secretion of cytokines including IL-1β, setting off an inflammatory cascade, or cytokine storm[150]. IL-1β has been shown to decrease collagen synthesis and increase collagen degradation[151], indicating that activation of NF-κB, whether via TNFα or IL-1β, can lead to modification of the extracellular matrix (ECM).

While the NF-κB signaling pathway has often been targeted to improve the function of an inflamed tendon/ligament [5], another pro-inflammatory cytokine, interleukin 6 (IL-6), activates the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling cascade. IL-6 can activate all the JAK family members (JAK1, JAK2, JAK3, and TYK2), and this potently activates STAT3 and to a minor extent STAT1. IL-6 has been shown to be elevated in patients with rheumatoid arthritis [100], in transected rat patellar tendon [101], and in ruptured rotator cuff [79]. While IL-6 has been extensively seen in response to inflammation and injury, the role of JAK/STAT in tendon injury and healing has conflicting reports. Recent studies suggest STAT3 activity may improve tendon healing [102]. However, this paradoxical pro-inflammatory vs anti-inflammatory role for STAT3 appears to be regulated by which cytokine activates the pathway [152]. For instance, IL-10 activation of STAT3 is mediated mostly through JAK1 [153] and this sets off an anti-inflammatory response, whereas IL-6 can activate STAT3 through JAK1, 2, or TYK2 and this leads to a pro-inflammatory response. This is all made even more complicated by co-activation and crosstalk between NF-κB and STAT signaling in a number of different models [154–157]. Together, these data suggest complex interactions at multiple levels between the JAK/STAT and NF-κB pathways.

The purpose of this study was to first determine the effects of TNF-α, IL-1β, and IL-6 on the mechanics, collagen content, and molecular signaling in a well characterized engineered ligament model [158]. We hypothesized that these cytokines would decrease the mechanical function and collagen content of engineered ligaments through the activation of NF-κB and STAT3. Since inflammation *in vivo* is never the result of a single cytokine, and inflammatory research performed *in vitro* always uses supraphysiological levels of cytokines, we asked

whether combining cytokines could result in a more physiological model of inflammatory tendinitis *in vitro*. Last, we used inhibitors of NF-κB and JAK1 to determine the role of these pathways in driving inflammatory tendinitis.

Methods

ACL Cells and Primary Culture

Cells were isolated from remnants of human anterior cruciate ligaments (ACL) collected during reconstruction surgery. The human ACL was obtained following informed consent, and all procedures and experiments were approved by the University of California Davis Institutional Review Board with IRB protocol number 779755. ACLs were digested in 0.1% collagenase Type II dissolved in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS) and 1% penicillin overnight at 37°C. The resulting cells were cultured in 15 cm plates containing 17 ml of growth media. Growth media consisted of high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin. Growth media was replaced every third day until cells were at 70-80% confluence before passage. Cells were frozen following the first passage.

For each experiment, one vial containing 1 ml human ACL cells was thawed, and the cells expanded to approximately 20-25 15 cm plates to provide sufficient cell number to produce the constructs needed for each experimental intervention.

Preparation of Engineered Ligaments

Brushite anchors were prepared by mixing 3.5M orthophosphoric acid with powdered β-tricalcium phosphate on ice at a 1:1 (μl:mg) solid:liquid ratio. This mixture was pipetted into 3D printed molds containing pins and then centrifuged at 2250 x g at 4°C for 1 minute. The anchors were left overnight to dry at room temperature. The following day, the anchors were removed from their molds and stored at room temperature until ready for use.

To prepare the ligaments, cells were suspended at a concentration of approximately 367,000 cells per ml in a solution containing: 953.8 µl growth medium, 40.6 µl thrombin, 2.8 µl aprotinin (20 mg/ml), and 2.8 µl aminohexanoic acid (100 mg/ml). The thrombin/cell mixture (714 µl) was added between brushite anchors and shaken to fully cover the bottom of a 35 mm plate containing two brushite anchors spaced 12 mm apart. Finally, 286 µl of fibrinogen (20 mg/ml) was added to each plate to initiate clotting of the fibrin gel. Plates were incubated at 37°C with 5% CO_2 for 15 minutes to allow gel formation before being fed 2 ml of growth media supplemented with 8 µl of 50 mM ascorbic acid, 2 µl of 50 mM proline, and 1 µl of transforming growth factor-β1 (10 µg/ml). The constructs were cultured for 14 days, and the media was replaced every other day throughout the experiment. For each feeding, media was aspirated and 2 ml of new growth medium supplemented with 8 µl of 50 mM ascorbic acid, 2 µl of 50 mM proline, and 1 µl of TGF-β (10 µg/ml) were added.

Box-Behnken Design of Experiments Determination of Cytokine Levels

The cytokine mixure was determined using a Box-Behnken design of experiments. Box-Behken design of experiments uses an initial incomplete factorial design and a subsequent validation step to establish the best concentration and combination of factors to elicit a specific outcome. For the incomplete factorial experiment, low, medium, and high levels of multiple test variables (in our case different cytokines) are combined together and specific outcomes (in our case UTS and collagen content) are quantitatively measured. In the model creation step, engineered ligaments were each treated with unique combination of cytokines and the UTS and collagen content were measured at day 14. To complete the incomplete factorial stage, 12 different combinations of cytokines were tested. We also performed 4 replicates at the middle dose of all cytokines to

establish the variability of the system. The UTS and collagen data were input into DesignExperts (Stat-Ease, Minneapolis, MN), and the program fit the data with a model that determines the significance of the relationship between the test variables and specific outcomes [159]. In the current case, the UTS produced a linear model that had a p-value of 0.0078 and the collagen produced a quadratic model that had a p-value of 0.0224. These models were then validated in an independent experiment by using the model to predict the effect of changing the test variables on the output variable of interest and then treating the ligaments with each combination and measuring the actual UTS (Figure 3C and D). To validate the current model, we used a combination of cytokines that were predicted to decrease UTS by 50%: Low (27.5 pg/ml IL-1β; 715 pg/ml TNF-α; 13.347 ng/ml IL-6); a second that was predicted to have little effect on UTS: High (0.05 pg/ml IL-1β, 2.5 pg/ml TNF-α, 20 ng/ml IL-6); and two combinations that were predicted to produce a UTS midway between the low and middle: Mid-Low (32.8 pg/ml IL-1β, 117 pg/ml TNF-α, 2.954 ng/ml IL-6); and middle and high: Mid-High (12.5 pg/ml IL-1β, 13 pg/ml TNF-α, 5.015 ng/ml IL-6) UTS values.

Treatment of Engineered Ligaments

On day 8, groups of constructs were treated with same feed media and the specific intervention being tested. The treatments were as follows: 0, 5, 10, 20 ng/ml TNF-α; 0, 20, 100, 500 pg/ml IL-1β; 0, 2, 20, 100 ng/ml IL-6. For this experiment, control constructs were treated with the vehicle (1 μl/ml 100% ethanol). Other treatments used included: cytokine mixture (714 pg/ml TNF-α, 13.343 ng/ml IL-6, 27.5 pg/ml IL-1β); 10 μM Itacitinib (dissolved in 100% ethanol); SC75741 (dissolved in 100% ethanol) or both cytokine mixture and Itacitinib or SC75741. The

cytokines were from Peprotech (Cranbury, NJ) and the Itacitinib and SC75741 were from Selleck Chemicals (Houston, TX).

Mechanical testing

Prior to testing, the length of each construct was measured in millimeters using a digital caliper. Constructs width and thickness was measured by spectral-domain optical coherence tomography using a OQ Labscope (Lumedica, Durham, NC). Constructs were placed in 3D printed grips and submerged in 37 °C 0.9% saline and mechanically tested using 10 cycles of preconditioning to 0.10 N at a rate of 0.25 mm/s prior to loading to failure at a rate of 0.25 mm/s using a Model 68SC-1 single column tensile tester (Instron, Norwood, MA). The cross-sectional area (CSA) was determined by multiplying the measured width and thickness of the ligament. The maximum tensile load (MTL) was the maximal load measured prior to failure of the tissue in Newtons. Ultimate tensile strength (UTS) was calculated by normalizing the MTL to the measured CSA of the tissue and the Young's modulus was calculated as the maximal slope of the stress-strain curve. After testing, ligaments were removed from anchors, dried on a glass plate at 120 °C for 30 min, and measured for dry mass.

Collagen Analysis

Collagen content was determined by measuring hydroxyproline levels. After testing, the constructs were dried in an oven at 120°C for 20 minutes, placed in 1.7 ml tubes, and weighed except for the CYTO + ITA samples from Figure 7, which were cut from the anchors and processed by DSC (see below) before drying and weighing. Following drying and weighing all tissues were submerged in 200 µl of 6 M HCl and placed on a heating block for 2 hours at 120°C

with tube lids carefully secured. After 2 hours, the tubes were removed from heat, centrifuged for 10 seconds, then placed back on the heating block within a chemical hood for 1 hour at 120°C with tube lids open. After 1.5 hours, the liquid had evaporated, and the samples were dry. They were then removed from heating block and suspended in 200 µl of a hydroxyproline buffer. Samples were further diluted 1:20 in the same buffer. 150 µl of 14.1 mg/ml chloramine T solution was added to each sample, vortexed, and incubated for 20 minutes at room temperature. Then, 150 µl of aldehyde-perchloric acid solution (1.5g of 4(dimethylamino)benzaldehyde, 6 ml 1-propanol, 2.6 ml 70% perchloric acid, 500 µl water) was added to each sample, vortexed, and incubated for 15 min on heating block at 60°C. Samples were then cooled for 10 min and read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) by placing 200 µl of each sample into two wells in a 96-well plate. Hydroxyproline was converted to collagen mass by assuming that collagen contains 13.7% hydroxyproline. Percent collagen was calculated by dividing the mass of collagen by the dry mass of the tissue.

Differential Scanning Calorimetry Measurements

Following mechanical testing, the CYTO + ITA samples mechanically tested for Figure 6 were removed from their anchors, cut using a 4 mm biopsy bunch, and stored in 1x DPBS until differential scanning calorimetry (DSC) was performed. Samples were removed from DPBS, lightly blotted to remove excess liquid, placed on the bottom of a Tzero® aluminum pan and sealed using an aluminum hermetic lid and Tzero® press, weighed, and analyzed using a Discovery DSC with autosampler (TA Instruments, New Castle, DE). Samples were then equilibrated at 40ºC and heated from 40°C to 90°C at a rate of 10°C/min. The temperature and

heat flow associated with material transitions as a function of time and temperature were determined.

24-well Plate Experiments

Human ACL fibroblasts were cultured in 24-well plates for collection and western blot analysis. Cells were plated into 24-well plates containing 1 ml of growth media. After two days, cells were treated at varying time points with growth media containing: control; cytokine mixture (714 pg/ml TNF-α, 13.343 ng/ml IL-6, 27.5 pg/ml IL-1β). Additionally, cells were treated at varying time points with growth media containing: vehicle (1 μl/ml 100% ethanol); cytokine mixture (714 pg/ml TNF-α, 13.343 ng/ml IL-6, 27.5 pg/ml IL-1β); 10 μM Itacitinib (dissolved in 100% ethanol); or both cytokine mixture and Itacitinib. After the specified treatment time, cells were removed from the incubator, placed on ice, and rinsed 3x with ice-cold 1x DPBS. Cells were then collected in 1x Laemmli sample buffer (LSB) (75 μl), sonicated, and boiled for 5 min at 100°C and stored at -30ºC before analysis.

Western Blotting

Samples (10μL) were loaded onto a 4-20% Criterion TGX Stain-Free Precast Gel and run for 45 min at a constant voltage of 200 V. The gel was then activated under UV-light to quantify total protein within each well. The total protein within the lane was then used to normalize all western blot quantifications [51–53]. Proteins were then transferred to an activated PVDF membrane for 30 min at a constant voltage of 100 V. Transfer was confirmed by Ponceau staining the membranes. Membranes were then rinsed and blocked in 1% fish skin gelatin in Tris-buffered saline w/ 0.1% Tween (TBST) for 30 minutes, washed in TBST, and then

incubated at 4ºC overnight with the primary antibody diluted in TBST at 1:1000 (MMP-1 used at 1:200). The following day membranes were washed 3x for 5 min with TBST and incubated for 1 hour at room temperature in a 0.5% skim milk in TBST solution containing peroxidase-conjugated secondary antibodies diluted at 1:10,000. Following incubation with secondaries, Immobilon Western Chemiluminescent HRP substrate (Millipore, Hayward, CA, USA) was then applied to the membranes to visualize the proteins by chemiluminescence. Images were taken using the ChemiDoc MP System and bands were quantified using Image Lab (Bio-Rad). The following antibodies were used: MMP-1 (sc-58377); pSTAT3 (cs 9145S); STAT3 (cs9139S); p-NF-κB (cs3033S); Procollagen (SP1.D8 from Hybridoma) (AB_528438).

Statistics

All statistics were performed using GraphPad Prism, version 9.5.1 (San Diego, CA, USA). For XY plots of morphological, mechanical, and collagen data, a simple linear regression and slope comparison was performed between drug concentration and outcome, e.g. MTL (Y). The correlation coefficient (R2) for the slopes are displayed directly on the graph. Effects were calculated either by t-test (control v. CYTO) or 2-way ANOVA and the analysis is shown on each graph. Data is reported as mean ± standard deviation and all technical replicates are shown in each graph. Each technical replicate represents an individual ligament that was engineered from the same donor cells on the same day. Each new experiment, using a different donor or a different vial of cells from the same donor is called a biological replicate, since it is biologically distinct from any other set of ligaments. All experiments were repeated with at least one other donor, and used at least one male and one female donor for each experiment. The results from each experiment were qualitatively similar so results of a single trial is reported.

Results

TNF-α and IL-1β Decreases Engineered Ligament Mechanics

Engineered human ligaments treated with increasing amounts of TNF-α and IL-1β had decreased mechanical function, whereas IL-6 treatment had no effect on mechanics (Figure 1). Maximal tensile load (MTL) was significantly decreased by TNF-α and IL-1β treatment, but was unaffected by IL-6 (Figure 1A-C). TNF-α and IL-1β also decreased tissue cross-sectional area (CSA), while IL-6 had no effect (Figure 1D-F). Ultimate tensile strength (UTS; MTL relative to size) was significantly decreased by both TNF-α and IL-1β, however, IL-6 again had no effect. (Figure 1G-I), whereas modulus was significantly decreased only by TNF-α, while IL-1β and IL-6 had no significant effect (Figure 1G-L).

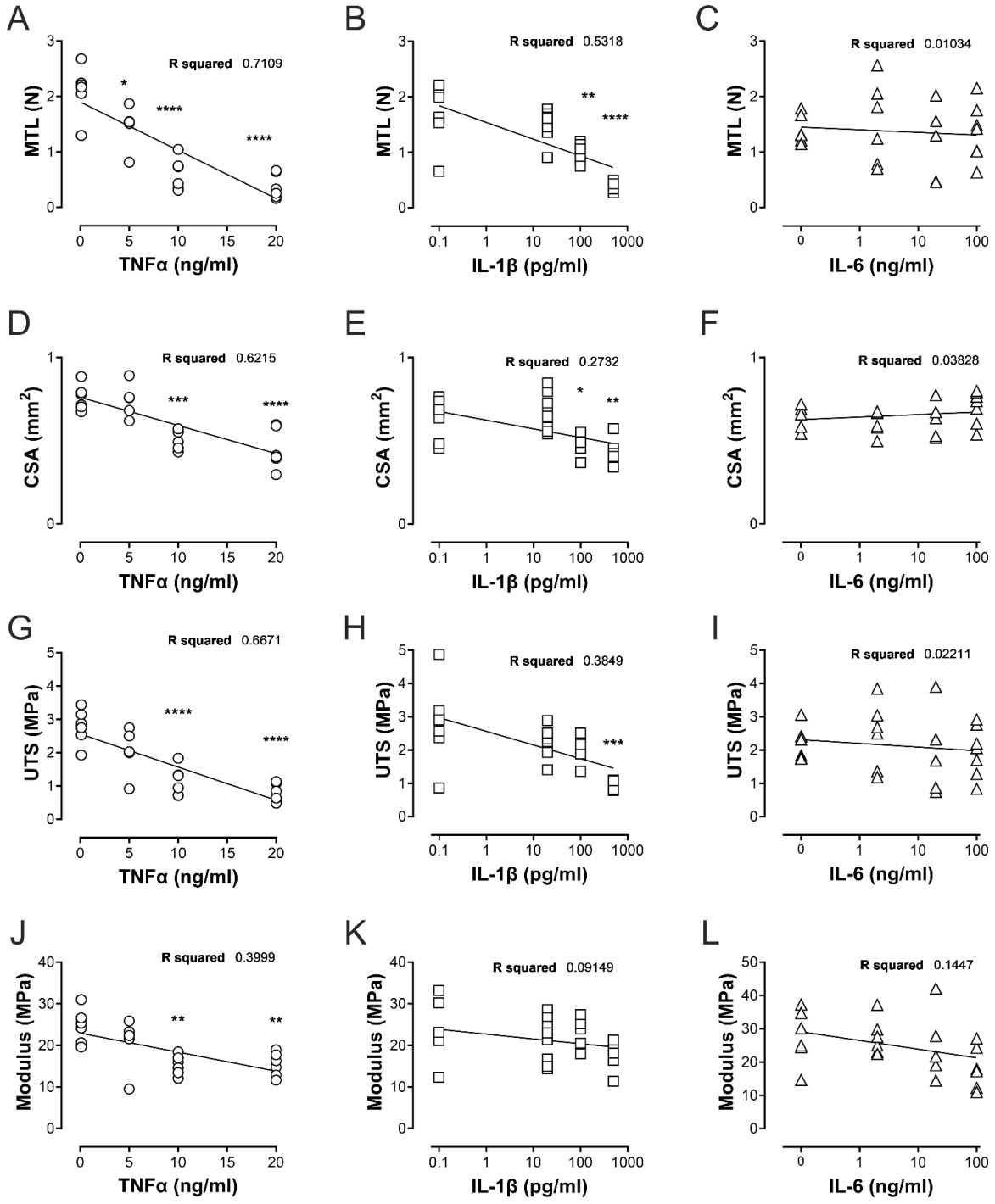

Figure 1: TNF-α and IL-1β decrease engineered ligament mechanics. (A-C) Maximum tensile load (MTL) is decreased by TNF-α and IL-1β, but is not affected by IL-6. (D-F) Cross-

sectional area (CSA) is decreased by TNF-α and IL-1β, but is not affected by IL-6. (G-I) Ultimate tensile strength (UTS) is decreased by TNF-α and IL-1β, but is not affected by IL-6. (J-L) Modulus is decreased by TNF-α but unaffected by IL-1β or IL-6. *p < 0.05, **p < .01, ***p < .001, ****p < .0001, Tukey's multiple comparison test compared to 0 group. n = 5-8.

TNF-α and IL-1β Decrease Engineered Ligament Collagen Content

As with mechanics, engineered human ligaments showed a dose-dependent decrease in collagen content when treated with TNF-α and IL-1β (Figure 2A-B), whereas IL-6 treatment had no effect on total collagen (Figure 2C). Interestingly, IL-6 treatment showed a significant decrease at the lowest dose, but higher doses did not decrease collagen (Figure 2C). Dry mass of the ligaments was decreased in a dose-dependent manner by TNF-α and IL-1β (Figure 2G-H), while IL-6 showed no effect (Figure 2I). The result of the drop in total collagen and dry mass was that only IL-1β significantly decreased the percent of tissue dry mass from collagen (% Collagen; Figure 2E), while TNF-α and IL-6 did not show a statistically significant effect (Figure 2D, F).

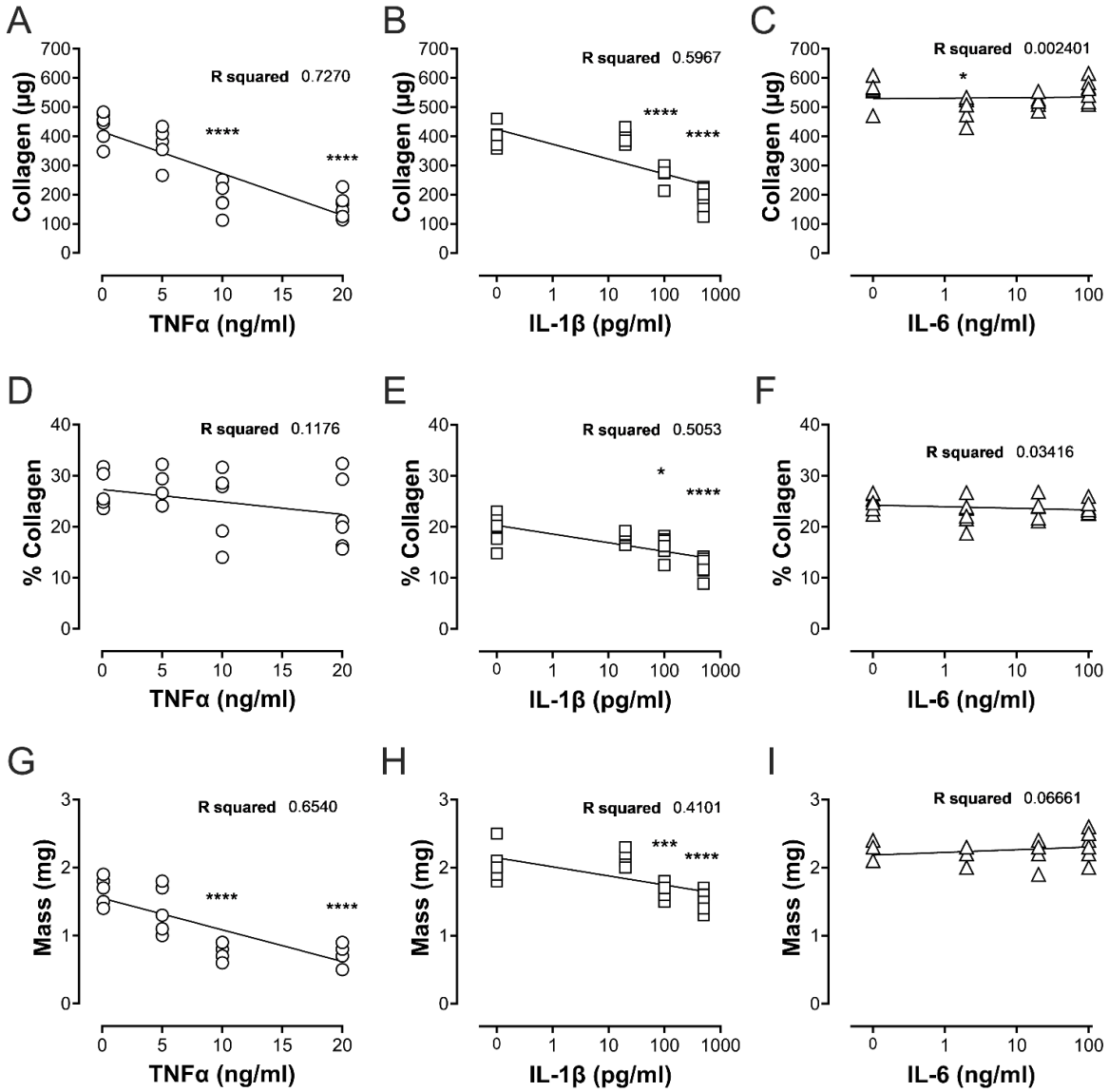

Figure 2: TNF-α and IL-1β decrease engineered ligament collagen content. (A-C) Collagen content is decreased by TNF-α and IL-1β decrease collagen content but is not changed by IL-6. (D-F) Percent collagen by dry mass is decreased by IL-1β but is not changed by TNF-α and IL-6. (G-I) Dry mass is decreased by TNF-α and IL-1β but is not changed by IL-6. *p < 0.05, **p < .01, ***p < .001, ****p < .0001, Tukey's multiple comparison test compared to 0 group. n = 5-8.

Cytokine Mixture Decreases Engineered Ligaments Mechanics and Collagen

Since *in vivo* cytokines never act alone, and the negative effects of individual cytokines were not observed until supraphysiological levels were used, we next determined whether combining multiple cytokines at more physiological levels could have a similar effect. To model this, we tested low, moderate, and high dosages of each cytokine using an incomplete factorial Box-Behnken design (see methods for details). Interestingly, the relationship between cytokine level and decreasing mechanics was linear and showed little interaction between the cytokines (Figure 3A). By contrast, the collagen content data showed significant interactions between the cytokines (Figure 3B). Most interestingly, at low levels of TNF-α, moderate levels of IL-1β actually increased collagen content. The linear model for mechanics ($p = 0.0078$), and the quadratic model for collagen content were both significant ($p = 0.022$). In both cases, TNF-α was the strongest component of the model.

Next, we validated the model by comparing the predicted vs measured outcome of different concentrations and combinations of the three cytokines on mechanics and collagen content. Since our goal was to decrease mechanics 50%, our "low" combination of cytokines was predicted by the model to decrease UTS by 50%, whereas the "high" combination was predicted to have little effect on either. We then spaced the other predicted combinations equally between the extremes. As can be seen in Figure 3C and D, the predicted and measured mechanical and collagen content values show a significant linear relationship ($p = 0.0001$), indicating that the Box-Behnken model is valid.

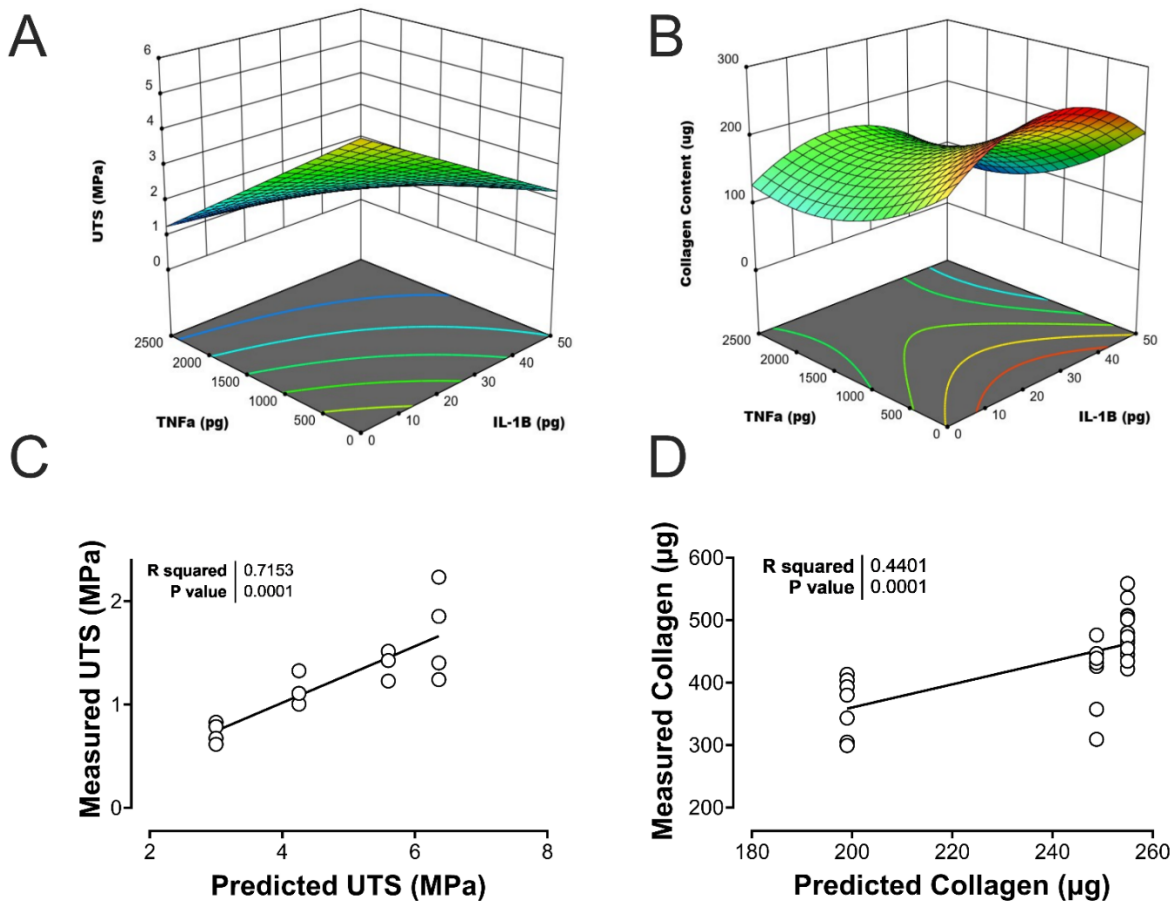

Figure 3: Box–Behnken design and cytokine cocktail validation. (A) Actual and space filling values of UTS based on varying levels of TNF-α, IL-1β, and IL-6. (B) Actual and predictive values of collagen content based on varying levels of TNF-α, IL-1β, and IL-6. (C) Measured vs predicted UTS shows nearly linear relationship. (D) Measured vs predicted collagen content, predicted low and predicted high treatments resulted in measured low and measured high values, respectively. Simple linear regression to measure goodness of fit (R^2). n = 3-7.

The optimal inflammatory mixture (termed CYTO) contained 715 pg/ml TNF-α, 27.5 pg/ml IL-1β, and 13 ng/ml IL-6. CYTO decreased MTL 40%, and UTS and modulus both were 39% lower

in the CYTO treated constructs (Figure 4A-C). Collagen content was down 22% and mass remained unchanged, meaning that the percent collagen also declined, but less than total collagen (12%; Figure 4D-F).

Next, the effect of CYTO on cell signaling in response to feeding was determined in 24-well plates. Treatment with CYTO resulted in increased phosphorylation of NF-κB at 1.5 and 3 hours and a slight elevation was maintained through 24 hours (Figure 4G). While phospho-NF-κB protein levels increased, phospho-STAT3 protein levels were lower in the cytokine treated group relative to controls at 24 hours (Figure 4H). As seen previously, feeding increased procollagen levels significantly at 24 hours and CYTO treatment did not significantly alter this response (Figure 4I).

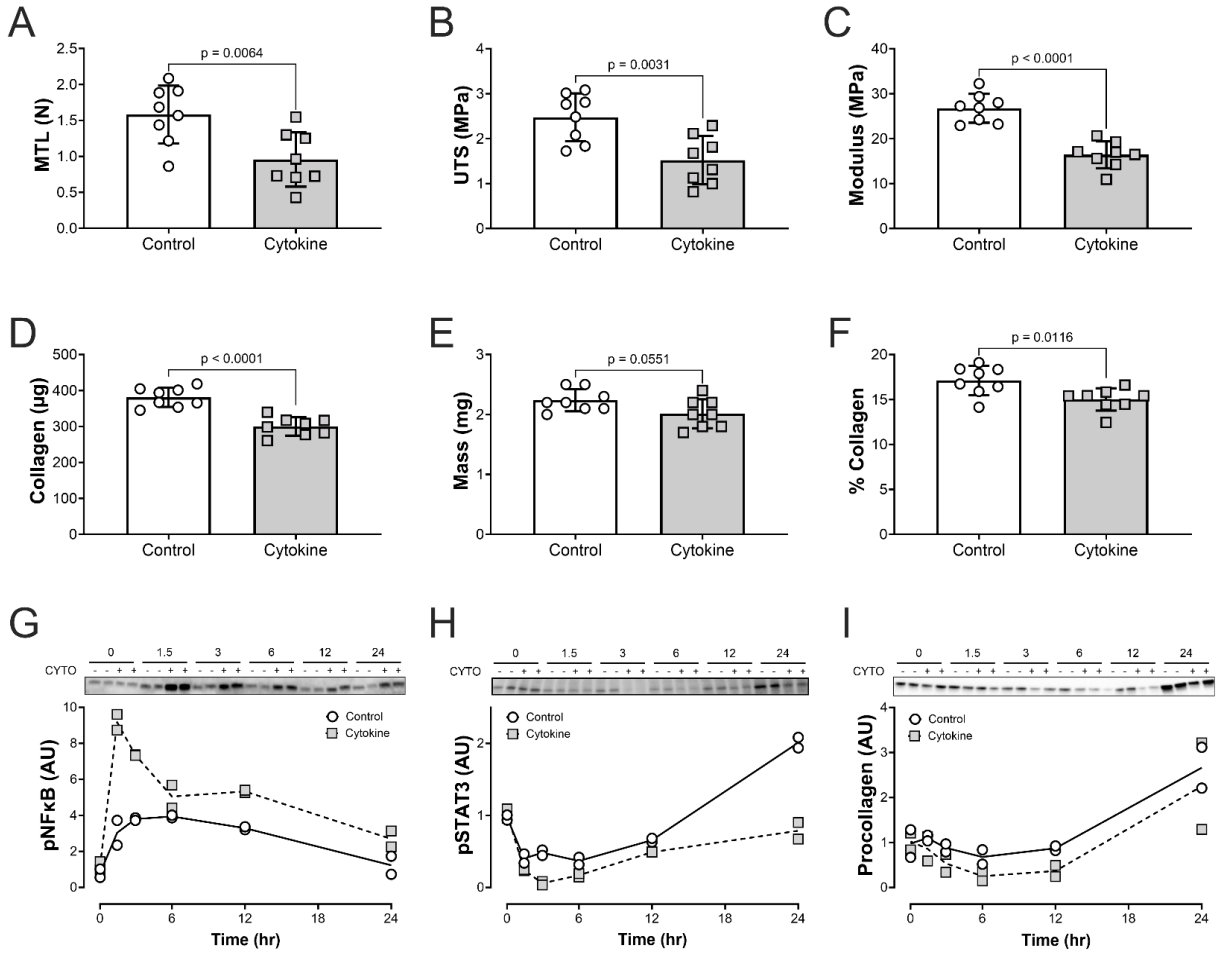

Figure 4: Cytokine cocktail decreases mechanics and collagen, activates NF-κB. (A) MTL, (B) UTS, (C) modulus, (D) collagen, and (E) percent collagen are significantly decreased by cytokine cocktail treatment ($p < 0.05$). (F) Dry mass is trends towards a decrease by cytokine cocktail treatment. (G) p-NF-κB is increased by cytokine treatment at 1.5 hours and remains elevated compared to control for 24 hours. (H) p-STAT3 is decreased after feeding, and is lower in cytokine treated group relative to control at 24 hours. (I) Procollagen is slightly lowered by cytokine treatment at 6 and 12 hours but returns to control levels at 24 hours.

Inhibition of NF-κB Decreases Engineered Ligament Mechanics and Collagen

Given the increase in phospho-NF-κB with cytokine treatment, we then looked to reverse the negative effects of CYTO treatment on engineered ligaments using the NF-κB inhibitor SC75741. Treatment with SC75741 on its own decreased ligament mechanics and collagen content (Figure 5). At low doses of SC75741, MTL, UTS, and modulus tended to decrease, and this effect reached statistical significance at 10μM (Figure 5A-C). In the presence of CYTO, SC75741 tended to increase mechanical and material properties at low doses, resulting in a significant interaction effect. However, once again the 10μM treatment resulted in the lowest mechanics. A significant main effect of both CYTO and SC75741 was also seen on total collagen, percent collagen and mass. Once again, low doses of SC75741 decreased collagen in the control group, and tended to increase it in the CYTO group (Figure 5E-G). The result was a significant interaction for total collagen and mass. The negative drug effect suggests that NF-κB inhibition is detrimental to the development of engineered human ligaments (Figure 4M). However, the ability of low dose SC75741 to improve collagen and mechanics in CYTO treated ligaments suggests that small amounts of active NF-κB improve ligament structure and function, but large amounts are detrimental (Figure 4G-L).

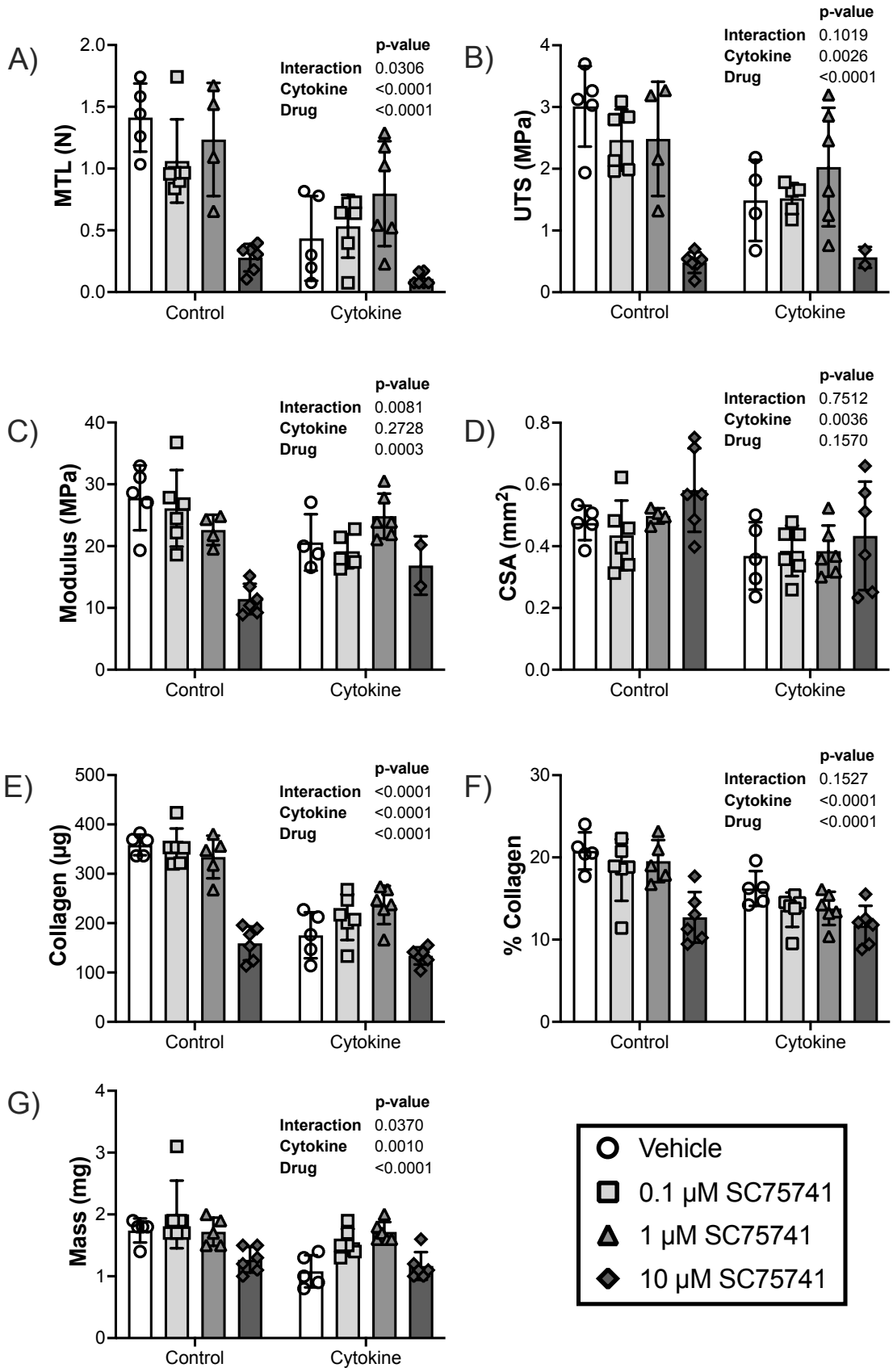
A)
MTL (N)
2.0
1.5
1.0
0.5
0.0
p-value
Interaction 0.0306
Cytokine <0.0001
Drug <0.0001
Control
Cytokine
B)
UTS (MPa)
4
3
2
1
0
p-value
Interaction 0.1019
Cytokine 0.0026
Drug <0.0001
Control
Cytokine
C)
Modulus (MPa)
40
30
20
10
0
p-value
Interaction 0.0081
Cytokine 0.2728
Drug 0.0003
Control
Cytokine
D)
CSA (mm2)
0.8
0.6
0.4
0.2
0.0
p-value
Interaction 0.7512
Cytokine 0.0036
Drug 0.1570
Control
Cytokine
E)
Collagen (µg)
500
400
300
200
100
0
p-value
Interaction <0.0001
Cytokine <0.0001
Drug <0.0001
Control
Cytokine
F)
% Collagen
30
20
10
0
p-value
Interaction 0.1527
Cytokine <0.0001
Drug <0.0001
Control
Cytokine
G)
Mass (mg)
4
3
2
1
0
p-value
Interaction 0.0370
Cytokine 0.0010
Drug <0.0001
Control
Cytokine
Vehicle
0.1 µM SC75741
1 µM SC75741
10 µM SC75741

Figure 5: NF-κB inhibition worsens engineered ligament function. (A-C) Mechanics are decreased by SC75741 by itself, while lower dose of SC75741 shows some improvement when combined with cytokines before being significantly decreased with high dose. (D) CSA is decreased by cytokines. (E) Collagen is decreased by high dose of SC75741 with or without cytokine treatment. (F) Percent collagen is decreased by both cytokines and SC75741. (G) Mass is decreased by cytokines. It is recovered by lower dose of SC75741 and unchanged by high dose. Statistics calculated by 2-way ANOVA. n = 5-6.

Itacitinib Improves Engineered Ligament Mechanics and Collagen

Phosphorylation of STAT3 surprisingly decreased following CYTO treatment relative to controls and this was especially evident 24 hours after feeding, when procollagen levels were highest (Figure 4H). The decrease in STAT3 phosphorylation was a surprise since we previously demonstrated that inhibiting one potential upstream STAT3 kinase, JAK1, with Itacitinib (ITA) resulted in stronger ligaments with more collagen. To determine the role of JAK1 in the response to CYTO, we tested how treatment with ITA affected CYTO ligaments. As observed previously, treatment with ITA had a positive main effect on MTL, UTS, and modulus (Figure 6A, C, D). Treatment with CYTO significantly decreased mechanics as described above. Interestingly, co-incubation of CYTO and ITA returned mechanics to control levels or higher. Most importantly, both UTS and modulus showed an interaction between CYTO and ITA, suggesting that ITA was blocking the negative effect of the cytokines.

To better understand how ITA was improving mechanics, we looked deeper into the effect of co-incubation on collagen content and the physical properties of the matrix. There was a main effect

of CYTO on collagen content and ITA showed a trend to reverse the loss of collagen. Beyond the total amount of collagen, we used digital scanning calorimetry to perform thermal analysis of the matrix as an indicator of collagen crosslinking and fibril size and density. Measures of both enthalpy and the onset temperature at denaturation mirrored what was seen for UTS and modulus, ITA increased, CYTO decreased, and the combination showed an interaction (Figure 6 F, G). This finding suggests that CYTO decreases collagen crosslinking, whereas ITA may increase crosslinking. Interestingly, the peak temperature was increased by CYTO and tended to decrease with ITA.

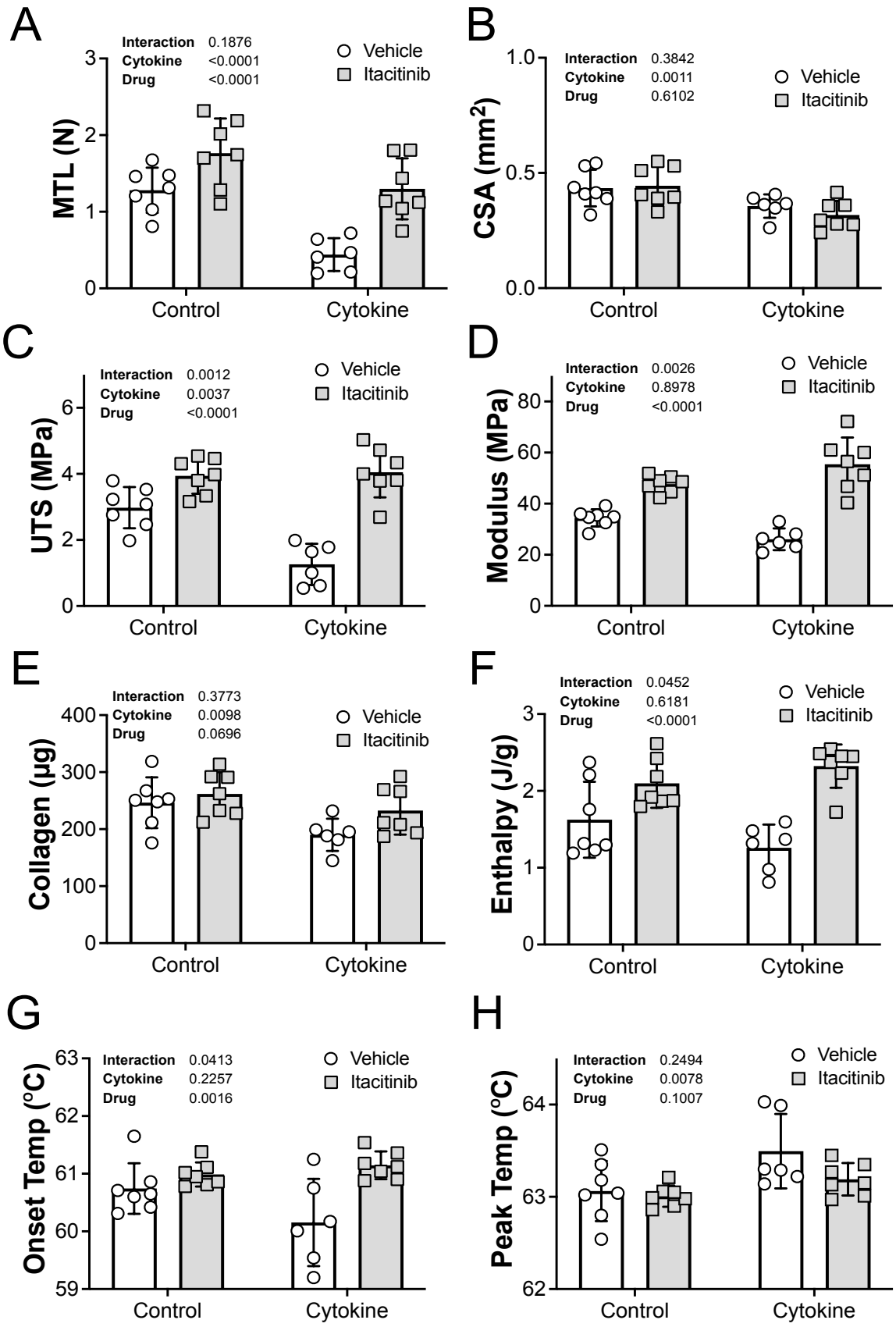
A
Interaction 0.1876
Cytokine <0.0001
Drug <0.0001
Vehicle
Itacitinib
MTL (N)
Control
Cytokine
B
Interaction 0.3842
Cytokine 0.0011
Drug 0.6102
CSA (mm2)
C
Interaction 0.0012
Cytokine 0.0037
Drug <0.0001
UTS (MPa)
D
Interaction 0.0026
Cytokine 0.8978
Drug <0.0001
Modulus (MPa)
E
Interaction 0.3773
Cytokine 0.0098
Drug 0.0696
Collagen (µg)
F
Interaction 0.0452
Cytokine 0.6181
Drug <0.0001
Enthalpy (J/g)
G
Interaction 0.0413
Cytokine 0.2257
Drug 0.0016
Onset Temp (°C)
H
Interaction 0.2494
Cytokine 0.0078
Drug 0.1007
Peak Temp (°C)

Figure 6: Itacitinib improves engineered ligament function. (A) MTL is decreased by cytokines and increased by Itacitinib. There is a trend towards an interaction effect. (B) CSA is decreased by cytokines. (C) UTS is decreased by cytokine treatment and improved with Itacitinib treatment. There is an interaction effect. (D) Modulus is increased by Itacitinib. (E) Collagen content is decreased by cytokines and there is a trend towards improvement with Itacitinib. (F-G) Enthalpy and onset temperature is increased by Itacitinib. (H) Peak temperature is increased by cytokine treatment. Statistics calculated by 2-way ANOVA. n = 6-7.

Itacitinib Decreases Matrix Metalloproteinase-1 Levels

To determine whether the negative effects of CYTO could be further elucidated, we measured inflammatory signaling, and markers of collagen synthesis and degradation by western blot. As in Figure 4, CYTO slightly decreased phospho-STAT3 and ITA completely prevented STAT3 phosphorylation without affecting total STAT3 levels (Figure 7A-B). CYTO increased phospho-NF-κB both 1 (Figure 7C) and 24-hours after treatment and this was not affected by ITA (Figure 7D). Neither CYTO nor ITA effected procollagen synthesis (Figure 7E). However, CYTO increased matrix metalloproteinase-1 (MMP-1) levels and this was partially prevented by ITA (Figure 7F). These results suggest that the decrease in collagen content with CYTO treatment (Figure 4J) may be the result of a greater increase in collagen degradation and this is partially prevented by ITA.

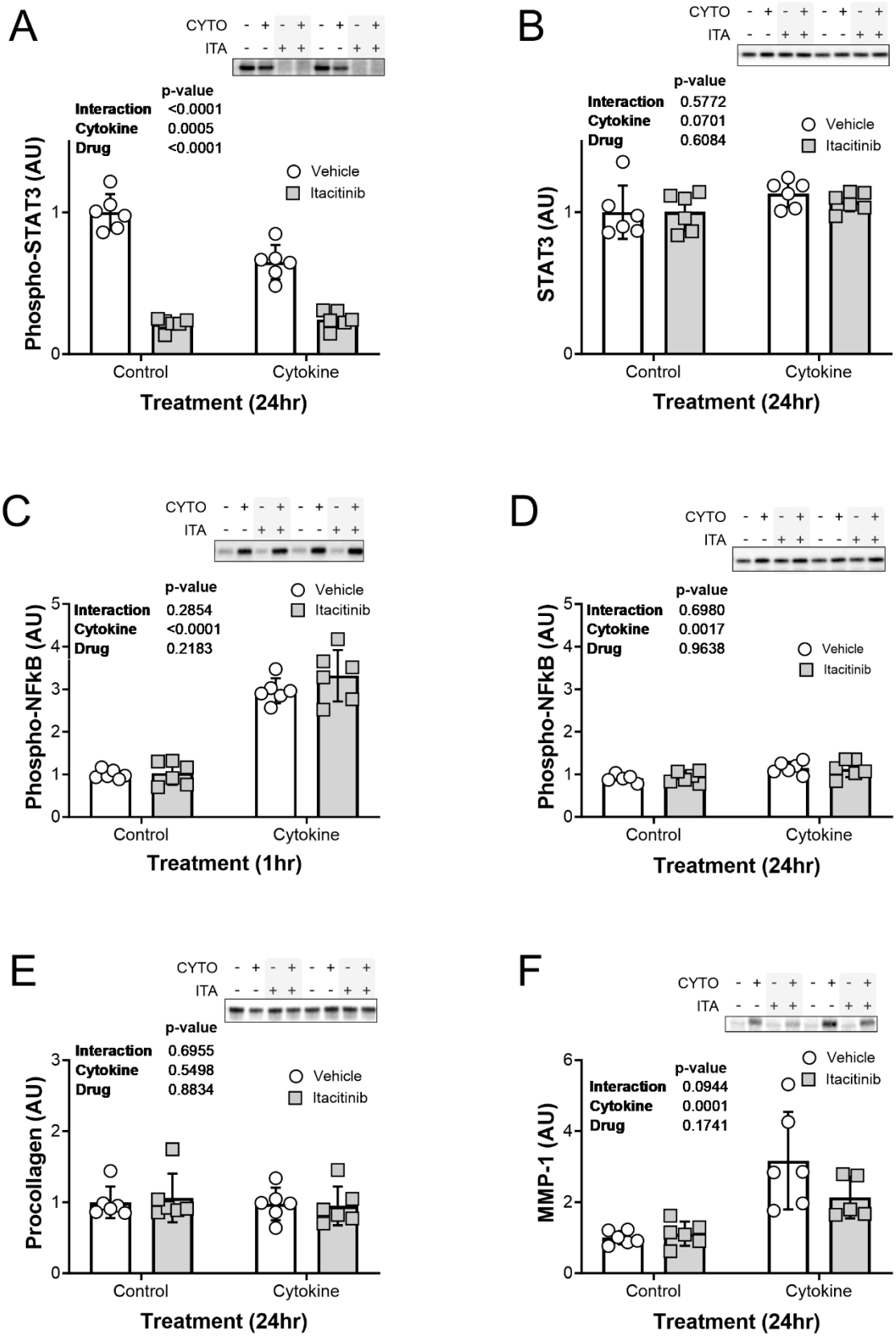
A
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction <0.0001
Cytokine 0.0005
Drug <0.0001
Vehicle
Itacitinib
Phospho-STAT3 (AU)
0
1
Control
Cytokine
Treatment (24hr)
B
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction 0.5772
Cytokine 0.0701
Drug 0.6084
Vehicle
Itacitinib
STAT3 (AU)
0
1
Control
Cytokine
Treatment (24hr)
C
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction 0.2854
Cytokine <0.0001
Drug 0.2183
Vehicle
Itacitinib
Phospho-NFkB (AU)
0
1
2
3
4
5
Control
Cytokine
Treatment (1hr)
D
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction 0.6980
Cytokine 0.0017
Drug 0.9638
Vehicle
Itacitinib
Phospho-NFkB (AU)
0
1
2
3
4
5
Control
Cytokine
Treatment (24hr)
E
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction 0.6955
Cytokine 0.5498
Drug 0.8834
Vehicle
Itacitinib
Procollagen (AU)
0
1
2
3
Control
Cytokine
Treatment (24hr)
F
CYTO - + - + - + - +
ITA - - + + - - + +
p-value
Interaction 0.0944
Cytokine 0.0001
Drug 0.1741
Vehicle
Itacitinib
MMP-1 (AU)
0
2
4
6
Control
Cytokine
Treatment (24hr)

Figure 7: Itacitinib inhibits p-STAT3 and decreases MMP-1 activity. (A) p-STAT3 is decreased by cytokines and completely inhibited by Itacitinib at 24 hours. (B) Total STAT3 is unchanged by cytokine or Itacitinib treatment at 24 hours. (C-D) p-NF-κB is increased by cytokines at 1 hour and 24 hours. (E) Procollagen is unchanged by both cytokines and Itacitinib. (F) MMP-1 is increased by cytokine treatment and trends towards a decrease when combined with Itacitinib at 24 hours. Statistics calculated by 2-way ANOVA. n = 6.

Discussion

Traumatic injuries to tendons can result in acute and/or chronic inflammation that can progress into degeneration. Using an engineered human ligament model, we have reproduced the effects of chronic inflammation on tissue structure/function using a more physiologic mixture of pro-inflammatory cytokines (CYTO). CYTO decreased collagen content without affecting procollagen synthesis. Instead, CYTO increases degradative enzymes like MMP-1 and appears to decrease crosslinking. As expected, CYTO increases NF-κB activity; however, completely preventing the activation of NF-κB decreases ligament function more than CYTO alone. Lastly, the negative effects of CYTO on ligament mechanics, MMP-1, and collagen content and thermal properties can be rescued by inhibition of JAK1.

The role of inflammation in tendinitis remains controversial. There are clearly times when inflammatory cells enter a tendon or ligament following acute injury. However, the role of inflammation in the degeneration of tendons or ligaments is poorly understood. For example, the cytokines TNF-α and IL-1β have been shown to activate the pro-inflammatory NF-κB pathway and increase collagen degradation while decreasing collagen synthesis [78,151]. At the same time, TNF-α signaling is vital in the early inflammatory response needed to regenerate tissue following injury [98]. Similarly, the IL-6/JAK/STAT signaling pathway has been a target for anti-inflammatory interventions [99] while also being suggested to play an important role in healthy tendon recovery following injury [102]. Combined, these data suggest a critical role for the timing, duration, and concentration of cytokines in a pro-inflammatory response. In order to improve our understanding of the effect of inflammation on tendon/ligament function, a better model is necessary. Traditionally, cytokine experiments *in vitro* use 10ng/ml TNF-α, 10 ng/ml

IL-1β, or 100ng/ml IL-6 [101,160–163]. By contrast, synovial fluid concentrations of TNF-α, IL-1β, and IL-6 two days after ACL rupture are 5, 5, and 20,000 pg/ml, respectively [164]. This means that cell culture uses levels of cytokines that are 2,000-, 2,000-, and 5-fold higher than after a traumatic injury. Here we describe a mixture of cytokines that decreases ligament function ~50% that contains 714 pg/ml TNF-α, 27.5 pg/ml IL-1β, and 13 ng/ml IL-6. Even though the TNF-α level is still ~100-fold higher than *in vivo*, it is 14-fold lower than a standard *in vitro* dose. On top of that, the dose of IL-1β is 360-fold lower (within 5-fold of *in vivo* levels) and IL-6 is at a physiological level. This is despite the fact that the cell culture media contains 10% fetal bovine serum, with ~2.5mg/ml of albumin, which is known to blunt the cellular response to TNF-α [165].

Treating engineered ligaments with this multi-cytokine cocktail resulted in a decrease in mechanical and material properties as well as the collagen content and concentration within the ligaments. Further, using DSC we show that CYTO also decreases the thermal properties of the collagen. An increase in enthalpy and onset temperature suggests that collagen crosslinking has increased. This has been elegantly demonstrated by Lee and Veres who used ribose to crosslink the collagen within decellularized bovine tail tendons [166]. Ribose treatment for 28 days increased advanced glycation end-product crosslinks and tendon stiffness concomitant with increases in enthalpy and onset temperature [166]. This suggests that CYTO decreases and ITA increases collagen crosslinking. The decrease in collagen content with CYTO treatment in the ligaments was not unexpected. TNF-α and IL-1β have previously been shown to decrease collagen synthesis and increase collagen degradation in fibroblasts *in vitro* [151]. In NIH 3T3 and hepatic stellate cells, treatment with TNF-α leads to an increase in NF-κB binding and a

subsequent decrease in procollagen Ia1 gene expression [167]. Further, transfection with the p65 isoform of NF-κB, in the absence of TNF-α, recapitulates the inhibition of collagen expression [167]. Interestingly, our CYTO mix did not affect procollagen protein synthesis. There was a slight transient decrease in procollagen at about 12 hours, but this was not significant. While we did not measure collagen Ia1 gene expression, measuring procollagen I protein levels over 24 hours should allow for changes in mRNA to be translated into differences in protein synthesis. CYTO was able to activate NF-κB in our model. An approximately 2-fold increase was measured in the 24-hour area under the curve for NF-κB (Figure 4G). It is unclear why the increase in NF-κB activity did not translate into a decrease in procollagen I synthesis. The most obvious explanations are that: 1) NF-κB regulates collagen I mRNA and translation through separate mechanisms; 2) human ligament fibroblasts respond differently to CYTO than embryonic mouse or liver fibroblasts; or 3) supraphysiological levels of individual cytokines signal differently than a mixture of cytokines at a lower concentration.

Though the decrease in collagen synthesis was not observed, an increase in matrix metalloproteinases (MMPs), specifically MMP-1, was seen following CYTO treatment. As the name suggests, MMPs degrade proteins in the extracellular matrix. We chose to measure MMP-1 levels because it is important in digesting triple-helical collagen and has a high affinity for fibrillar collagen [168–170]. The ability of CYTO to increase MMP-1 may again be mediated by the activation of NF-κB since inhibition of NF-κB by overexpression of its inhibitor IκBα reduces MMP expression [171]. However, here we found that inhibition of JAK1 could blunt the effect of CYTO on MMP-1 levels. Further, this blunting of MMP-1 levels was related to the ability of ITA to prevent the decrease in percent collagen within the engineered ligaments

(Figure 6 and 7). Together, the lack of change in procollagen I levels, the blunting of MMP-1 activation, the improved percent collagen, the improved thermal properties of the matrix, and the normalization of mechanics in engineered ligaments treated with ITA suggests that JAK1 activation may play an important role in the molecular regulation of tissue collagen turnover and molecular structure in response to cytokines.

Interestingly, while CYTO treatment decreases mechanical function roughly 40-50%, we typically only see a 20-25% decrease in collagen. These data highlights the fact that cytokines are affecting more than just collagen content. The most likely explanation for the discrepancy between mechanics and collagen is a reduction in collagen crosslinking. Consistent with this hypothesis, TNF-α, IL1-β, and IL-6 activity have all been suggested to decrease lysyl oxidase activity[172–174]. Lysyl oxidase is the primary enzyme that produces collagen crosslinks through a copper (Cu)-dependent reaction that catalyzes the crosslinking of lysine residues in neighboring collagen molecules[175]. Blocking LOX activity is known to decrease the stiffness of developing tendons without affecting collagen content[176] and we have previously demonstrated that estrogen inhibits this enzyme and decreases the stiffness of engineered human ligaments without affecting collagen content[129]. Most interestingly in this regard, the thermal properties of the engineered human ligaments better reflected the changes in mechanics caused by CYTO or inhibition of JAK1 than collagen content. As mentioned above, matrix enthalpy and temperature at the onset of denaturation reflect the packing density and crosslinking of the collagen in the matrix [166]. This is best seen when comparing embryonic and older tendons. In the bicep tendon, enthalpy increases 7-fold and melting temperature increases ~6°C as tendon stiffness, collagen packing density, and crosslinking increase from birth to midlife [177].

Interestingly, enthalpy and onset temperature of engineered ligaments followed similar trends with CYTO and ITA treatments as what was seen mechanically. However, the peak temperature did not follow these same trends. Instead, CYTO treatment increased peak temperature, while ITA treatment had no effect. These data suggest that cytokines have a greater effect on mechanics than total collagen, likely by decreasing the content, packing density, and crosslinking of collagen fibrils within the tissue and this is reversed by inhibition of JAK1. Further study is needed to understand the effects of CYTO and ITA on collagen ultrastructure.

Despite overwhelming data both in this study and across the literature suggesting NF-κB plays a major role in the negative effect of cytokines, treating engineered ligaments with the potent NF-κB inhibitor SC75741 did not improve ligament mechanics or collagen content. Instead, even small amounts of SC75741 decreased mechanics and collagen. Interestingly, combining low doses of SC75741 with CYTO tended to improve engineered ligament function compared to CYTO alone. However, high doses of SC75741 were toxic in the presence or absence of CYTO. One possible explanation for this is that small amounts of NF-κB activity are required during the developmental stage [158] of the engineered ligament (days 8-14), even though high levels are detrimental. If this were true, low levels of SC75741 in control media would not completely block NF-κB allowing near normal development, whereas high levels would be more detrimental. However, in the presence of CYTO the low level of SC75741 would bring NF-κB activity back towards the level needed for engineered human ligament development, resulting in improved function. Regardless, complete inhibition of NF-κB by high levels of SC75741 prevents engineered human ligament development. Inhibition of NF-κB results in apoptosis [178], which could result in fewer fibroblasts available to produce the ECM at a time point

where there is an exponential increase in collagen [158]. Furthermore, increased canonical NF-κB activity has been shown to improve tendon healing [179], despite other studies suggesting otherwise [5,93,180,181]. Taken together, the conflicting information in the literature as well as our conflicting results suggest that both the duration and timing of NF-κB activation/inhibition may play a critical role in the development and healing of tendons and ligaments. Further characterization of the downstream targets of NF-κB is needed to better understand why complete inhibition in this model resulted in poor quality ligaments.

Equally confusing is the STAT signaling. In human ACL fibroblasts, phospho-STAT3 goes up with feeding, a stimulus that increases procollagen synthesis and improves ligament mechanics, and CYTO treatment decreases phospho-STAT3 despite decreasing ligament mechanics. However, two stimuli that have similar effects on STAT3 signaling (CYTO and ITA both decrease phospho-STAT3) have completely opposing effects on engineered human ligament mechanics. This contradiction likely reflects an incomplete understanding of the actions of other JAK and STAT family members. The Janus kinase family (JAK1, 2, 3, and TYK2) members interact with the seven members of the STAT family (STAT1, 2, 3, 4, 5A, 5B, and 6) to signal cytokine levels. This complex and highly intertwined network is likely dependent on the type and the state of differentiation of the target cell. In tendon precursor cells (TPCs) for example, Chen and colleagues have elegantly demonstrated that inhibition of STAT3 returned the stem-like properties of these stem cells with aging [182]. The results of that study suggested that STAT3 activity was the driving force behind the negative effects of aging on TPCs. There are two ways in which the work of Chen and colleagues differ from the work presented here. First, in our study blocking phosphorylation of STAT3 using ITA resulted in stronger engineered

ligaments and tended to increased collagen. If the effect of ITA was to increase TPCs, an increase in collagen content and mechanics would not be expected since, as stem cells, TPCs produce less collagen and increasing cell number does not improve ligament mechanics in this model [4]. Second, STAT3 cannot be the driving force behind the mechanical changes induced by CYTO or ITA treatment. Both ITA and CYTO decreased STAT3 phosphorylation, even though they had opposing effects on ligament mechanics. Together, these data suggest that JAK1 inhibition is preventing the negative effects of CYTO in a novel, STAT3-independent, manner. It is interesting to note that JAK inhibitors are used clinically to treat dermatologic diseases and arthritis, both collagen-dependent diseases. Further, the patent that the makers of Itacitinib, Novartis, were granted includes the use of Itacitinib in the possible use for "treating tendon and/or ligament injury". That, together with the data from this manuscript, and that of our previous work showing JAK1 inhibition is associated with developmental tendon growth [136], suggests that the role of JAK/STAT signaling in tendon/ligament function needs much more investigation.

In conclusion, while other *in* vitro models have been used to explore the complex role of inflammation on tissue [183], we have used a 3D engineered human ACL ligament model to develop a novel multi-cytokine model of inflammatory tendinitis that decreases the concentration of cytokines to the lowest *in vitro* amounts reported. Using this model, we show that cytokines cause a decrease in mechanical and material properties that is likely the result of both a decrease in content, through increased degradation, and crosslinking of the collagen within the tissue. Lastly, we show that inhibition of JAK1 can partially reverse the effect of cytokines on MMP-1 and prevents the negative mechanical effects of CYTO treatment. Together, our data suggest that

JAK1 inhibition may be an effective treatment for inflammatory tendinitis and that our model can be used to effectively screen for potential treatments.

Acknowledgements

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Author Contributions

A.A. and K.B. conceptualized the study. A.A., F.D., A.R., and E.E. contributed to data collection. A.A., F.D., A.R., E.E., and K.B contributed to data analysis. A.A. and K.B. contributed to manuscript. All authors approved the final manuscript.

Chapter 4: Effect of Isometric Loading on *In Vitro* Engineered Human Ligaments

A. Avey, K. Tam, K. Baar

Abstract

Loading both causes and is used to treat tendinopathies, raising the possibility that load provides both positive and negative signals. For example, isometric loading has previously been shown to reverse central core patellar tendinopathy resulting from overuse in a professional basketball player. Acute inflammation may provide one of the loading signals; however, it is unclear whether this acute inflammatory response has positive or negative effect on tendon health. To test the positive effect of isometric loading and clarify the role of inflammation in response to load, we isometrically loaded engineered human ligaments over the course of 6 days with or without the JAK1 inhibitor Itacitinib (ITA). Isometric loading increased mechanical and material properties of ligaments without changing the collagen content or percent collagen, suggesting improved alignment, collagen fibrin packing density, or crosslinking. ITA improved mechanical properties and increased collagen, independent of load. These results demonstrate that the engineered human ligament model reproduces the positive effects of isometric loading seen *in vivo*. Furthermore, the data also indicate that JAK1 inhibition in combination with an isometric loading may improve the response to loading and improve tendon/ligament function.

Introduction

Tendinopathy is the degeneration of tendon and is prevalent among athletes and active non-athletes across age groups [184]. Tendinopathy can be further characterized as tendinitis, tendon pain with signs of inflammation, and tendinosis, tendon pain without inflammation [147]. Classically, tendinitis is thought to be the result of an acute injury resulting in an inflammatory response to initiate the repair process [185–187], while tendinosis is the result of chronic overuse, or a failed inflammatory process, and as a result active inflammation does not play a role [188]. However, more recent work has demonstrated that even in individuals with Achilles tendinosis there are signs of chronic inflammation [93], fueling the growing support for further research into the role of inflammation in chronic tendinopathy [92,148,149].

The pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway has been shown to be activated in tendinopathy [95]. This pro-inflammatory pathway has previously been targeted as a possible mechanism to prevent the inflammation that has been more recently associated with chronic tendinopathy [5]. However, a recent study from our laboratory demonstrated that inhibiting NF-κB was detrimental to engineered ligaments and instead identified inhibition of the Janus kinase 1 (JAK1), signal transducer and activator of transcription (STAT) signaling pathway as a potential route to improve tendon/ligament function [189]. The JAK/STAT signaling pathway is activated by the pro-inflammatory cytokine IL-6, which is elevated in ruptured rotator cuff [79] and transected rat patellar tendons [101]. Furthermore, IL-6 expression in tenocytes increases with loading [190], suggesting it may play a role in the response to load, whether positive or negative.

Despite the poorly defined and complicated role of inflammation, a more well-characterized sign of chronic tendinopathy is a disorganized collagen matrix [188,191]. Tendons/ligaments are primarily comprised of collagen fibrils aligned with the direction of loading [192]. In the absence of load, collagen fibril alignment and mechanical properties decrease significantly [193]. This suggests that it is critical to apply load to a newly synthesized matrix, especially following acute inflammation [194] to facilitate proper collagen alignment. However, in an injured tendon biomechanical stress shielding can occur. Stress shielding is where the healthy aligned collagen, which is stiffer than the poorly aligned injured matrix, shields the injured region from load to prevent catastrophic failure of the tissue. Prolonged stress shielding can result in increased tendon thickness around the injured site, without relieving the pain of tendinopathy [195]. To combat stress shielding and apply load to the less organized matrix (scar), isometric exercises that allow stress relaxation of the aligned matrix [196] can be useful. In fact, isometric loading has been shown to decrease tendon pain in those suffering from tendinopathies [6,8,197]. Beyond pain, a case study utilizing isometric loading and complimentary nutrition showed that a central core patellar tendinopathy could be reversed, as determined by both decreased pain and improved structure by magnetic resonance imaging (MRI), resulting in improved athletic performance [7]. Lastly, in an injured patellar tendon loaded using dynamic or isometric exercise (matching time under tension) Steffen and colleagues showed that the isometric load increased expression of tendon markers, whereas the dynamic load increased expression of cartilage associated genes [198].

The purpose of this study was to determine whether we could model the positive effect of isometric load using an engineered human ligament model [158]. Furthermore, we investigated

the effect of JAK1 inhibition alongside isometric load to determine whether the previously reported positive effects of JAK1 inhibition [189] contributed to the loading effect or were additive with load. We hypothesized that isometric loading would improve the mechanical function of engineered ligaments and JAK1 inhibition would augment these effects.

Methods

Cell Culture

Human anterior cruciate ligaments (ACL) were collected during reconstruction surgery and obtained following informed consent, and all procedures and experiments were approved by the University of California Davis Institutional Review Board. Cells were isolated from remnants of these human ACLs as previously described [158]. Briefly, human ACL remnants were digested overnight at 37°C in a 0.1% Type II Collagenase solution dissolved in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin. The isolated cells were then cultured in 15 cm plates containing growth media consisting of DMEM supplemented with 10% FBS and 1% penicillin. Growth media was replaced every three days until cells were at 70% confluence before passage. Cells were frozen following the first passage. For each experiment, one vial of cells was thawed, and the cells were cultured to approximately 20-25 15 cm plates to provide enough cells for constructs.

Engineered Ligaments

In vitro ligament constructs were engineered as previously described [158]. Briefly, brushite anchors were prepared by mixing a 1 μl to 1 μg liquid to solid ratio of 3.5 M orthophosphoric acid with β-tricalcium phosphate on ice. This was added to a reverse molded silicon cast made as the negative of 3D printed teardrop posts and centrifuged at 2250 x g for 1 min at 4ºC. The anchors were left overnight to dry and then kept at room temperature until used.

Ligament constructs were made by preparing sterile 35 mm plates with brushite anchors spaced 12 mm apart. A 714 μl solution containing $2.5x10^5$ cells, 5.8 Units of thrombin, 20 μg aprotinin,

and 200 μg 6-aminohexanoic acid was added to each 35 mm plate between the brushite anchors. The plates were shaken until the liquid completely covered the bottom of the plate. Then, 286 μl of fibrinogen (20 mg/ml) was added to each plate, the plates were shaken, and then incubated for 15 minutes at 37°C with 5% CO_2 to allow the gel to form completely. After 15 minutes, constructs were fed 2 ml of feed media consisting of growth media supplemented with 200 μM ascorbic acid, 50 μM proline, and 5 ng of transforming growth factor-β1 (TGF-β1). The constructs were cultured for 14 days, and the media was replaced every other day throughout the experiment.

Loading and Itacitinib Intervention

Constructs were treated with feed media described previously from day 0 to day 6. On day 7, constructs were transferred either into custom designed, 3D printed grips within bespoke bioreactors (Figure 1A-C), or new 35 mm control plates containing immobile grips spaced 10 mm apart. Both groups were fed 5 ml of media to ensure full submersion of the ligament (Figure 1). On day 8, ITA was added to half of the constructs, and loading began. The treatment groups consisted of normal feed media supplemented as follows: vehicle (1 μl/ml 100% ethanol); or ITA (10 μM) dissolved in ethanol and diluted to 1μl/ml final volume. The loading intervention was as follows: a 30 second isometric hold at 4% strain (0.4mm based on 10 mm resting length), and a 2-minute rest, repeated 4x for a total time under tension of 2 minutes and a total active period of 8 minutes. The loading protocol was a repeated every 6 hours for 6 days. Constructs were fed on days 8, 10, 12, and were mechanically tested on Day 14, 5 hours after the final loading bout.

Mechanical Testing

On day 14, constructs were mechanical tested as previously described [158]. Briefly, constructs were removed from the incubator, and the length measured using digital calipers as the distance between the inside points of the teardrop anchors. The width and thickness of the ligament was determined by spectral-domain optical coherence tomography using a OQ Labscope (Lumedica, Durham, NC). Following anthropomorphic measurements, the constructs were placed into 3D printed grips attached to a 10 N load cell in a Model 68SC-1 single column tensile tester (Instron, Norwood, MA). The ligaments were submerged in a bio-bath containing 37°C saline and mechanically tested. Testing consisted of 10 cycles of preconditioning to 0.10 N followed by loading to failure at a constant rate of 0.25 mm/s. The maximum tensile load (MTL) was determined as the maximal load measured in Newtons on the tensile tester before failure, giving a mechanical property of the tissue. The cross-sectional area (CSA) was determined by multiplying the measured width and depth of the tissue. The ultimate tensile strength (UTS) was calculated by normalizing the MTL to CSA, giving a material property of the tissue relative to size. The Young's modulus was calculated as the maximal slope of the stress-strain curve, giving another material property of the tissue relative to size.

Differential Scanning Calorimetry

Following mechanical testing ligaments were removed from anchors and placed in 1x DPBS until ready for differential scanning calorimetry (DSC). Ligaments were removed from DPBS, lightly blotted to remove excess moisture, cut in the midsection using a 4 mm biopsy punch, and hermetically sealed in a Tzero® aluminum pan. The remaining tissue was saved for hydroxyproline assay. Samples for DSC were weighed and then tested using a Discovery DSC

with autosampler (TA Instruments, New Castle, DE). Samples were equilibrated at 40ºC and then heated from 40°C to 90°C at a rate of 10°C/min. The enthalpy normalized to mass (J/g), onset temperature, and peak temperature were measured. Following analysis, lids were punctured, tissue was removed and dried at 120ºC for 30 minutes along with the tissue that was not used for DSC. Dried engineered human ligaments were placed in 1.7 ml tubes, weighed for dry mass, and stored at room temperature.

Hydroxyproline Assay

Hydroxyproline assay was performed as previously described [133,158]. Briefly, dried constructs were hydrolyzed in 200 µl of 6 N HCl at 120ºC for 2 hours, and then left for 1.5 hours at 120ºC to allow for evaporation of HCl in a chemical fume hood. Dried samples were then removed from heat and resuspended in 200 µl of hydroxyproline buffer. The samples were further diluted 1:20 in the same hydroxyproline buffer. Following dilution, chloramine T solution was added to each sample and standard, then left at room temperature for 20 minutes. Then, aldehyde-perchloric acid solution was added to each sample and standard, and incubated at 60ºC for 15 minutes. Samples were removed from heat and left to cool at room temperature for 10 min. 200 µl of each sample and standard was pipetted into 96-well plate in duplicate. The 96-well plate was read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). A standard curve was used to calculate total hydroxyproline in samples, which was converted to collagen mass by assuming that 13.7% of collagen is hydroxyproline. Collagen content was divided by the dry mass of the original tissue sample to determine percent collagen.

Statistics

All data are presented as mean ± standard deviation with each individual ligament (technical replicate) represented by a point in all graphs. All experiments were performed multiple times using different cell donors (biological replicates). Figures represent one biological replicate, but all biological replicates showed qualitatively similar data. Data was analyzed by 2-way ANOVA, with load and ITA as the main effects, using GraphPad Prism 10.0. All data showed equal variance, therefore Tukey's honestly significant differences posthoc tests were performed when the ANOVA was significant. All main effects and interactions are shown on the figures.

Results

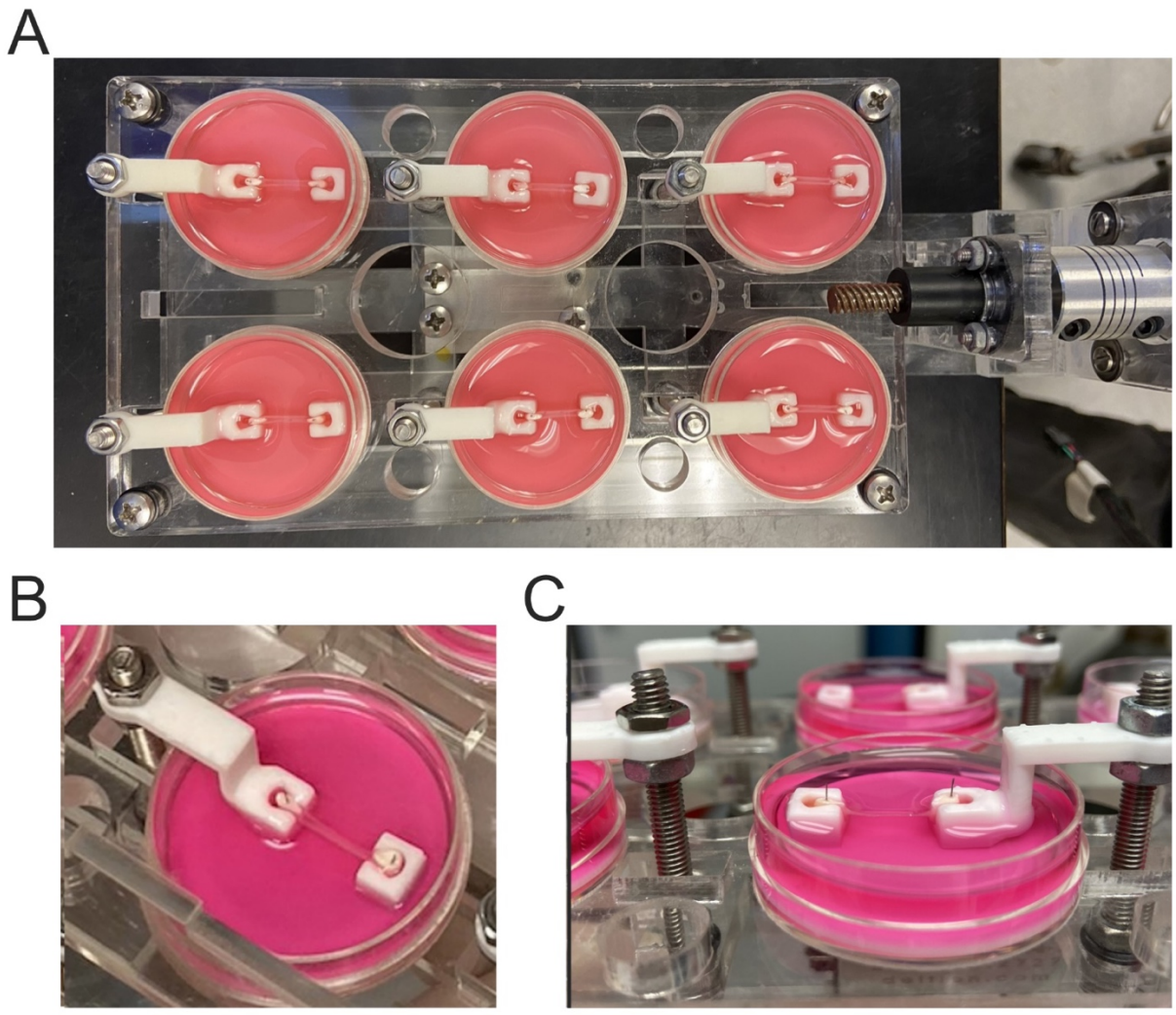

Figure 1: *In vitro* bioreactors. (A) Top view of engineered human ligaments loaded into 3D printed grips in bioreactor. (B-C) Engineered human ligament loaded into 3D printed grips in bioreactors and submerged in construct feed media.

Isometric Loading Improves Engineered Ligament Mechanics

Isometric loading and JAK1 inhibition improved ligament mechanics through independent and additive mechanisms (Figure 2). There was no significant increase in MTL with isometric

loading (p = 0.133) or ITA treatment (p = 0.134) (Figure 2A). Isometric loading did not change CSA of ligaments (p = 0.105), while ITA also had no effect on CSA (Figure 2B). There was a significant main effect of loading on UTS and ITA treatment did not reach statistical significance (p = 0.072) (Figure 2C). However, this improvement in material properties with loading was not seen with modulus, but ITA treatment showed a nearly significant effect (p = 0.0558) (Figure 2D). This suggests that isometric loading of engineered human ligaments may improve material properties (i.e. tissue quality) more than mechanical properties (i.e. tissue size).

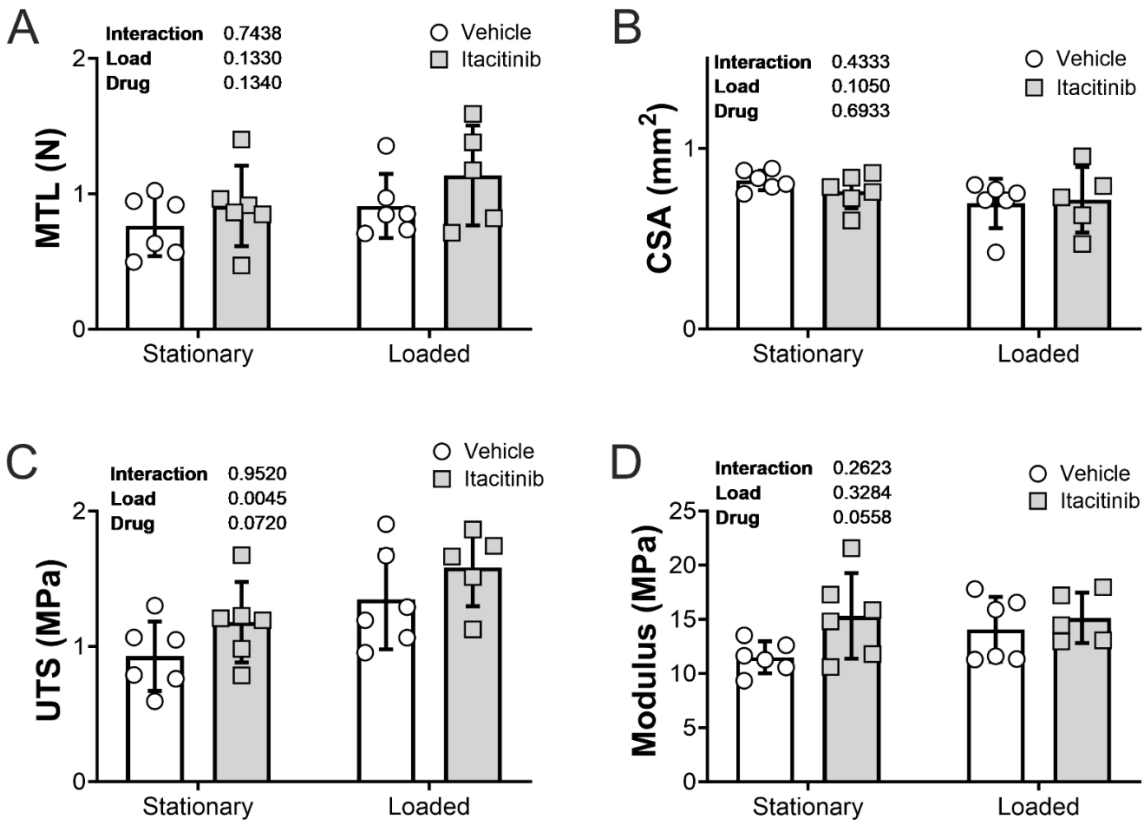

Figure 2: Isometric loading and Itacitinib improve ligament mechanics independently. (A) MTL trends towards an increase with isometric loading and ITA. (B) CSA trends towards a decrease with load but is unchanged by ITA. (C) UTS is significantly increased by isometric loading and nearly significantly increased by ITA independent of each other. (D) Modulus is

increased by ITA but is unchanged by isometric loading. Statistics calculated by 2-way ANOVA. n = 6.

Isometric Loading Does Not Affect Collagen Content

ITA treatment (p = 0.071) and isometric loading did not significantly affect collagen content (Figure 3A). There was also no significant interaction effect between isometric loading and ITA (p = 0.0546) (Figure 3A). There was no effect on dry mass with ITA or isometric loading alone as well as with isometric loading and ITA together (Figure 3B). The percent of the tissue dry mass that was collagen (% Collagen) was unaffected by either intervention (Figure 3C). The fact that there is not a main effect of load on collagen concentration, combined with the main effect of load to increase UTS (Figure 2C), suggests that loading may improve collagen organization or crosslinking.

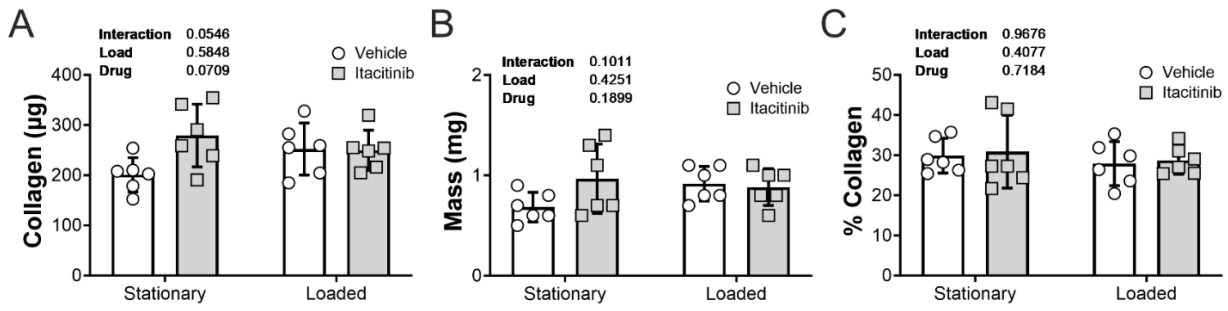

Figure 3: Isometric loading does not affect collagen content. (A) Collagen content trends towards an increase by ITA but is unchanged by isometric loading. There is a trend for an interaction affect where isometric loading prevents increased collagen content when combined with ITA. (B) Dry mass is unchanged by isometric loading and trends towards an increase with ITA and an interaction effect. (C) Percent collagen of dry mass is not affected by isometric loading or ITA. Statistics calculated by 2-way ANOVA. n = 6.

Isometric Loading Increases Enthalpy and Peak Temperature

Isometric loading increases enthalpy and peak temperature at denaturation while ITA tends to increase onset and peak temperature (Figure 4). There is a main effect of isometric loading on enthalpy, no effect of ITA, and no interaction (Figure 4A). ITA shows a strong trend towards a main effect of increasing onset temperature ($p = 0.0514$) and there is a trend ($p = 0.18$) for isometric loading to do the same (Figure 4B). Peak temperature shows a main effect of increasing with isometric loading and a strong trend ($p = 0.0535$) to increase with ITA (Figure 4C). The onset and peak temperature data mirrors of UTS, suggesting that the improved material properties with both load and ITA may result from increasing the thermal properties (i.e. collagen fibril size, packing density, or crosslinking) of the matrix.

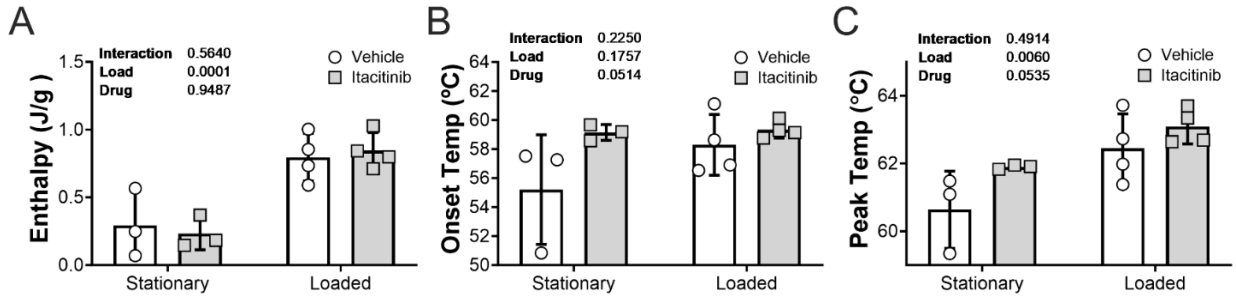

Figure 4: Thermal properties are increased by isometric loading and Itacitinib. (A) Enthalpy normalized to mass is increased with isometric loading. (B) Onset temperature is increased by ITA and trends towards an increase with isometric loading. (C) Peak temperature is increased by isometric loading and ITA independent of each other. Statistics calculated by 2-way ANOVA. n = 3-4.

Discussion

Isometric loading has been shown to decrease pain associated with chronic tendinopathy and even return tendinopathic tissue to normal [6,7,197]. In the current work, we have reproduced the positive effect of isometric loading on tendon using an engineered human ligament model. Using this model, the combined effect of isometric loading and JAK1 inhibition resulted in a greater improvement in the material properties of ligaments than with loading alone. This data suggests that targeted JAK1 inhibition combined isometric loading may be a novel therapeutic for chronic tendinopathy.

Tendinopathy can be associated with diseases such as obesity and diabetes [199], drugs such as fluoroquinolones or corticosteroids [199,200], and overuse [201]. The mechanism underlying the origin of the tendinopathy may vary, but tendinopathic tissue tends to show regions of poor collagen organization. As the organization of the collagen matrix decreases, this alters the thermal properties of the tissue [177]. In cadaveric studies, tendinopathic tissues show smaller collagen fibrils, decreased packing density, and lower crosslinking and these properties with associated with lower enthalpy and denaturation temperature. Therefore, it was interesting to note that the isometric loading significantly increased both enthalpy and peak temperature and tended to increase onset temperature. This suggests that loading increases matrix organization and possibly improves collagen fibril orientation, size, packing density, and/or crosslinking. In support of this finding, a collagen matrix will orient in the direction of force even in the absence of cells [202]. Load is also known to be important in the development of larger collagen fibrils, producing the bimodal distribution of fibril size that is associated with normal tissue mechanics in adult tendons [203]. Beyond alignment and fibril size, Heinemeier and colleagues

demonstrated that lysyl oxidase gene expression increases with loading [204]. These data suggest that load improves collagen fibril orientation, size, and crosslinking, consistent with the improved thermal properties observed in the current work. Future studies using transmission electron microscopy, transcriptomic data, and HPLC analysis of crosslinks will be needed to establish whether one, or all, of these processes are improved with isometric loads.

Loading of tendon *in vivo* results in an acute inflammatory response [190,205]. While a pro-inflammatory response in muscle following load is believed to be necessary for adaptation [206], the pro-inflammatory response in tendon has been generally thought to have a negative effect [183]. Here we found that JAK1 inhibition using the drug Itacitinib tended to improve engineered ligament mechanics independent of loading. Interestingly, the improvement in mechanics with isometric loading and ITA was not associated with in proportional increase in collagen. The improved mechanical and material properties associated with combined isometric loading and ITA best related to the thermal properties of the engineered ligaments. These data suggest that isometric loading and ITA both increase the thermal stability of the matrix; however, they likely do so through different mechanisms. It would be interesting to combine load with cytokine treatment [189] to determine whether loading could overcome some of the negative aspects of the pro-inflammatory markers that are elevated in chronic tendinopathy [93]. Although we previously reported that NF-κB inhibition was detrimental to engineered ligament function [189], and combining cytokine treatment with inhibition of NF-κB decreased ligament function more than either treatment alone, we did not investigate the role of NF-κB with isometric loading. Given that NF-κB has been targeted to treat chronic tendon injury [5], whether loading can directly alter NF-κB activity needs to be addressed.

It is important to note that the loading intervention was performed during a rapid developmental phase of the engineered human ligaments that is similar to what would be expected in a neonatal tendon or a tendon following an acute injury [158]. These tissues all have relative low collagen content, small fibrils, and low crosslinks. It will be interesting to compare the effect of this loading intervention when it is performed on day 8 engineered human ligaments to the response seen in more mature (day 14) ligaments that already have a higher collagen content and better mechanical strength. Whether loading can improve mechanics, collagen content, and/or the thermal properties of a more mature matrix needs to be addressed. Furthermore, in the current work we did not investigate different loading protocols, such as comparing isometric with dynamic loading movements that better mimic the eccentric and heavy resistance training protocols that have previously been reported to improve pain and function in tendinopathic patients [207].

In conclusion, we have demonstrated an *in vitro* engineered human ligament can replicate the positive effects of isometric loading seen *in vivo*. Further, JAK1 inhibition resulted in improved ligament mechanics independent of loading. Despite the lack of an interaction effect, the combined intervention of load and Itacitinib resulted in the greatest increase in mechanical properties. The results from this study indicate JAK1 inhibition combined with loading should be investigated as a novel intervention to treat tendinopathy.

Acknowledgements

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Author Contributions

A.A. and K.B. conceptualized the study. K.T. and K.B. designed and constructed bioreactors. A.A. contributed to data collection. A.A. and K.B contributed to data analysis. A.A. and K.B. contributed to manuscript. All authors approved the final manuscript.

Chapter 5: Discussion

Engineered Ligament Model

A dense extracellular matrix that turns over slowly along with low cellularity can make it difficult to biochemically analyze mature tendon/ligament *in vivo*. An *in vitro* ligament model that responds to growth factors [128,208], hormones [129], and exercise [132] was originally developed by the Baar lab group [4]. The high cellularity and increased rate of collagen turnover allows for quicker data collection and easier use in biochemical assays. I set out to further characterize this model to validate its continued use and expand our understanding of the different developmental stages of this model.

We validated the use of this model at multiple passages. There was no significant decrease in tenoblast markers and constructs showed no change when engineered at different passage numbers ranging from 4 to 11 across different biological donors. Furthermore, we found two distinct phases in the development of the engineered ligaments characterized by an exponential increase from Day 7 to 14 followed by a plateau in total collagen and percent collagen of dry mass. We hypothesize that we can use these phases to model different developmental stages of the tissues. The effects of interventions from Day 7 to Day 14 would be representative of the effects on a developing or regenerating tissue after injury, while the effects of interventions beginning after Day 14 would be representative of the effects on a mature, healthy tissue. Overall, this part of the dissertation resulted in a better understanding of the most widely used engineered ligament model and the identification of important time points along its development.

Although the results of this study further validated this engineered ligament model, limitations still exist. Outside of the inherent limitations of an *in vitro* model compared to *in vivo*, an important distinction with these engineered ligaments is that it uses a single cell type – human ACL fibroblasts. Progenitor cells have been identified as a small cell population that exists within ACLs that may help with tissue renewal [209]. Introducing mesenchymal stem cells (MSCs) as another cell population within this model could further improve the validity of its response to cytokines, hormones, and loading during its developing stage. Another current limitation within this model is the significantly lower percent collagen of dry mass compared to an *in vivo* tissue. While native tendon/ligament dry mass is approximately 80% collagen, the engineered ligaments in this model plateaued at approximately 25%. A future study utilizing histology and transmission electron microscopy to measure cellularity, collagen fibril diameter, and packing density would increase understanding of how this model compares to *in vivo* tissue.

Tendinitis

The role of inflammation in tendinopathy continues to be controversial. However, studies have indicated inflammation plays a significant role in chronic tendinopathy [93,148,149] and there is a critical need to better understand the mechanism of inflammation in tendon injury and healing[210]. Although the inflammatory response *in vivo* is incredibly complex and still poorly understood, I hoped to develop an *in vitro* model for tendinitis using pro-inflammatory cytokines that had been found to be elevated in synovial fluid in chronic inflammatory diseases such as rheumatoid arthritis and following ACL injury [211,212].

I originally planned to work primarily with TNF-α since it activates the NF-κB pathway [160] which had been identified as a target for treatment of chronic inflammation [5]. Treatment with increasing concentrations of TNF-α showed a repeatable dose-dependent decrease in mechanical function and collagen content in engineered ligaments. However, I received feedback during this project that expanding to multiple cytokines would lead to a more physiologically relevant model for ligament response to inflammation. Therefore, I expanded treatments to IL-1β and IL-6. Interestingly, IL-6 alone did not have the same individual effect on ligament function as TNFα and IL-1β. Still, considering the IL-6/STAT3 pathway had been identified as a potentially important aspect to the inflammatory response in tendon/ligament [79,100,101] we moved forward with IL-6 included in the cytokine cocktail treatment. This cytokine cocktail resulted in a novel *in vitro* model for tendinitis using cytokines at more physiologically relevant concentrations.

An important limitation to this model of tendinitis is the lack of immune cells. While fibroblasts both respond to and secrete cytokines [213], both innate and adaptive immune cells reside within tendon/ligament and play a critical role in response to injury [214]. To expand on this model, introducing co-culture with immune cells such as macrophages could better mirror an *in vivo* response. Co-culture experiments of myoblasts, myofibroblasts, and MSCs with M1 and M2 macrophages have already been conducted in 2D cell culture environment [215,216]. This co-culture method with a 3D ligament model would allow for a project determining the specific timing for a proper immune response. I would look to determine if there is an ideal timeline where transitioning from M1 macrophage co-culture to M2 macrophage co-culture improves ligament quality and function. Going a step further, mimicking the endogenous inflammatory

response using serum or synovial fluid taken from people suffering from a chronic inflammatory disease such as rheumatoid arthritis or an unresolved injury could provide a more physiologically relevant intervention. However, this would introduce biological variability between each subject. Overall, increasing the physiological relevance of a pro-inflammatory intervention would serve to increase the validity of future screenings for potential treatments using this *in vitro* model of tendinitis.

NF-κB and STAT3 Signaling

My original plans for my dissertation were in hindsight overly ambitious. As was highlighted in Chapter 1, there are many signaling pathways within tendon/ligament that are understudied and represent current gaps in the field. Whether FGF treatment could improve the development of engineered ligaments or a what is the detailed role of SPARC in tendon/ligament function remain exciting questions. Meanwhile, a project testing the effect of exosomes derived from human serum following an intense exercise bout is already underway (Paulussen and Baar, unpublished). Ultimately, the combination of overwhelming interest, failed experiments, and the complexity of an inflammatory response led to my dissertation work focusing on the NF-κB and STAT3 pro-inflammatory pathways.

As we had hypothesized, treatment with a cytokine cocktail resulted in the phosphorylation and activation of NF-κB. This led me to pursue natural products (substances found in nature) that could inhibit NF-κB activation. While I successfully identified natural products that inhibited NF-κB, treatment with these substances actually resulted in a dramatic decrease in ligament function and quality (Avey, unpublished). To ensure these negative effects were not the result of

off-target effects and actually caused by NF-κB inhibition, I treated ligaments with the known NF-κB inhibitor presented in Chapter 3 of this dissertation. Again, we found that a high dose of this NF-κB inhibitor significantly decreased the function of the ligament while low to mid doses had no effect. These results demonstrated NF-κB activity is essential to the development of engineered ligaments, a surprising but incredibly interesting finding. These data suggest that NF-κB inhibition may be detrimental to the regeneration of an injured tendon/ligament, but further exploration of this is needed.

Another confusing aspect of my results is the STAT3 signaling. After NF-κB inhibition was unsuccessful, I turned my attention to STAT3. Although IL-6 treatment alone did not have a significant effect on ligament mechanics and collagen, the potential cross-talk between the NF-κB and STAT3 pathways meant the cytokine cocktail may still be working through the STAT pathway [156,157]. Inhibition of JAK1 resulted in increased mechanics and collagen in constructs both with and without cytokine treatment. This was exciting data as I turned my attention to investigating the molecular signaling involved. However, to my surprise western blot data revealed that cytokine treatment alone *decreased* the phosphorylation of STAT3. Therefore, STAT3 activation appears to not be driving the negative effects of the cytokine treatment, but complete inhibition of STAT3 activity using a JAK1 inhibitor improved ligament mechanics and collagen. These data suggest that cytokines that activate JAK1 may target a different STAT protein. There are 7 STAT proteins (1, 2, 3, 4, 5A, 5B, and 6). It is possible that cytokines increase and JAK1 inhibition decreases the activity of one or more of the other STAT proteins and this is how Itacitinib works. While this leads to more questions regarding the complex signaling involved in an inflammatory response, it does deliver alongside a concurrent project

[136] a key finding that inhibition of the JAK1 improves engineered ligament quality and is potentially the first new drug to treat tendinopathy in decades.

Many questions remain regarding the roles of NF-κB and JAK1 signaling in tendinitis. To advance this work, I would first look at the effect of cytokine treatment and subsequent NF-κB and JAK1 inhibition at later points in the development of the engineered ligaments. This dissertation work focused entirely from Day 8 to Day 14, given an inflammatory response would typically follow injury where the tissue will be entering a regenerating stage. However, given the rise in chronic inflammatory diseases such as obesity and diabetes in the United States as well as the identification of elevated inflammatory markers in chronic tendinopathies [93], it is relevant to determine whether a more mature tissue responds differently to inflammation. Both NF-κB and JAK1 pathways have been identified as having detrimental effect on tendon [4,120,127,129,157,158], while also potentially playing an important role in improving tendon healing [130, 156]. Therefore, it is possible that the effects of NF-κB and JAK1 inhibition are different at different stages of ligament development and regeneration. Treating engineered ligaments starting at Day 14 may lead to entirely different results. Furthermore, engineered ligaments lacked the dynamic load that occurs *in vivo* during treatments with pro-inflammatory cytokines and the JAK1 and NF-κB inhibitors. Utilizing the bioreactors pictured in Chapter 4 Figure 1 to introduce dynamic loading alongside both pro- and anti-inflammatory interventions could provide a more physiologically relevant model.

Isometric Loading

Isometric loading has been identified as an effective intervention for tendinopathy [6,7,197]. However, these positive effects of isometric load had not yet been replicated in an engineered ligament model. Therefore, I investigated the effects of isometric load on an engineered ligament as well as the combination of load and a JAK1 inhibitor to determine whether preventing an inflammatory response prevented adaptation to load.

Ligament mechanics were improved by isometric loading; however, collagen content was not changed. Since there was an increase in mechanical function without a corresponding increase in collagen, we used differential scanning calorimetry to quantify differences in matrix quality. Here we found a significant loading effect on enthalpy, suggesting that isometric loading increases collagen mechanics through improved collagen alignment or crosslinking. However, future studies are needed to understand these factors further. Interestingly, combining isometric loading with the JAK1 inhibitor resulted in the highest average MTL and UTS within the study, yet collagen content was still unchanged. In fact, collagen content in the unloaded group treated with JAK1 inhibitor increased relative to controls but the introduction of isometric load prevented this increase. These results suggest that isometric loading may prevent the decrease in collagen degradation that was previously shown to occur with JAK1 inhibition in Chapter 3. Ultimately, the work in Chapter 4 of this dissertation shows the first replication of the positive effects of isometric load on an *in vitro* engineered ligament and suggests introducing a JAK1 inhibitor to an isometric loading protocol may further improve tendon/ligament health.

There are several studies that would build upon this research. First, repeating these interventions with engineered ligaments and collecting the tissues for qPCR, western blotting, histology, and

transmission electron microscopy. Due to limitations in the number of ligaments that can fit into the bioreactors and time constraints, these were analyses I was unable to complete during my doctoral research. Adding this data to the work already presented in Chapter 4 would result in a more complete understanding of how these interventions are working to improve ligament mechanics. The next step would be to translate this work to an *in vivo* model. Steffen, Mienaltowski, and Baar previously developed a rodent model of patellar tendinopathy as well as an isometric loading protocol [198]. Introducing the JAK1 inhibitor to this model would answer two important questions. One, does the positive affect of JAK1 inhibition with isometric loading translate to an *in vivo* healthy tissue? Second, what are the effects of using an anti-inflammatory on an already injured tissue with regards to its regeneration? Answering the first question would serve to further validate the use of the engineered ligament as an effective model for *in vivo* tissue. Answering the second question would determine whether JAK1 inhibition is truly the next major breakthrough in tendon/ligament rehabilitation protocols.

Key Takeaways

1. The engineered ligament model is a reliable *in vitro* model across multiple passages to test ligament response to a multitude of interventions.
2. Timing of interventions allows for testing of tissue at a developmental/regenerating stage (Day 7-14) or a matured stage (Day 14-21).
3. Pro-inflammatory cytokines significantly reduce ligament function and matrix quality.
4. Inhibition of JAK1, not NF-κB, improves ligament function during the developmental/regenerating stage.

5. Use of a JAK1 inhibitor alongside a targeted isometric loading protocol may further improve tendon/ligament health.

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