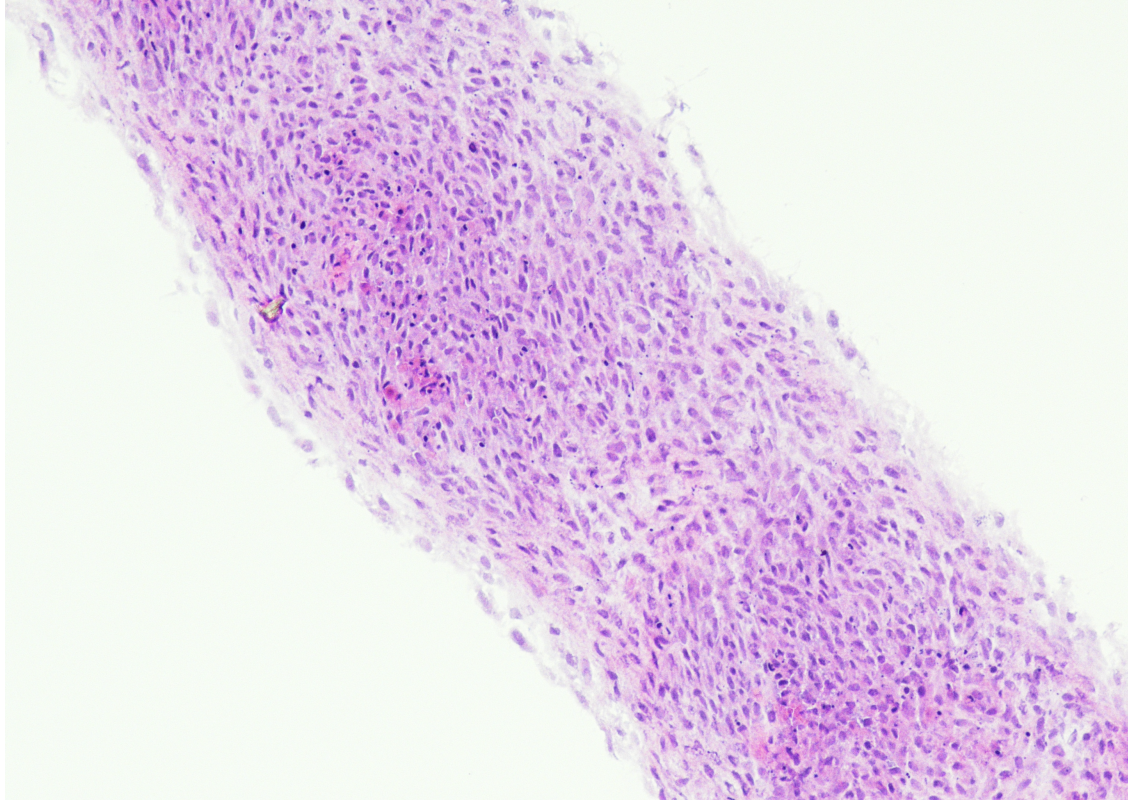




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UNIVERSITY OF TECHNOLOGY



Macrophage-Driven Regulation of Collagen Formation in a 3D Human Tendon Model

Master of Science thesis for the Biotechnology MSc degree

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Cover: A construct stained with hematoxylin and eosin and imaged at 10x magnification.

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Abstract

Tendon injuries affect millions of people annually and tendons rarely regain full mechanical function after healing. Macrophages are key regulators of tendon healing, shifting between pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes across the phases of healing, yet their specific effects on tenocyte behavior and extracellular matrix formation remain unclear. This project aimed to investigate how M1 and M2 macrophage phenotypes affect the biomechanical properties, tenocyte phenotype and activity, and extracellular matrix formation in 3D human tendon constructs. Primary human tenocytes isolated from the semitendinosus tendon were embedded in a fibrin-based hydrogel, where cell-mediated contraction led to formation of a 3D *in vitro* tendon construct. Macrophage–tenocyte crosstalk was established using a Transwell-based paracrine co-culture system. The effects of macrophage–tenocyte crosstalk on tendon constructs were evaluated using uniaxial tensile testing, reverse transcription quantitative PCR, and hematoxylin and eosin staining. M1-stimulated constructs exhibited significantly increased expression of MMP-1, MMP-13, TIMP-1, and IL-6, together with decreased scleraxis expression, indicating enhanced matrix remodeling, increased inflammatory signaling, and an altered tenocyte phenotype. M2-stimulated constructs exhibited no significant changes in the evaluated mechanical properties and displayed tissue organization comparable to controls. However, significant increases in TIMP-1 and IL-6 expression were observed, indicating altered matrix remodeling and inflammatory signaling despite the absence of detectable mechanical effects. Histological assessment demonstrated no significant differences in histological scores between both groups and the control. These findings suggest that pro-inflammatory M1 macrophage signaling negatively affects tendon construct mechanical properties and extracellular matrix integrity, while M2 macrophage signaling exerts a more moderate influence. These results highlights the importance of macrophage phenotype balance during tendon healing and may contribute to future development of improved regenerative strategies for tendon repair.

Keywords: Tendon injury, tendinopathy, macrophages, tendon repair, 3D hydrogel model, tendon constructs, mechanical testing, gene expression, histology.

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Clara Hammelev Jörgensen and Frida Hannius, Gothenburg, June 2026

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

ACTA2	Actin alpha 2, smooth muscle
CSA	Cross-sectional area
CXCL2	C-X-C motif chemokine ligand 2
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
IFN- γ	Interferon-gamma
IL-13	Interleukin-13
IL-4	Interleukin-4
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
PBS	Phosphate buffered saline
Pen-Strep	Penicillin streptomycin
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S100a4	S100 calcium-binding protein A4
SCX	Scleraxis
TIMP	Tissue inhibitor of metalloproteinase
TGF- β	Transforming growth factor beta
TNMD	Tenomodulin

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1

Introduction

Each year, 6 to 7 million cases of tendon ruptures are reported worldwide [1]. Highlighting that, despite being one of the strongest components of the body, tendons are a common source of injury. The injuries range from chronic to acute, and for adults, the likelihood of complete regeneration of the torn tissue is low. The pre-injury state is rarely recovered [2, 3], and the tendon composition, structure, and strength are changed, even after treatment and rehabilitation [4].

Tendon healing is an interdependent process involving inflammation, proliferation, and remodeling, where immune cells and tendon cells coordinate to repair the injured tissue. Macrophages, among other cells, play a crucial role in tendon healing. As macrophages exist in different polarized states, increasing attention has recently been paid to understand how these different phenotypes influence the different stages of tendon healing [5]. Despite the significance of macrophages in healing, there is limited knowledge on how macrophages affect tendon cell behavior [6]. While prior research has characterized macrophage behavior in wound healing broadly, and animal models have demonstrated that depleting or modulating macrophages affect tendon repair outcomes [7, 8, 9], the effect of specific macrophage phenotypes on tenocyte behavior and collagen formation remain poorly understood. It is unclear whether the mechanical and structural weaknesses seen in healed tendons are driven by macrophage-induced changes in tenocyte gene expression and ECM remodeling, a gap this study aims to address.

To investigate this, an *in vitro* co-culture model consisting of 3D human tendon constructs and macrophages were used to evaluate how different macrophage phenotypes influence biomechanical properties, tenocyte phenotype and activity, and ECM formation. Improving the understanding of how the immune responses regulate tendon healing, particularly via tenocyte-macrophage cross-talk, is important for further progress in tendon healing research.

1.1 Aim

This project aimed to investigate how different macrophage phenotypes, M1 and M2, influence tendon construct biomechanical properties, tenocyte phenotype and activity, and ECM formation in a 3D *in vitro* human tendon construct.

1.2 Contribution

This master's thesis was carried out as a collaborative project between two students, with the workload divided equally throughout the study. Both students have the same academic background and experience of laboratory work. The laboratory work carried out during this project was shared in order for both students to gain experience with all methods used in the project, while also allowing for efficient collaboration during the experimental phase of this project. The experimental plan required work during weekends. During these occasions, the workload was divided between the students when the presence of both was not necessary. Since many of the laboratory procedures consisted of repeated experimental steps that both students had performed multiple times, the work could be shared efficiently while maintaining consistency throughout the experiments. In some cases, parts of the laboratory work outside regular working hours was done by the supervisor.

The report is the result of both students writing and working an equal amount before joint revision. Although different sections of the written report were assigned with an initial area of responsibility, all parts of the thesis were discussed, reviewed and revised by both students. This approach was chosen due to the extensive amount of literature that needed to be read, making it more efficient for one student to take primary responsibility for summarizing and structuring specific topics at first.

The planning of the project and experimental design was carried out together with the supervisors. Decisions related to data analysis and interpretation were discussed and made jointly with the supervisors. Throughout the project, continuous discussions were held between both students to ensure a shared understanding of the results, conclusions and discussion presented in the thesis.

2

Theory

This chapter presents the theoretical background relevant to tendon biology, injury mechanisms, immune response and the analytical methods used in this study. Included is theory regarding the biology and structure of tendons, followed by a description of tendon injuries, the following inflammatory response and tendon healing processes. Furthermore, the principles underlying the mechanical and biological testing used to evaluate tendon construct properties are introduced, including information on gene expression analysis and histological staining.

2.1 Tendon Structure and Biology

Tendons connect muscles to bones and enable movement by transmitting mechanical forces. Their mechanical competence depends on a highly organized extracellular matrix (ECM) which is predominantly composed of collagen. Collagen molecules are assembled into a hierarchical structure of fibrils, fibers, and fascicles [10], as illustrated in Figure 1. The fascicles are enclosed within a tissue called endotenon, which is surrounded by the epitenon and an outer paratenon sheath [11]. Tendon strength is determined by the density and orientation of collagen fibers, the degree of intermolecular cross-linking, and the overall dimensions of the tissue [12].

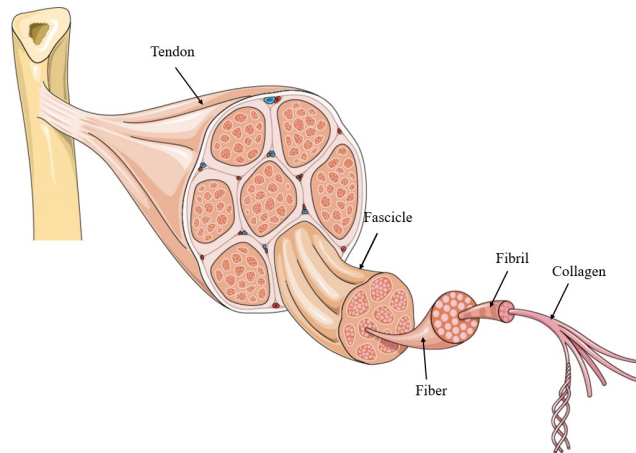


Figure 1: Tendon anatomy showing the hierarchical organization of collagen fibers into fibrils, fibers, and fascicles. Adapted from Servier Medical Art [13], licensed under CC BY 3.0.

Collagen type I is the predominant ECM component and is primarily responsible for the tensile strength of the tendon. The second most prevalent type is collagen type III which plays an important role in regulating fibril diameter [14], and is particularly associated with tendon healing, as it can stabilise the injury site by forming rapid cross-links [15]. Type III collagen fibers are weaker and have a higher turnover rate compared to type I, but are responsible for tendon elasticity as well as the maturation of type I fibers [16, 17]. Other less prevalent types include collagen V and collagen VI, which are believed to have roles in regulating the assembly and coordination of collagen molecules [18]. Beyond collagen, the tendon ECM contains water, which accounts for more than half of the total tendon mass and is essential for maintaining the structural stability of collagen molecules [19, 20].

Up to 5% of the tendon dry weight is composed of proteoglycans, including decorin, biglycan, and fibromodulin, all of which are important in collagen fibrillogenesis. Of these, decorin is the most abundant [14]. The ECM also contains numerous glycoproteins that are fundamental for achieving the necessary cellular and mechanical functions. These include, but are not limited to, tenomodulin (TNMD), the thrombospondin family, tenascin-c, and elastin. TNMD and thrombospondins are important for tendon stem/progenitor cell proliferation and fibril formation [18]. Tenascin-C is crucial for the generation of tendon tissue and is also believed to contribute to elasticity [21]. Elastin, accounting for less than 2% of the dry weight, maintain tenocyte-ECM crosstalk within the tissue and assists in tendon elasticity [22].

Tendons are characterized by a low cell density, contributing to their poor healing capacity following injury [23]. The most prevalent cells are tenocytes, a type of fibroblast that act as regulators to sustain homeostasis [24]. Post-injury, tenocytes travel to the site of injury and upregulate their production of ECM components to try to restore the tissue [21]. They are primarily located in the fascicles, and express tendon-associated markers and transcription factors such as scleraxis (SCX) and TNMD [24]. As the low cell density and dependence on tenocyte-driven collagen synthesis make tendons particularly vulnerable to injury, an understanding how the cellular environment regulates collagen formation is essential for improving healing outcomes.

2.2 Tendon Injuries and Healing

Tendon injuries are among the most common musculoskeletal disorders, affecting a broad range of the population and representing a significant clinical challenge worldwide [1]. Tendon injuries are classified as chronic or acute. Chronic tendon pathology, known as tendinopathy, is the most prevalent form and is characterized by pain and degenerative changes in the tendon tissue. It is commonly associated with repetitive mechanical loading or overuse and risk factors include age, extreme exercise and oxidative stress, [25, 26], although the underlying pathogenesis remains poorly defined [27]. Acute tendon injuries include full or partial ruptures of the tendon [10]. They typically result from sudden trauma and are associated with an

immediate loss of mechanical function. Regardless of injury type, the biomechanical properties of the tendon, including its structure and composition, remain altered even after treatment and rehabilitation [4], and the pre-injury mechanical state is rarely fully recovered.

Tendon healing is a complex, interdependent process that proceeds through three overlapping phases called inflammation, proliferation, and remodeling [27]. Unlike most tissues, tendons heal primarily by scar formation rather than true tissue regeneration, resulting in mechanically inferior repair tissue [26]. The low cellularity and vascularization, as well as slow ECM remodeling, contributes to the poor healing capacity that tends to lead to scar formation and fibrosis [23]. The outcome of healing is highly dependent on the anatomical location of the tendon, the severity of the injury and the cellular and molecular environment at the injury site [28].

The inflammatory phase is initiated immediately following injury through the release of damage-associated molecular patterns (DAMPs) from damaged cells, which activate the innate immune system and recruit circulating immune cells to the injury site [29]. The white blood cells normally circulate in the bloodstream and includes monocytes, lymphocytes and a group of cells called granulocytes, such as neutrophils. Neutrophils are the first cells to arrive to the injury site, contributing to the clearance of debris and necrotic tissue [30, 27]. They are rapidly followed by macrophages, which has a regulatory role in coordinating the transition between healing phases through the secretion of cytokines, chemokines and growth factors [31], including C-X-C motif chemokine ligand 2 (CXCL2), contributing to further recruitment of neutrophils to the injury site as tendon repair progresses [32].

The proliferation phase begins a few days after injury [27] and is characterized by increased cellular activity, ECM production and absorption of water [33]. During this stage, tenocytes and tendon-derived fibroblasts proliferate and migrate into the injured area, where they synthesize large amounts of collagen, primarily type III collagen, to provide temporary structural support [33, 27]. Angiogenesis is stimulated through the release of growth factors, leading to the formation of new blood vessels that supply oxygen and nutrients to the healing tissue [30, 33]. Matrix metalloproteinases (MMPs) tightly regulate ECM turnover and remodeling by degrading matrix components such as collagen, fibronectin and laminin, thereby creating space for the deposition of newly synthesized collagen. The balance between MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are of importance during tissue repair [34], and they are both influenced by the immune microenvironment.

The third phase is the remodeling phase, which is characterized by decreased cellularity and matrix reorganization [33]. Collagen type III is gradually replaced by collagen type I, as the tissue matures and mechanical loading increases [27]. This transition is often incomplete and the repaired tissue contains a higher proportion of collagen type III compared to native tendon tissue [33]. The reason for this is the production of scar tissue rather than healthy tendon tissue, which contributes to its reduced tensile strength and stiffness [10].

The three overlapping phases are tightly modulated by the secretion of signaling molecules. The initial inflammatory phase is driven by pro-inflammatory cytokines from the immune cells, while tissue repair is initiated by growth factors released by cells located at the site of injury. Tendon cells are both activated to degrade and produce ECM [33], which underscores the importance of balance that drive the different stages of healing.

2.3 The Role of Macrophages in Tendon Healing

Among the immune cells that are recruited to the injury site, macrophages are the most influential on tendon healing. Understanding of their impact on tendon healing is crucial for the overall understanding of the tendon healing process and its different phases. They derive from circulating monocytes that migrate into the tissue and differentiate in response to cytokines secreted from damaged cells and adopts distinct states that evolve across the different stages of healing [7]. Macrophages regulate the behavior of resident tendon cells and the composition of the ECM, making them central determinants in whether healing proceeds toward a functional remodeled tissue or fibrotic scar formation.

2.3.1 Macrophage Polarization

Macrophages can broadly be categorized into two polarization states, the pro-inflammatory state M1, or anti-inflammatory state M2 [5]. This polarization is driven by signals in the local microenvironment. M1 polarization is induced by pro-inflammatory stimulus such as lipopolysaccharide (LPS) and interferon-gamma (IFN- γ), while M2 polarization is promoted by anti-inflammatory cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) [35], which reflects the polarization conditions used in the present study. Examples of markers associated with M1- and M2 macrophage activation includes cluster of differentiation 16 (CD16) and 163 (CD163), respectively [36, 37].

During the early inflammatory phase of tendon healing, M1 macrophages predominate and drive the initial immune response [2]. They clear pathogens, cellular debris, and necrotic tissue from the wound environment [7], and sustain the inflammatory response through the secretion of pro-inflammatory cytokines and mediators [38, 6]. Although the inflammatory activity is necessary to initiate tissue repair, prolonged or excessive M1 activation is detrimental. It suppresses the ability of fibroblasts to synthesize organized collagen, promotes ECM degradation, and can lead to the formation of disorganized collagen fibrils and fibrotic scar tissue [39, 5].

As healing progresses into the proliferative and remodeling phases, a timely transitioned polarization from M1 to M2 is critical [30]. M2 macrophages suppress excessive inflammation and promote tissue repair through the secretion of anti-inflammatory cytokines and growth factors that support ECM synthesis and remodeling [39]. However, excessive or prolonged M2 signaling has also been associated with fibrosis, in which the newly deposited tissue fails to regain the mechanical

properties and collagen organization of a healthy tendon [2, 5]. A well-regulated balance between M1 and M2 activity is therefore essential for achieving functional tendon repair.

2.3.2 Macrophage-Tenocyte Crosstalk

The interaction between tenocytes and macrophages are primarily mediated through paracrine signaling. Macrophages secrete signaling molecules, cytokines and growth factors, to act on tenocytes to modulate their gene expression and tenocyte behavior. This cross-talk goes in both directions, as tenocytes also releases signals that influence macrophage behavior.

Upon recognition of DAMPs, M1 macrophages secretes pro-inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 [40]. This shapes the environment to pro-inflammatory, as well as activates MMPs, such as MMP-1, MMP-3, and MMP-13, that are involved in tendon and ECM degradation [40, 41]. MMPs cleaves collagen fibrils and it is established that an imbalance between collagen synthesis and degradation is a key factor in many diseases. However, MMP activity can also be an indicator of remodeling during repair [42]. The expression of MMPs are inhibited by TIMPs, and the MMP/TIMP balance is important for maintaining ECM homeostasis. M1 and M2 macrophages produce varying levels of MMPs and TIMPs, thereby affecting inflammatory responses and tissue repair processes [34, 43].

The pro-inflammatory environment also suppress the expression of key tenocyte markers such as SCX, which is an early transcription factor, and TNMD, which is a marker of mature tendons [44, 45]. SCX is crucial for initiating tendon repair and acts as a marker of tenogenic progenitor cells and mature tenocytes. It has a critical role in regulating collagen synthesis and thus facilitating healthy tendon development and homeostasis [45]. SCX is expressed continuously through all stages of tendon development until the tendon is mature. It upregulates the expression of TNMD, which regulates proliferation and collagen maturation, as well as acts as an anti-angiogenic factor [45, 44]. These combined effects of the M1 induced pro-inflammatory environment leads to impaired collagen synthesis and organization.

As the process proceeds and macrophages shift to the M2 phenotype, there is an increase in the secretion of factors including, but not limited to, IL-4 , IL-10 , IL-13 and transforming growth factor beta ($\text{TGF-}\beta$). This disrupts the inflammatory process, aids in tissue repair, and contributes to the deposition of collagen type I and III [40]. More specifically, M2 macrophages release $\text{TGF-}\beta 1$, which helps limit excessive inflammation and supports tendon healing. $\text{TGF-}\beta 1$ regulates angiogenesis by controlling vascular cell proliferation and modulating inflammatory responses. These immune cells also recruit tenocytes and other cells, initiating production of matrix factors. Furthermore, macrophage-derived products, especially $\text{TGF-}\beta 1$, are known to facilitate in the differentiation of tissue fibroblasts into myofibroblasts during tissue repair [6, 46]. This is associated with an increase in actin alpha 2, smooth muscle (ACTA2) expression in tenocytes, a myofibroblastic marker associated with contractile activity and fibrotic remodeling [47]. Additionally, S100 calcium-binding

protein A4 (S100a4) expression in tenocytes has been associated with fibrotic tissue remodeling [48], and has been linked to increased content of myofibroblasts at sites of tendon injury. Together, this drives a scar-like repair characterized by disorganized and excessive ECM deposition, which provides limited mechanical support but fails to restore native tendon structure, resulting in reduced function [49].

2.4 Experimental Model

A variety of different experimental models are available when studying tendon biology, and range from *in vivo* to *in vitro*, all with their own advantages and disadvantages. Animal models are common when studying tendon biology, for example using rats or rabbits, but they can not completely reflect human properties and presents ethical challenges [50]. *In vitro* models offer the advantage of having a system to study responses in without the disruption of other components like *in vivo* studies have. Human cell-derived models allows for the usage of human cells with fewer ethical considerations. In specific, using a 3D human tendon model over a 2D model better represents the tendon collagen structure [51, 52], and allows for uniaxial tensile testing to evaluate mechanical properties.

This study uses an established 3D fibrin-based hydrogel model with tenocytes. Fibrin hydrogels have a composition of water that reflects real soft tissue and can be combined with cells, creating a biologically compatible system. Thrombin cleaves fibrinogen, resulting in a fibrin gel that can attach to surfaces [53]. Additionally, aprotinin, an inhibitor of proteolytic enzymes, is included to prevent fibrin degradation [54]. The fibrin gel is seeded onto a silicone-coated well with two sutures that acts as anchor points. As the edges of the gel are released from the walls, the tenocytes exert tension on the sutures, driving the gel to contract and the cells to align along the longitudinal axis. After approximately 14 days, this results in tendon-like constructs that reflects structural features of a real tendon [55]. To support collagen synthesis [56, 57], L-ascorbic acid and L-proline are supplemented in the culture media.

While the 3D construct provides a biologically relevant platform for tenocyte behavior and collagen formation, studying how macrophages influence this system requires a method that allows paracrine signaling without direct cell-to-cell contact. For this purpose, this study uses Transwell co-culture systems to study the cross-talk between macrophages and tenocytes. A representative image of the contraction of the 3D tendon construct and the complete Transwell co-culture system is illustrated in Figure 2.

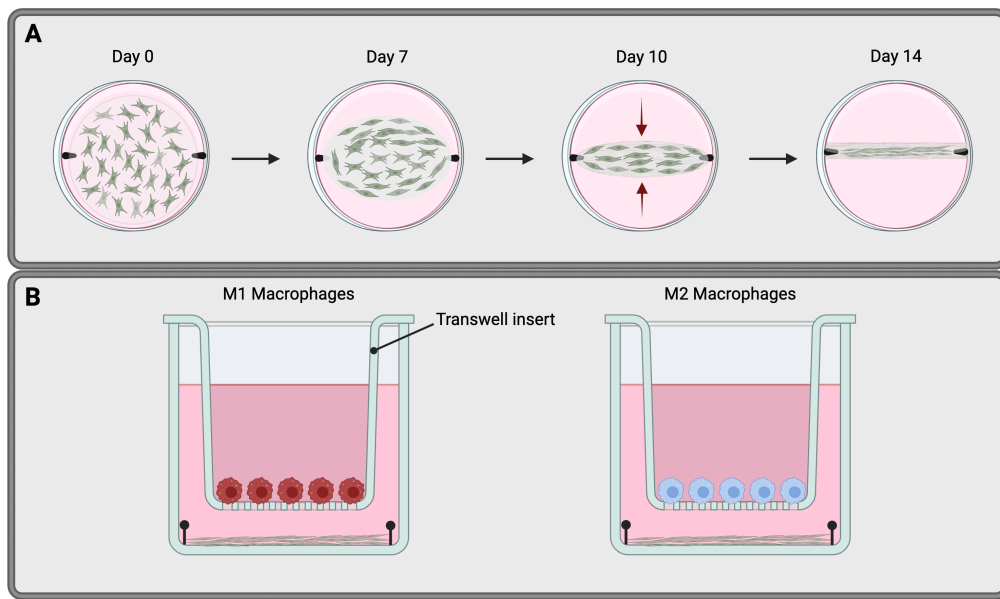


Figure 2: (A) Schematic overview of the fibrin-based 3D construct over time (Day 0–14), showing the progressive contraction of the tenocyte-laden hydrogel. (B) Schematic representation of the Transwell co-culture system used to investigate the effects of M1 and M2 macrophages on the 3D tendon constructs. Created with BioRender.com.

Primary tenocytes were used instead of cell lines to maximize biological representation, as primary cells better reflect the gene expression and behavior of native cells. While it introduces some donor-to-donor variability [58, 59], it also generates a system that is more representative of the human tendon environment compared to using established cell lines. However, it has been declared that tenocytes undergo phenotypic changes at higher passages in terms of genetic profile and morphology [60], and usually passage numbers below 5 is used [61, 62, 63]. The macrophages used were differentiated from the THP-1 monocyte cell line, which is commonly used in macrophage-related research. The cells are originally isolated from a child with leukemia and exhibits cellular properties similar to primary monocytes [64]. Compared to using macrophages derived from primary monocytes, THP-1 cells are more homogeneous, reproducible, and not limited by donor-to-donor variability or short lifespan [65].

2.5 Analytical Methods

The following sections presents the theory of the methods used to evaluate the mechanical and biological properties of the tendon constructs. The methods include mechanical testing used to provide measurements of the structural and material properties, and gene expression analysis, and histological assessment, that was used to evaluate the cellular and molecular responses.

2.5.1 Mechanical Testing

Mechanical testing is used to evaluate the structural and material properties of biological tissues and constructs. It refers to stretching a material until failure while recording the displacement and force, giving information on several mechanical parameters.

Stress (σ) is defined as the load normalized to the cross-sectional area, and strain (ϵ) is the deformation relative to the initial length. Stress and strain is independent of the geometry [66], allowing for comparisons between constructs of varying dimensions. Young's modulus (E), also known as modulus of elasticity, characterizes the stiffness of a material in the linear elastic region of the stress-strain curve. It is calculated as the slope of this linear region, as shown in Equation 1 [67]. Young's modulus is a material property and in the context of this study, it means that Young's modulus only takes the collagen quality into consideration, independent of the amount.

$$E = \frac{F/A}{\Delta L/L_0} = \frac{FL_0}{A\Delta L} \quad (1)$$

In contrast, stiffness (k) is dependent on both material and geometry and refers to an objects resistance to deformation [68]. Stiffness is calculated either from the slope of the force-displacement curve, or from Young's modulus equation, as shown in Equation 2. Stiffness is dependent both on how much collagen has formed, as well as the quality.

$$k = E \times \frac{A}{L_0} = \frac{FL_0}{A\Delta L} \times \frac{A}{L_0} = \frac{F}{\Delta L} \quad (2)$$

Maximum load refers to the maximum force sustained by the construct before failure, and elongation at maximum load describes the extent of deformation at the point of failure. The parameters defined provides a comprehensive characterization of construct mechanical properties. In this study, mechanical testing provides insight on how macrophages affect stiffness and load-bearing capacity of the construct, which are indicators of collagen quantity, quality, organization, and cross-linking.

2.5.2 Gene Expression Analysis

Gene expression analysis refers to the quantification of messenger RNA (mRNA) to assess what genes are up- and downregulated in response to a stimulus or condition. This study conducted gene expression analysis to evaluate the effects of M1 and M2 macrophage phenotypes on tenocyte gene expression.

There are several methods for conducting such an analysis. One alternative is reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In brief, RNA is extracted before reverse transcription into complementary DNA (cDNA). Subsequently, the cDNA is amplified, and the amount of RNA is quantified during the amplification using fluorescence emitted from dyes or probes. The output is cycle thresholds (Ct), which is the number of cycles needed for the signal to reach a threshold. A Ct value in the lower range indicate higher RNA levels, while a high Ct value indicate lower RNA levels. In relative quantification, which is used in this study, the Ct or standard quantity (Sq) value is normalized against a housekeeping gene [69]. This approach is appropriate when comparing differential gene expression between experimental conditions rather than determining absolute RNA quantities.

The genes analyzed in this study were selected to capture the key biological processes that is relevant to collagen formation regulated by macrophages (Table 1). Genes coding for collagen type 1 and collagen type III were included as direct measurements of structural matrix production [14]. MMPs were included as markers of matrix degradation, alongside the MMP inhibitor TIMP-1 to evaluate the balance between matrix degradation and synthesis [34]. SCX, a tenocyte-associated transcription factor, and TNMD, a tendon maturation marker, were included to evaluate the tenocyte phenotype [24]. ACTA2 and S100a4 were included as indicators of myofibroblast formation and fibroblastic phenotype shift, respectively, to assess the fibrotic state [47, 48]. Additionally, genes related to immune activation were included to characterize the inflammatory environment produced by each macrophage phenotype. These include IL-6, a cytokine involved in both pro- and anti-inflammatory signaling [70, 41], the anti-inflammatory cytokine IL-10 [40], CXCL2, a chemokine used to evaluate neutrophil recruitment pathways [32], and CD163 as a macrophage-specific marker [37]. TGF- β 1 and HSPB-1 was included to evaluate the regulation of ECM production and fibrosis, and as a marker of cellular stress, respectively [46, 71]. Finally, the housekeeping genes used for normalization were GusB and YWHAZ.

Table 1: Genes included in the gene expression analysis and their corresponding function.

Collagen	
COL1A1	Codes for collagen type I [14].
COL3A1	Codes for collagen type III [14].
Matrix Remodeling	
MMP-1	Codes for matrix degrading enzymes [34].
MMP-13	Codes for matrix degrading enzymes [34].
TIMP-1	Enzymatic inhibitor of MMPs [34].
Tenocyte Phenotype and Activation	
SCX	Tenocyte-associated transcription factor [24].
TNMD	Tendon-specific marker associated with tendon maturation [24].
ACTA2	Associated with myofibroblast formation [47].
S100a4	Associated with fibrotic tendon healing [48].
Immune Response and Cellular Stress	
IL-6	Associated with pro- and anti-inflammatory signaling [70, 41].
IL-10	Anti-inflammatory cytokine [40].
CXCL2	Macrophage-produced recruiter of neutrophils [32].
CD163	Macrophage-specific marker [37].
TGF- β 1	Associated with fibrosis and ECM regulation [46].
HSPB-1	Cellular stress marker [71].

2.5.3 Hematoxylin and Eosin Staining

Hematoxylin and eosin Staining (H&E staining) is a common histological approach used to assess tissue morphology. Hematoxylin stains the nucleus purple-blue and eosin stains the cytoplasm and ECM pink [72]. In tendon constructs, H&E staining gives an overview of cell distribution, cellular density, and the overall tissue organization including collagen structure and alignment. Stained sections can further be evaluated with histopathological grading systems such as Bonar Score, which is utilized in this project. Bonar score is a semi-quantitative grading system based on features such as collagen arrangement, collagen stainability, cellularity and cell morphology. The grades are from 0 to 3, reflecting normal to abnormal features [73]. The maximum possible Bonar score in this study is 12, and a higher score represents the presence of features related to tendinopathy.

3

Methods

To achieve the stated aims of this project, several methods were used to investigate how different macrophage phenotypes influence tendon construct properties in a 3D *in vitro* model. THP-1 monocytes were expanded and differentiated into M1 and M2 macrophages to establish the cross-talk with tenocytes. Primary human tendon cells were isolated and cultured before being incorporated into fibrin-based 3D tendon constructs, enabling the investigation of macrophage-tenocyte cross-talk in a 3D co-culture environment without direct cell-to-cell contact. To assess biomechanical properties, the constructs were analyzed using mechanical testing. RT-qPCR was used to evaluate gene expression related to tenocyte phenotype and activity, as well as ECM remodeling and immune responses. H&E staining was used to examine the construct morphology and collagen organization. This section includes brief explanations of the methods. The exact protocols as well as the formulations of the different media used can be found in Appendix A.

3.1 Experimental Setup

A schematic overview of the experimental design and analysis workflow is shown in Figure 3. The cells utilized were seeded and cultured approximately two weeks prior to the start of the experiments (day 0). Four donors were included in the final analysis, with six constructs formed for each donor and treatment. Of these, two to four constructs were used for mechanical testing, one of which was often the construct well called "extra". One construct was used for gene expression and one for histological staining and grading.

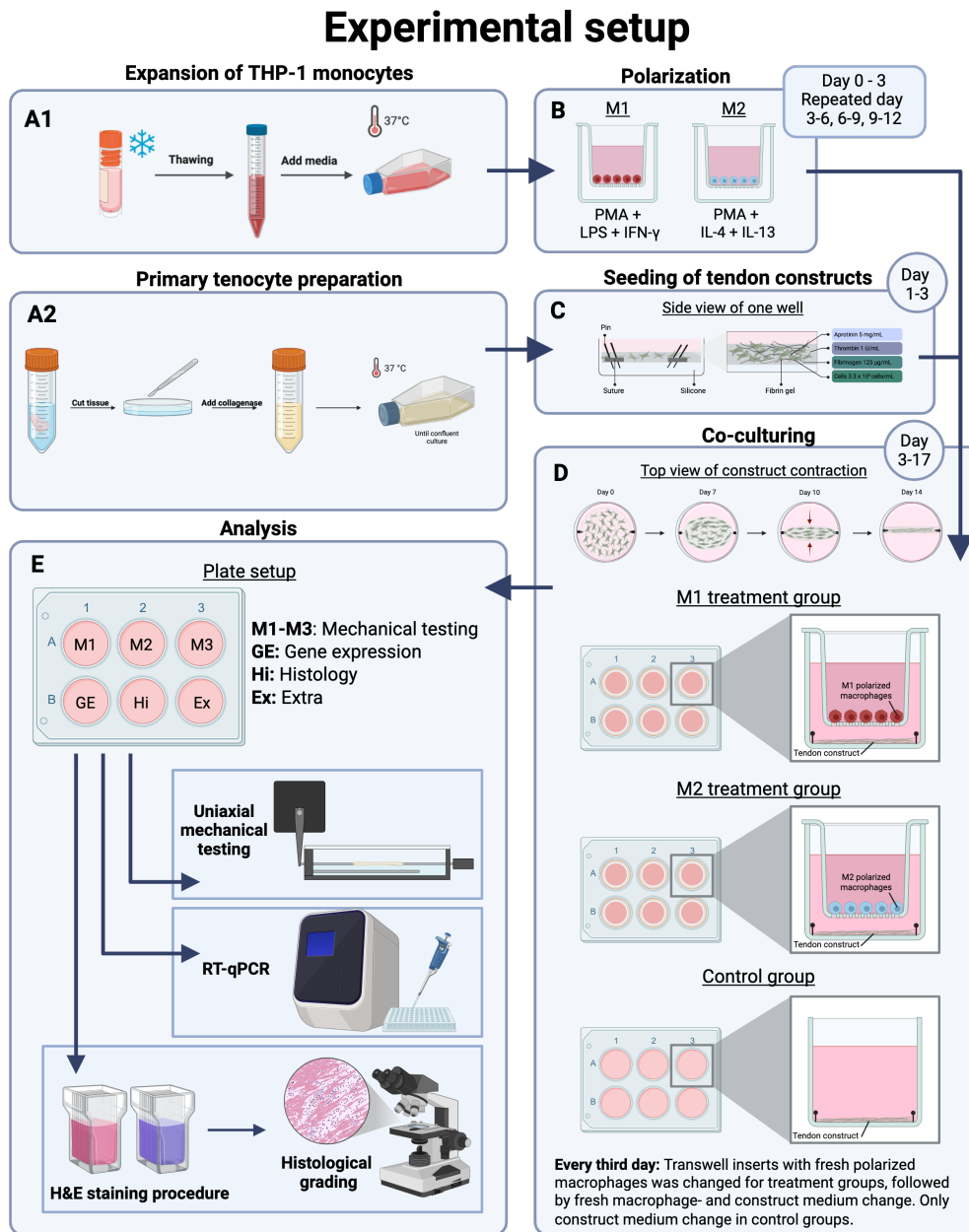


Figure 3: A schematic overview of the experimental setup performed for four donors. (A1) THP-1 monocytes were expanded and passaged as needed throughout the experiment. (A2) Donor tendon tissue was prepared, degraded, and cultured to confluency before tendon construct seeding. (B) Monocytes were differentiated into macrophages using PMA (day 0), polarized to M1 or M2 (day 2), and added to the co-culture at day 3. Fresh macrophages were generated every third day (days 3–6, 6–9, and 9–12). (C) Tendon constructs were seeded in 6-well plates with mix of cells, fibrinogen, thrombin, and aprotinin. (D) Tendon constructs were co-cultured with M1 or M2 macrophages, while controls received medium only. Medium and macrophages were replaced every third day during days 3–17. (E) Constructs were analyzed by uniaxial mechanical testing, RT-qPCR, and histological assessment using H&E staining and grading.

3.2 Monocyte Expansion, Macrophage Culture, and Differentiation

The macrophages used in this project were derived from the THP-1 monocyte cell line. Following monocyte expansion, THP-1 cells were seeded in Transwell inserts (pore size $0.4\ \mu\text{m}$, clear PET membrane (semipermeable), diameter 24 mm) at a density of 5×10^5 cells/mL. The culture medium used consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep), from now on referred to as macrophage media. Cells were treated with 50 nM phorbol 12-myristate 13-acetate (PMA) and incubated overnight to induce differentiation into macrophages. The cells were washed with phosphate buffered saline (PBS) and re-supplemented with media overnight.

Differentiation into M1 macrophages were induced by supplementing the medium with LPS (100 ng/mL) and IFN- γ (20 ng/mL). For M2 differentiation, the medium was supplemented with IL-4 (20 ng/mL) and IL-13 (20 ng/mL), followed by incubation overnight. Detailed protocols for expansion, subculturing of THP-1 cells and differentiation into M1 and M2 phenotypes are provided in Appendix A.2.1.

To allow paracrine cross-talk between macrophages and tenocytes in the 3D human tendon constructs, the Transwell inserts were placed above the constructs. The cell inserts were replaced every third day with freshly prepared cells. The initial addition of macrophages coincided with the loosening of the construct edges as described in Section 3.3.

3.3 Tenocyte Culture and Formation of Tendon Constructs

The preparation and formation of tendon constructs began with the isolation of primary tendon cells from tendon tissue obtained from eight donors. Due to experimental issues, data from four donors were excluded from further analysis, which is described in Section 5.4. Tissue samples were collected from the operating theater in PBS supplemented with 1% Pen-Strep. All visible non-tendon tissue was removed prior to cutting the tissue into smaller pieces with a scalpel, which then was transferred to tubes containing Dulbecco's Modified Eagle Medium (DMEM/F12) supplemented with 20% fetal bovine serum (FBS) and collagenase type II for digestion overnight. The following day, the samples were washed and resuspended in fresh DMEM/F12 medium supplemented with 10% FBS and 1% Pen-strep (from now on referred to as culture medium) before being seeded out in culture flasks. The culture medium was replaced the following day to remove debris, after which the cells were cultivated to confluency with medium changes every third day. Detailed protocols for extraction, culturing and maintenance of primary tendon cells are provided in Appendix A.3.

Upon reaching confluency, the tenocytes were trypsinized by adding trypsin for 5

minutes to detach the cells from the flasks surface, before transferred to Falcon tubes containing culture medium and centrifuged. The resulting pellet was resuspended in new medium, and cell counting was performed by mixing 10 μ L of cell suspension with 10 μ L trypan blue. Subsequently, 10 μ L of the mixture was loaded onto a cell counting chip. The cell concentration was set to 333 333 cells/mL by dilution with growth medium.

The artificial tendon constructs were formed in 6-well plates with 200 000 cells/well. This cell density was selected based on a previous optimization described by Bayer et al. [74]. The bottom of each well had been coated with approximately 3 mm of silicone (Dow SYLGAARD 184) and sutures were positioned 15 mm apart and secured with insect needles to act as anchoring points for the constructs. The plates were sterilized by submersion in ethanol for 1 hour, and washed twice with PBS. For each plate, six Eppendorf tubes were prepared. Aprotinin (5 mg/mL) and cells were added to each tube and mixed, followed by the addition of fibrinogen (125 μ g/mL) and thrombin (1 U/mL). The mixture was then immediately transferred to the corresponding well. After all wells were prepared, the plates were incubated for 1 hour before adding 3 mL of DMEM/F12 medium supplemented with 10% FBS, 1% Pen-Strep, 200 μ M ascorbic acid and 50 μ M L-proline (from now on referred to as construct media) to each well. A detailed protocol for the preparation of the wells and formation of artificial tendon constructs are provided in Appendix A.4.

After 48 hours, the construct medium was removed and the constructs edges were loosened from the well walls to allow contraction. Fresh construct medium was added, and the constructs were left to contract for 17 days. Every third day, the Transwell inserts containing macrophages were removed and the construct media was aspirated. Freshly polarized macrophages in new Transwell inserts, together with fresh macrophage media, were added above the constructs, followed by addition of fresh construct media to the wells.

Control constructs were grown from the same donors and maintained under the same experimental conditions as the treatment groups, with the exception of having no Transwell inserts and thus no addition of macrophage media. This allowed the evaluation of cross-talk between macrophages and tenocytes to be evaluated independently of biological variability between donors.

3.4 Gene Expression Analysis

To evaluate gene expression changes in response to the treatments, RT-qPCR was performed. RNA extraction was initiated by transferring the tendon construct to a tube with 1 mL TRIzol together with silicon carbide beads and stainless steel beads. The tissue was then homogenized using a bead beater, before adding 100 μ L 1-Bromo-3-chloropropane (BCP) and centrifuged for 15 minutes to ensure phase separation. The upper aqueous phase containing the RNA was transferred to a new tube with 4 μ L glycogen. An equal amount of isopropanol was then added to each sample to precipitate the RNA, followed by centrifugation and washing with ethanol solutions of different concentrations. After letting the pellet air-dry for 5 minutes, it was resuspended in 10 μ L RNase-free water. RNA concentration was then measured using the NanoDrop spectrophotometer. The detailed protocol for RNA extraction is provided in Appendix A.7. The RNA was then reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems and the Bio-Rad T100 Thermal Cycler.

The plate layout was designed without sample replicates, but included a standard curve with duplicate samples and a five-step 1:4 serial dilution along with a negative control. The CFX Opus 96 Real-Time PCR System from Bio-Rad was used for qPCR. The qPCR cycles program and detailed RT-qPCR protocol are provided in Appendix A.7. Primer sequences used for qPCR analysis targeted MMP-1, MMP-13, TIMP-1, COL3A1, SCX, ACTA2, S100A4, IL-6, CXCL2, HSPB1, IL-10, CD163, and TGF- β 1. COL1A1 and TNMD was not included in the analysis due to technical issues with the primer and gene expression below cycle threshold.

3.5 Mechanical Testing

Mechanical testing was performed using the *UniVerts S - Universal Uniaxial Mechanical Tester*. The sutures of each construct was glued between two sandpapers before it was mounted in the machine. A moist gauze was used during this time to prevent the construct from drying. The assembled constructs were subsequently mounted in the testing machine, and the test was initiated at a displacement of 4 mm/min until failure. A detailed protocol for the mechanical testing procedure is provided in Appendix A.6. In Figure 4 are representative images integrated onto a graph at selected time points to visualize changes in the tendon constructs throughout the testing process. Images are taken every 0.2 second during testing and was used for measurements of the CSA using ImageJ.

Force (N) vs. Displacement (mm) Curve

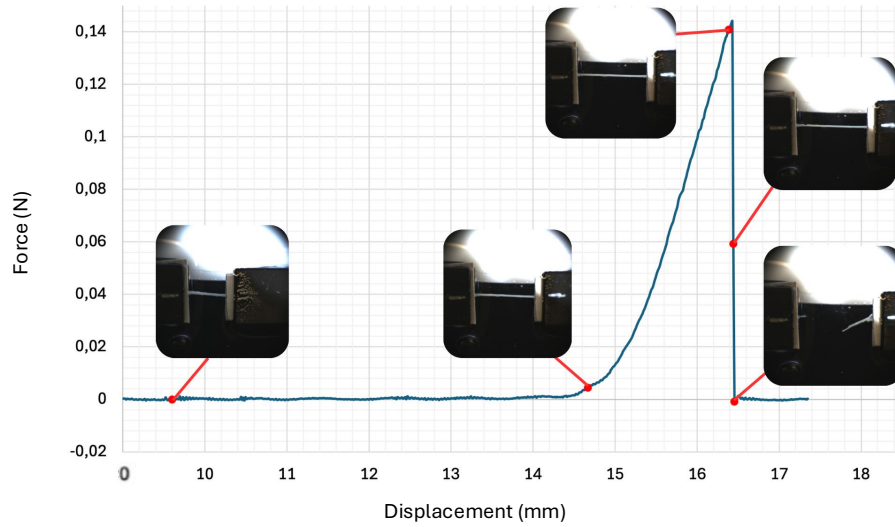


Figure 4: Force (N) - displacement(mm) curve with representative images at selected time points illustrating changes of the tendon construct during mechanical testing and the corresponding effect on the force-displacement curve.

3.6 H&E Staining

Prior to staining, the constructs were submerged into an optimal cutting temperature (OCT) embedding matrix and frozen in liquid nitrogen. The samples were sectioned into 8 μm slices using a cryostat and mounted onto glass microscope slides. H&E staining was initiated by air-drying the sections at room temperature for 15 minutes, followed by fixation in 4% formaldehyde for 3 minutes. The sections were then washed under running water, and stained with Harris hematoxylin, followed by an additional wash with water and differentiation in acid alcohol. After an additional wash under running water, dehydration was performed by sequential immersion in 70%, 95% and 100% ethanol, followed by two washes in xylene. The slides were then mounted using ProLong Gold Antifade Mountant, followed by imaging. A detailed protocol for H&E staining can be found in Appendix A.8. The images were subsequently histologically graded using a modified scoring approach based on the histopathological grading system Bonar Score, which is used for evaluating tendinopathy. It was performed by visually evaluating the histological sections and assigning each sample a score by comparison to reference images. The scoring was based on four parameters: collagen arrangement, collagen stainability, cellularity, and cell morphology. Each histological section was scored independently by both students. For each sample, the scores assigned by the two students were averaged for each parameter, and the resulting parameter scores were then averaged to obtain a final score for each donor and condition. The reference images used for scoring are shown in Figure B.1 in Appendix B. The scoring was not performed blinded, as

the filenames contained information about the constructs (control, M1, and M2).

3.7 Data Analysis and Processing

Mechanical testing data was analyzed using the UniVert data from CellScale and included two to four technical replicates per donor and treatment. Technical replicates were first averaged, and donor means were subsequently used for statistical analysis. During testing, the software continuously recorded force and displacement data, which were converted into stress-strain curves from the manually entered length and diameter. The dimensions of each sample were determined from the recording during the mechanical testing. Sample dimensions, area and gauge length, were measured using ImageJ. A length of 12 mm was selected as the reference length for measurements of the constructs. Young's modulus was calculated by the testing software by manually choosing two points on the linear part of the slope on the obtained stress-strain curve, as seen in Equation 1 in Section 2.5.1. Stiffness for each sample was calculated using the value on Young's modulus, CSA and the initial length, using Equation 2.

For the gene analysis, one technical replicate per donor and condition was analyzed and donor averages were used for statistical analysis. Genes where a majority of the Ct values exceeded 35 were excluded from the analysis. Several reference genes were assessed to evaluate which gene to normalize against. YWHAZ and GusB was selected by using RefFinder, a tool that assists in choosing what reference genes to use. The standard curve was based on human reference RNA. The Sq values of each gene obtained from the standard curve was normalized against the Sq average of the two to adjust for variations in initial sample RNA amount and pipetting. For the histological grading, scores were averaged between the two students for each parameter, and these means were further averaged to produce a final total score per donor and treatment, which was used in the statistical analysis.

Statistical analysis and graph generation for mechanical testing, gene expression analysis, and histology was performed using GraphPad Prism. Under the assumption of a non-parametric distribution and a limited sample size of the datasets, non-parametric statistical analyses were applied throughout the study. All data were analyzed using a Friedman test followed by a Dunn's uncorrected post hoc test to account for repeated donor measurements and to preserve statistical power in the limited sample size.

3.8 Ethical Aspects

This project utilized pseudonymous tendon tissue obtained from surgical waste collected during ACL surgery from consenting donors, with approval from the Ethical Review Authority in Sweden (2024-04117-02). All handling of human material followed Biosafety Level 2 standards. The current ethical approvals fully cover the use of the de-identified human tissue.

Musculoskeletal disorders, such as tendon injuries, represent a significant societal challenge affecting individuals as well as bringing burden to the healthcare system. The injuries are often associated with prolonged pain, long rehabilitation periods, and remaining impaired function, which can lead to increased societal costs due to sick leave, long-term treatments, and decreased work capacity. This project contributed to increased understanding of the underlying mechanisms of tendon healing, with the focus on the interaction between macrophages and tenocytes. Improved healing outcomes could reduce the risk of chronic tendon disorders and re-tears, thereby decreasing the long-term burden on healthcare systems.

The project used an established THP-1 monocyte cell line, which allowed for controlled and reproducible experiments while reducing the ethical concern related to repeated human sampling. Furthermore, this project was conducted as an *in vitro* laboratory study and therefore had limited direct environmental impact. By using a 3D human tendon model, the need for animal experiments was reduced, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement). One ethical aspect that had to be considered was the use of FBS in the cell culture media. It is considered inhumane, as it is taken from bovine fetuses during slaughter, and it is assumed to be painful for the fetuses [75]. However, as of now, it is the option that allowed for tendon construct formation, and is considered the best alternative for mammalian cell cultures [76]. The project included the use of antibiotics, which was necessary to use in this model to minimize contamination as it requires extensive handling. However, the amounts of antibiotics used were small. Additionally, there have been a substantial use of disposable glass and plastic supplies. It was necessary due to the importance of sterility, and all experiments were carried out with established laboratory guidelines, including proper chemical and waste handling and disposal.

4

Results

Results are presented across the following four sections covering mechanical testing, gene expression, histological staining, and morphological observations.

4.1 Mechanical Testing

Results for the mechanical testing are presented in Figures 5 and 6

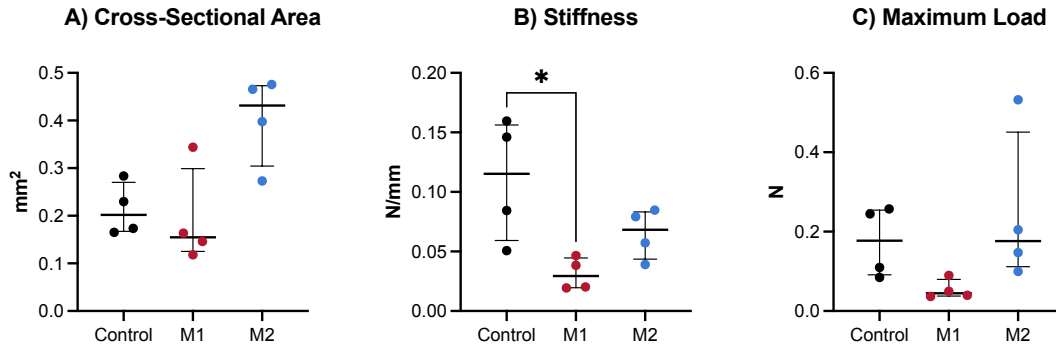


Figure 5: Results of sample dimensions and structural parameters from mechanical testing. Each data point represents an individual donor, with the plotted value corresponding to the mean of two to four replicate measurements for that donor. (A) Cross-sectional area (mm²), exhibiting no significant differences between groups and control. (B) Structural stiffness (N/mm), showing a significant reduction in the M1 group compared to the control. (C) Maximum load (N), demonstrating no significant differences. Statistical significance is indicated by asterisks (* $p < 0.05$).

M1-stimulated constructs showed significantly reduced stiffness compared to control ($p = 0.0133$), with a median of 0.0294 N/mm (range 0.0194–0.0465 N/mm) versus 0.115 N/mm (range 0.0508–0.160 N/mm). M2-stimulated constructs, by contrast, were not significantly different from control in stiffness ($p = 0.480$), with the median 0.0682 N/mm and range 0.0390–0.0847 N/mm. None of the treatment groups differed significantly from control in CSA or maximum load. Median maximum load was 0.0450 N ($p = 0.0771$, range 0.0370–0.090 N) for M1 and 0.176 N ($p = 0.724$, range 0.100–0.533 N) for M2, compared to a median of 0.178 N in the control group,

with values ranging between 0.0850–0.258 N. Median CSA was 0.202 mm² for control, 0.155 mm² for M1, and 0.432 mm² for M2. CSA ranged from 0.165–0.284 mm² in the control group, 0.118–0.344 mm² in the M1 group, and 0.273–0.476 mm² in the M2 group. The comparison with control showed $p = 0.724$ for M1 and $p = 0.158$ for M2.

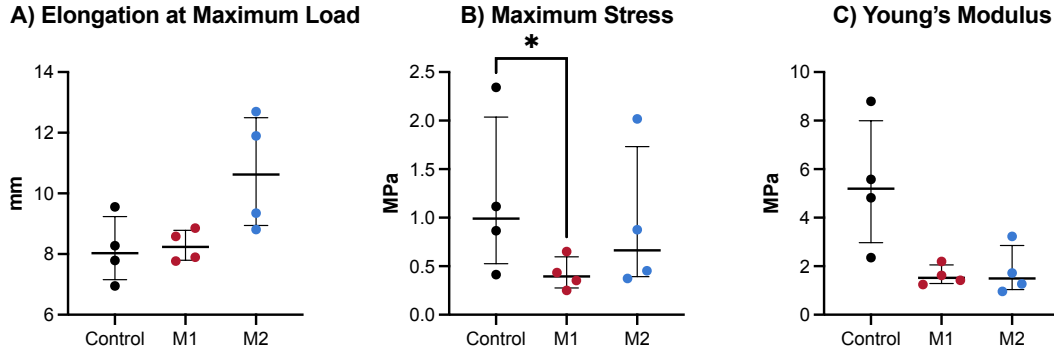


Figure 6: Evaluation of intrinsic biomechanical properties derived from mechanical testing. Each data point represents an individual donor, with the plotted value corresponding to the mean of two to four replicate measurements for that donor. (A) Elongation at peak load (mm), showing no significant differences between the treatment groups and control. (B) Maximum stress (MPa), demonstrating a statistically significant reduction in the M1 group compared to control. (C) Young's Modulus (MPa), showing no significant differences between M1 or M2 and control. Asterisks denote statistically significant differences (* $p < 0.05$).

Only M1 differed significantly from control in maximum stress ($p = 0.0339$), with a median maximum stress of 0.394 MPa (range 0.250–0.650 MPa), compared to 0.990 MPa for control (range 0.413–2.34 MPa). Median maximum stress was 0.664 MPa for M2, ranging between 0.373–2.02 MPa, with $p = 0.289$. Elongation at maximum load and Young's modulus were comparable between treatment groups and control. The M1- and M2 group had a median elongation at peak load of 8.24 mm (range 7.77–8.85 mm) and 10.62 mm (range 8.81–12.7 mm), respectively, both with $p = 0.289$. This compared to median 8.03 mm for control (range 6.95–9.56 mm). The M1 group demonstrated a median Young's modulus of 1.52 MPa, with values ranging from 1.24 to 2.20 MPa, with $p = 0.157$. The M2 group ranged from 0.960 to 3.23 MPa, with a median value of 1.49 MPa and $p = 0.0771$. Control demonstrated a median Young's modulus of 5.12 MPa (range 2.35–8.78 MPa).

4.2 Gene Expression Analysis

The results from the gene expression analysis is presented in the figures below.

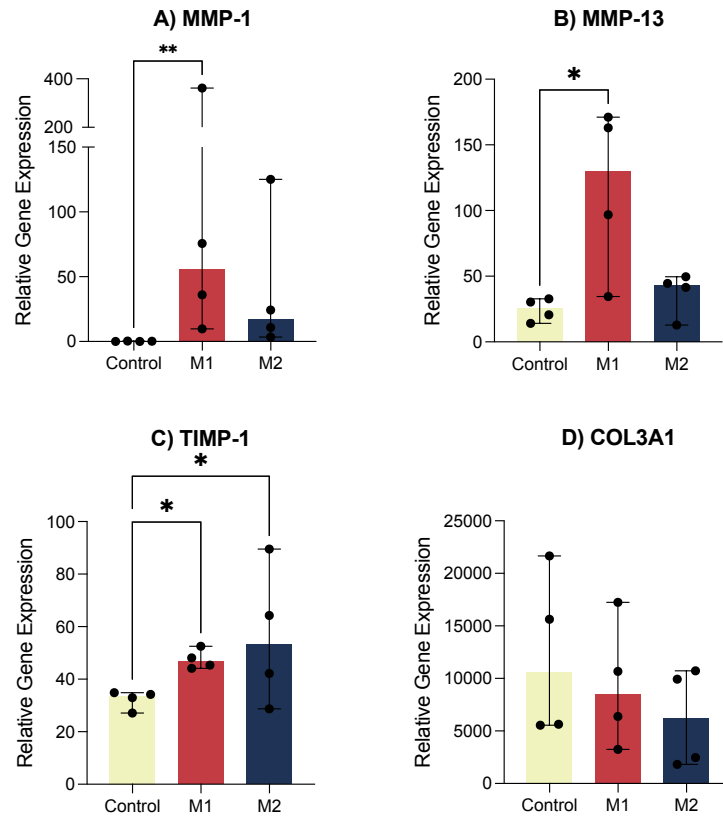


Figure 7: Gene expression data for ECM remodeling genes and collagen type III. Individual data points represent a single donor, evaluated in replicates of one. (A) MMP-1, exhibiting a significant increase in gene expression for M1 compared to control. (B) MMP-13, showing a significant increase in gene expression for M1 compared to control. (C) TIMP-1, demonstrating a significant increase in gene expression for both the M1 and M2 groups compared to the control. (D) COL3A1, demonstrating no significant differences between the treatment groups and the control. Asterisks denote statistically significant differences (* $p < 0.05$, ** $p < 0.01$).

The mRNA levels for MMP-1 was significantly increased in the M1 group ($p = 0.00470$) compared to the control, whilst an insignificant increase smaller than M1 was observed for M2 ($p = 0.157$). MMP-1 displayed a median relative expression of approximately 56.0 in the M1 group, with considerable inter-donor variability, including one donor exhibiting an approximately 360-fold increase, compared with near-baseline expression in the control group. MMP-13 expression was significantly increased in the M1 group ($p = 0.0339$), with a median relative expression of approximately 130 compared to approximately 25.5 in the control group. No significant difference in MMP-13 expression was observed in the M2 group relative to control ($p = 0.289$). Both the M1 and M2 groups exhibited a significant increase in TIMP-1

expression ($p = 0.0339$ for both), with median expression values of 46.7 and 53.2 for M1 and M2, respectively, compared to 33.6 for control. No significant difference in the expression of COL3A1 was demonstrated for M2 or M1 compared to control ($p = 0.0771$ and $p = 0.724$, respectively), control had a median expression value of 10 633, whilst M1 had a median of 8531 and M2 a median expression of 6203, respectively (Figure 7).

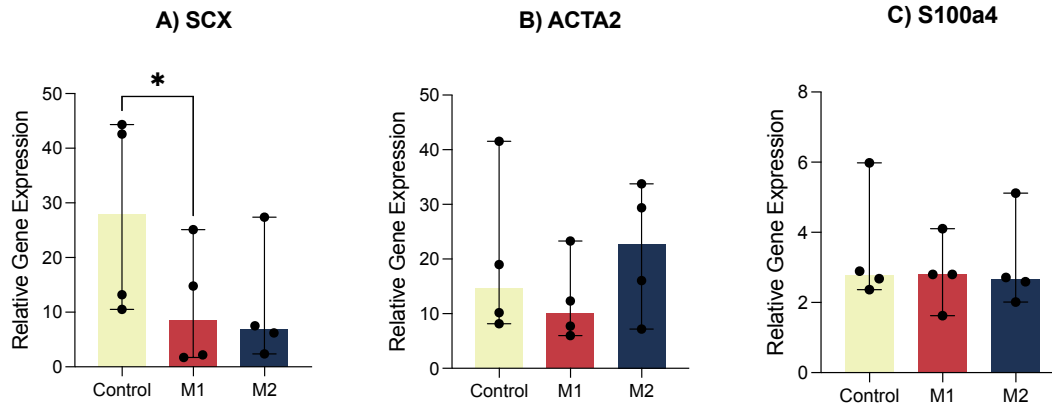


Figure 8: Gene expression of genes related to tenocyte phenotype and activation. Individual data points represent a single donor, evaluated in replicates of one. (A) SCX, demonstrating a significant decrease in expression for M1 compared to control. (B) ACTA2, showing no significant difference between M1 or M2 group and control, and (C) S100a4, demonstrating no significant differences between the treatment groups and control. Asterisks denote statistically significant differences (* $p < 0.05$).

SCX showed a significant decrease in expression in the M1 group relative to control ($p = 0.0133$), alongside a non-significant trend toward reduced expression in the M2 group ($p = 0.0771$), with the control group displaying the highest inter-donor variability. Median relative expression was approximately 28.0, 8.50, and 6.90 for the control, M1, and M2 group, respectively. For ACTA2, no significant differences were demonstrated between the M1 group ($p = 0.0771$) or the M2 group ($p = 0.724$) and the control. A trend toward elevated ACTA2 expression was observed in M2-stimulated constructs compared with the control and M1 group. No significant differences between M1 or M2 compared to control in S100a4 expression were demonstrated ($p = 0.0724$ and $p = 0.470$, respectively), with medians across all groups ranging from approximately 2.70 to 2.80 (Figure 8).

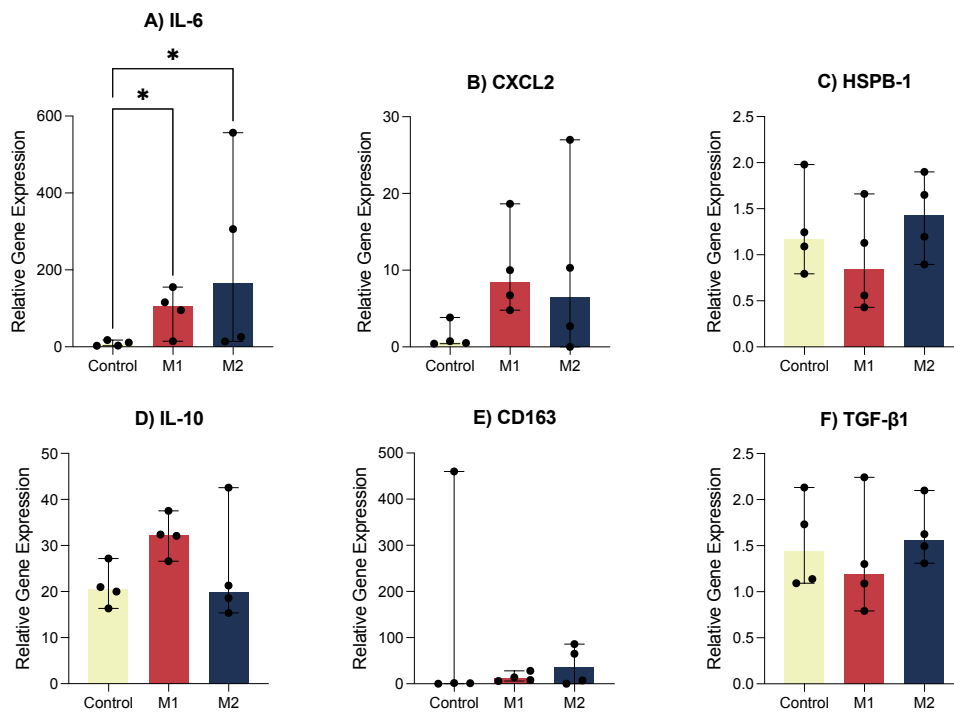


Figure 9: Results in gene expression for immune- and cellular stress-related genes. Individual data points represent a single donor, evaluated in replicates of one. (A) IL-6, demonstrating a significant increase in gene expression for both the M1 and M2 groups compared to the control. No significant differences are demonstrated between neither of the stimuli groups compared to control for (B) CXCL2, (C) HSPB-1, (D) IL-10, (E) CD163, and (F) TGF- β 1. Asterisks denote statistically significant differences (* $p < 0.05$)

Analysis of immune- and cellular stress-related gene expression demonstrated a significant increase in IL-6 expression for both M1 and M2 ($p = 0.0339$ for both groups) compared to control (Figure 9). Median relative expression was 105 and 166 for M1 and M2, respectively, and 7.10 for the control group. The expression level for CXCL2 was close to baseline for the majority of data points in the control group, whilst the M2 group had major inter-donor variability, with gene expression values ranging from 0 to approximately 27.0. Both the M1 and M2 group showed an insignificant trend towards increased CXCL2 expression ($p = 0.0771$ and $p = 0.724$, respectively) compared to control. The expression of HSPB-1 showed a trend of slightly decreased values for M1 ($p = 0.157$) and slightly increased values for M2 ($p = 0.734$) compared to control, with median expression values for control, M1, and M2 of approximately 1.17, 0.845, and 1.42, respectively.

IL-10, while insignificant, showed a trend towards increased expression for M1 and minimally decreased levels for M2 relative to control, with $p = 0.480$ for both. Major inter-donor variability was demonstrated for the control group in the relative gene expression of CD163, with expression values varying from 0 to approximately 560. Similarly to the expression of IL-6, the levels was slightly higher for M2 than M1,

with $p = 0.480$ for M1 and $p = 0.724$ for M2. mRNA levels was similar for all groups in TGF- $\beta 1$ ($p = 0.289$ for M1, and $p > 0.999$ for M2), with median values of 1.43, 1.20, and 1.56 for control, M1, and M2, respectively.

4.3 H&E Staining

Representative images of the H&E staining are shown at 4x magnification in Figure 10, 12, and 14 and at 10x magnification in Figure 11, 13, and 15.

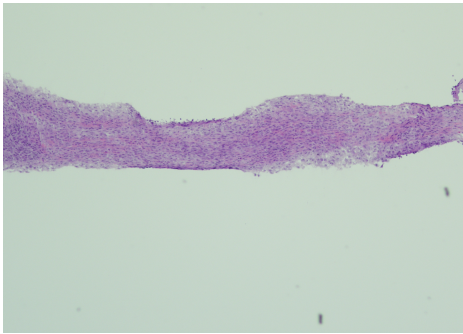


Figure 10: Control construct from donor 2 that is stained with H&E staining and imaged at 4x magnification.

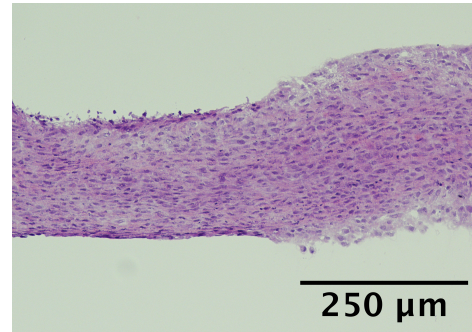


Figure 11: Control from donor 2 that is stained with H&E staining and imaged at 10x magnification.

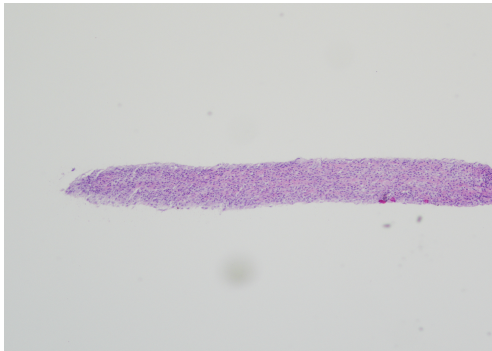


Figure 12: M1-construct from donor 2 that is stained with H&E staining and imaged at 4x magnification.

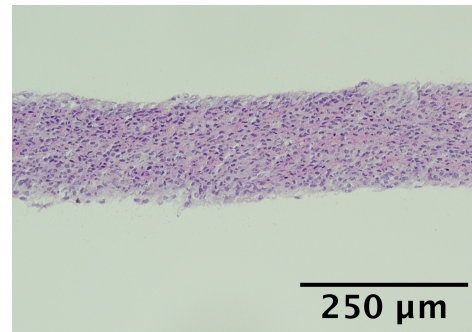


Figure 13: M1-construct from donor 2 that is stained with H&E staining and imaged at 10x magnification.

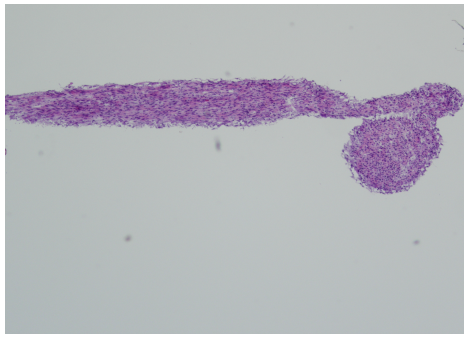


Figure 14: M2-construct from donor 2 that is stained with H&E staining and imaged at 4x magnification.

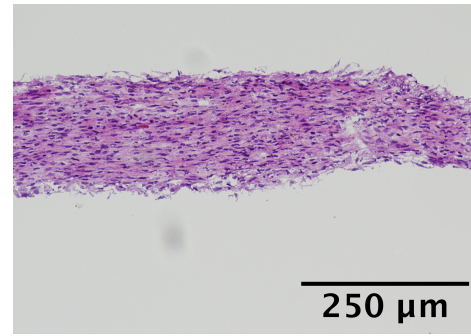


Figure 15: M2-construct from donor 2 that is stained with H&E staining and imaged at 10x magnification.

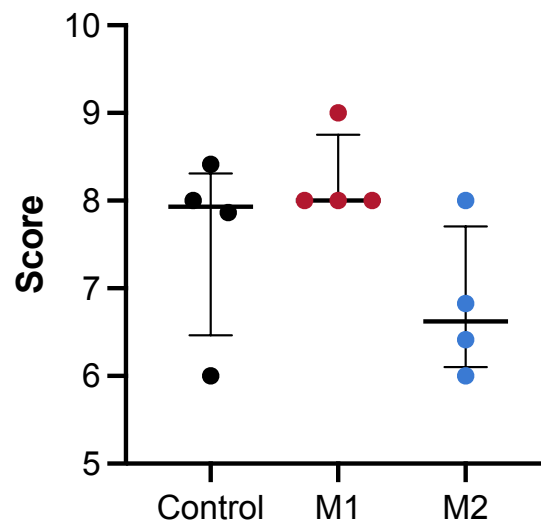


Figure 16: Total histological scoring for the control, M1, and M2 groups, with no statistically significant differences observed between the stimuli groups.

The maximum possible score was 12. The grading showed relatively high scores for both the control and M1-treated constructs, with mean scores of approximately 7.57 and 8.25 respectively (Figure 16). The M2-treated constructs displayed a lower mean score of approximately 6.81. No statistically significant differences were observed between the groups. The chart used for grading, as well as representative donors with their corresponding scores, are shown in Figures B.1 and B.2.

4.4 Morphological and Contraction Differences

Differences in construct contraction and morphology were observed between the groups throughout the experiments. M1-treated constructs contracted more rapidly and developed a thinner morphology compared to the other groups. In some cases, M1-treated constructs appeared to be fully contracted in approximately 7 days. The M2-treated constructs showed slower contraction and maintained a thicker morphology. Figure 17 shows representative images of constructs from donor 3 at day 17 to show the visual differences in morphology and contraction between the treatment groups.

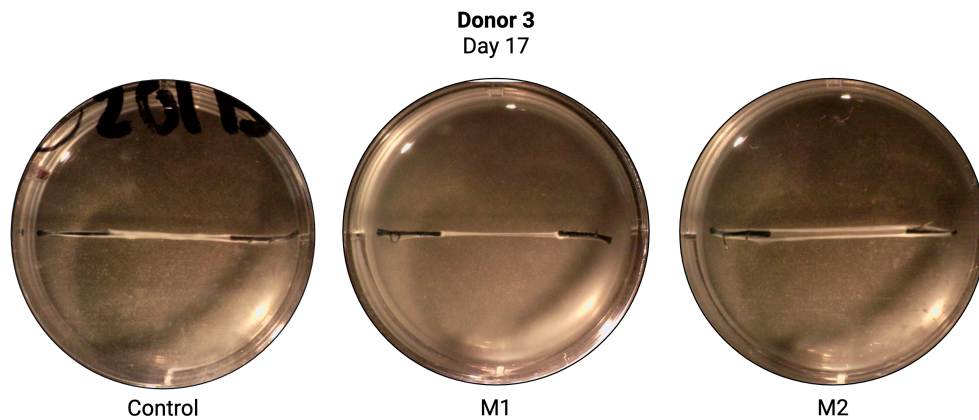


Figure 17: Constructs from donor 3 at day 17 with control, M1-treated and M2-treated constructs from left to right. Visual morphological and contraction differences can be observed between the groups. M1-treated constructs appear thinner compared to control- and M2-treated constructs.

The contraction behavior and morphology of the M2-constructs were comparable to those in the control group and generally followed a more expected contraction time of approximately 14 days. The total culture period was extended from 14 to 17 days due to slower contraction observed in constructs for some donors. These observations were not part of the original master's thesis aims and were therefore assessed through visual inspections only. Consequently, no quantitative measurements or statistical analyses of construct contraction were performed.

5

Discussion

Macrophages are known to play an important role in tendon healing and remodeling [5]. However, how different macrophage phenotypes influence tenocyte behavior remains poorly understood [6]. This study therefore investigated the effects of M1 and M2 macrophage phenotypes on biomechanical properties, gene expression, histological characteristics, and contraction behavior in a 3D *in vitro* tendon construct model. Overall, macrophage phenotype was found to influence both mechanical and biological properties of the constructs. M1-treated constructs exhibited reduced mechanical strength, faster contraction, and altered gene expression profiles characterized by increased MMP-1, MMP-13, TIMP-1, and IL-6 expression, and reduced SCX expression, together with insignificantly higher histological scores compared to control. In contrast, M2-treated constructs showed fewer deviations from control, with preserved mechanical properties and a gene expression profile more similar to the control group.

These findings provide a basis for the following discussion of how macrophage phenotype may regulate tendon construct remodeling and tenocyte behavior.

5.1 Mechanical Testing

This study investigated how M1 and M2 macrophage phenotypes influence the biomechanical properties of 3D human tendon constructs through paracrine signaling, using uniaxial tensile testing as the primary readout.

The most pronounced mechanical differences were observed in constructs co-cultured with M1 macrophages, which showed significantly reduced stiffness and maximum stress compared to control, as observed in Figures 5 and 6. This suggests that the pro-inflammatory environment created by M1 macrophages compromises the overall structural integrity of the constructs, reducing their capacity to resist and bear loads. These findings are consistent with the established role of M1 macrophages in promoting a catabolic environment during the early phase of healing, where excessive M1 activation is known to alter collagen synthesis and promote ECM degradation [5, 39]. It was also observed that constructs stimulated with M1 macrophages contracted considerably faster and formed visibly thinner constructs compared to both M2 and control groups (Figure 17). A likely contributing mechanism is the upregulation of

MMPs driven by pro-inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 [77]. MMPs facilitate the degradation of ECM components including collagen, and their elevated activity may have resulted in a loss of matrix integrity. Excessive ECM remodeling, combined with overproduction of inflammatory proteins and secretion of matrix-degrading enzymes, may result in a weaker and less organized collagen matrix [78, 79], resembling the degenerative changes observed in tendinopathy [25]. This likely contributed to the thinner morphology and inferior mechanical properties observed in M1 constructs, suggesting that the rapid contraction represents excessive and disorganized remodeling rather than functional tissue remodeling.

M2-stimulated constructs showed no significant differences from control in any of the mechanical parameters and closely resembled controls in overall morphology and contraction behavior, suggesting that M2 macrophage signaling supports a more regenerative environment that does not substantially disrupt normal matrix remodeling [39]. Although insignificant, an increased elongation at maximum load is observed for M2 constructs compared to both control and M1 (Figure 6). This may reflect that constructs subjected to M2-induced environments creates a more compliant structure that allows for larger deformations, commonly associated with collagen type III deposition during the proliferative phase in healing [27].

Both M1- and M2-stimulated constructs had a reduced Young's modulus relative to the control group (Figure 6), although insignificant. This despite their markedly different effects on properties such as maximum stress and stiffness. This dissociation between both material and structural properties requires careful consideration, as it suggests that M1 and M2 macrophages may share a common effect on intrinsic matrix quality while differing in their broader influence on construct integrity. Young's modulus, as a geometry-independent material property, reflects the stiffness of the material itself rather than the amount or dimensions of the construct, and is highly dependent on collagen fiber alignment and organization [80]. A reduced Young's modulus in both treatment groups therefore may suggest that macrophage paracrine signaling, regardless of phenotype, alters collagen organization, density, or cross-linking in the constructs. However, the underlying mechanisms likely differ between the two phenotypes. In the M1 condition, ECM degradation mediated by MMP activity [81] may disrupt collagen architecture, resulting in a less organized matrix. In the M2 condition, a possible explanation is increased synthesis of collagen III and other ECM components, as M2 macrophages are associated with ECM deposition [82] and a previous study reported a reduction in Young's modulus in response to an increased COL3:COL1 ratio [83]. This would be consistent with a shift toward a more remodeling or fibrotic state, which is further discussed in Section 5.2.

The fact that M2 constructs had a reduced material stiffness while maintaining load-bearing capacity comparable to control is interesting. It raises the possibility that M2 signaling promotes matrix remodeling and turnover in a way that only temporarily reduces material stiffness without compromising the overall load-bearing architecture of the construct, perhaps reflecting an early remodeling state in which the matrix is being reorganized rather than degraded. This stands in contrast to

the M1 environment, where remodeling appears to come at the expense of structural integrity, likely due to the smaller CSA observed in M1-stimulated constructs attributed to the potential matrix degradation. M2-stimulated constructs demonstrates larger CSAs (Figure 5), further suggesting that M2 macrophages stimulates tenocytes to produce substantial amounts of ECM. However, as previously mentioned, this synthesized ECM is characteristic of an early remodeling state resulting in a immature and more compliant tissue. But this remains speculative as no significant differences was demonstrated. This may reflect a true lack of biological effect, or alternatively be attributable to poor statistical power resulting from the limited sample size, which reduces the ability to detect true differences between groups. It is also worth noting that the CSA may potentially be overestimated for a few replicates in the M2 and control groups, which might have influenced the results.

Given the relatively short culture period and the absence of mechanical stimulation during construct formation, it is also possible that the reduced material stiffness in both groups partly reflects a general immaturity of the matrix that is further compounded by macrophage signaling, rather than a phenotype-specific biological effect. However, these findings could have relevance in the context of chronic tendinopathy, which is associated with prolonged inflammatory signaling, and the characteristics such as ECM disorganization and impaired mechanical properties are associated with degenerative tendon tissue [2, 25].

5.2 Gene Expression Analysis

To investigate the molecular effects of macrophage paracrine signaling on tenocyte behavior and ECM remodeling, gene expression analysis was performed on tendon constructs co-cultured with M1 or M2 macrophages and compared with unstimulated control constructs.

The most remarkable findings found was for constructs stimulated with M1 macrophages. Evaluation of expression levels demonstrated a significant increase in both MMP-1 and MMP-13 (Figure 7). This follows the established role of M1 macrophage signaling elevating the expression of MMPs via the secretion of pro-inflammatory cytokines, which contributes to ECM degradation [5]. In contrast, both treatment groups demonstrated a significant increase in the expression of TIMP-1, an inhibitor of MMPs [34]. For the M1 group, the elevated TIMP-1 expression is likely a cellular effort to limit excessive matrix degradation caused by the high MMP levels. In M2 constructs, the upregulation of TIMP-1 occurred without a corresponding increase in MMPs, suggesting a more protective environment favoring matrix preservation as a response to M2 signaling. This was accompanied with a reduction in SCX expression in both the M1 and M2 group (Figure 8), though this decrease was only statistically significant for M1. SCX is a transcription marker related to tendons and is considered essential for mature tendon repair [24, 84]. Consequently, the significant downregulation of SCX suggests a change in tenocyte phenotype, implying that the pro-inflammatory M1 environment may drive the cells toward a less mature or de-differentiated state. Moreover, a trend of

decreased COL3A1 expression was found for both M1 and M2 relative to control, where the M2 group demonstrated the lowest expression levels, this suggests that both macrophage phenotypes minimally reduce collagen type III production. The uniform expression of S100a4 and ACTA2 across all groups suggests that the environment do not push the tenocytes towards a fibrotic phenotype, as S100a4 and ACTA2 activation is associated as drivers of fibrotic healing and myofibroblast formation [48, 47]. All this combined, these results indicate that M1-treated constructs exhibit a matrix environment dominated by active remodeling and degradation, with some evidence of decreased maturation, suggesting an overall dysregulated rather than an entirely fibrotic tissue response.

Moreover, the expression of IL-6 was significantly upregulated in both the M1 and M2 group compared to control (Figure 9). IL-6 is considered a cytokine with dual roles depending on the signaling environment. *In vivo*, IL-6 has been proposed to act as a driver of structural adaptations within the ECM [41]. Additionally, studies on tendon-derived stem cells suggest that IL-6 can improve cellular proliferation while simultaneously suppressing tenogenic differentiation pathways [70]. In the context of this study, the widespread upregulation of IL-6 across both groups likely reflects its complex role. In the M1 environment, this signaling may work alongside the elevated MMPs to drive matrix turnover while pushing the tenocytes toward a changed phenotype, whereas in the M2 environment it might influence structural matrix remodeling without triggering a catabolic cascade.

None of the other immune related genes differed significantly in neither of the treatment groups (Figure 9). An interesting finding is that IL-10, commonly associated with M2 signaling [40], is more expressed for the M1-stimulated constructs. This suggests that macrophage phenotypes exist along a dynamic spectrum, and that specific secretion profiles are not necessarily limited to one phenotype in 3D tendon constructs. It could also reflect the dynamic nature of macrophage polarization or the influence of the tenocyte environment on macrophage behavior. It is further worth to note that the division of macrophages into M1 and M2 phenotypes is sometimes regarded as a highly simplified system, and that macrophage polarization being in a binary state does not reflect the complexity of biological systems [85, 86]. An additional interesting trend is the increased expression of CXCL2 in the M1 group compared to control. Although this did not reach significance, it might still imply that there is a hidden biological effect. CXCL2 is an inflammatory marker produced by macrophages, and facilitates the recruitment of neutrophils [32]. This combined with moderate levels of S100a4 further suggests that M1 signaling puts tenocyte in a more acute inflammatory phase, rather than reflecting a fibrotic state. A similar increase in CXCL2 was also observed in the M2 group, further supporting the idea that macrophage-signaling is not strictly phenotype-specific and may overlap between treatment groups in this 3D tendon environment. The implication that the tenocytes were not pushed towards a fibrotic phenotypes is further supported by the moderate levels of TGF- β 1 across all groups [46]. HSPB-1 was evaluated to assess if the different macrophages induced cellular stress [71], and the uniform expression of the gene across all three groups implies that the cells were not subjected to any stress that caused enhanced induction of this heat shock protein in specific.

The detection of CD163 expression within the constructs were not expected, as it is highly specific to macrophages [37] and in this experimental model macrophages are not supposed to reside in the tendon constructs. This may indicate some technical error, such as contamination or leaking Transwell inserts during the co-culture allowing migration of macrophages into the constructs. Alternatively, resident macrophages may have been present within the primary human tissue used to isolate the tenocytes. However, since all data points in the control group except one is near zero, macrophage migration from the inserts appears to be the more likely explanation. The outlier in the control group (Figure 9) is probably attributed to a technical artifact during qPCR preparation. Regardless of source, the presence of macrophages directly inside the constructs may have influenced the biological outcome of the study, this is further discussed in Section 5.4.

The significant findings for M1 highly connects to the significant decrease in major mechanical metrics for M1, specifically structural stiffness and maximum load. The M1-environment trigger a collapse in the ECM, driven by the upregulation of MMP-1 and MMP-13, ultimately leading to weaker and softer constructs. On the other hand, the M2 environment drove a different outcome, with no significant differences in any of the mechanical parameters evaluated. This lack of mechanical change matches the trends observed in the gene expression markers, with only increased expression of IL-6 and TIMP-1 without elevated MMP levels. Instead of breaking down the matrix, the M2 environment appears to maintain matrix stability, allowing the constructs to better preserve their structural integrity without the major mechanical decline seen in the M1 group.

An additional meaningful insight would be the analysis of COL1A1 expression to evaluate the ratio between collagen type I and III to back up hypotheses established from the mechanical testing data. However, due to several reasons this was not achieved, this is further discussed in Section 5.4.

5.3 Histological Staining

The use of H&E staining enabled visualization of the overall tissue morphology and cellular distribution within the constructs. At 4x magnification, all constructs displayed a continuous tissue-like structure which indicated successful formation of the engineered tendon tissue. Hematoxylin stained the cell nuclei dark blue, confirming the presence and survival of tenocytes throughout the construct, while eosin stained the cytoplasm and ECM pink. The cells appeared relatively evenly distributed.

At 10x magnification, the constructs demonstrated a dense cellular distribution with slightly elongated nuclei embedded within a pink-stained matrix, which indicates collagen deposition and active ECM formation. Despite the successful formation of early tendon-like tissue, the collagen organization appeared immature and lacked the highly aligned fibrillar collagen characteristics of native healthy tendon tissue.

The histological grading was based on four features, and had a maximum score of 12. Higher scores indicate a less organized extracellular matrix. Relatively high scores were observed across all treatment groups, especially in the control and M1-treated constructs. All groups showed similar cellularity and relatively comparable collagen stainability. Even though the highly aligned fibrillar characteristics could not be observed, signs of early alignment could be seen, and differences in scores were primarily associated with variations in early collagen arrangement and cell morphology, even though the overall differences did not vary a lot. The lower scores observed in the M2-treated constructs suggests a more regulated remodeling response with better tissue organization, which is consistent with the overall morphology, contraction behavior and gene expression results, which all more closely resembled the control group. However, no statistically significant differences were observed.

These results suggest that the constructs more closely resembled early remodeling and exhibited a less functional tenocyte phenotype. The immature collagen organization may be explained by the relatively short culture period. Tendon maturation, ECM alignment and collagen I replacing collagen III, are a process that require several months [27]. In addition, mechanical stimulation is known to promote collagen alignment in the direction of mechanical loading [27], and since the constructs were maintained under static conditions the whole culture period, it may have limited the potential development of a more mature structure and collagen organization.

5.4 Methodological Considerations and Limitations

The authors of this master's thesis are aware of the limitations of the study. Initially, this study planned to include six donors in total, and during the course of the project, two more donors were added due to technical errors in mechanical testing. But ultimately, only four out of eight donors were used. The appearance was transparent and gel-like, they were considerably weaker than the first four, and most of them had not contracted after 17 days. These observations suggested that unidentified experimental or technical factors may have affected construct formation, thereby reducing the reliability of the affected samples. The deviations observed were therefore considered to be associated with experimental or technical factors rather than biological variation alone. To avoid biased selection, all samples from the affected donors were excluded from the final analysis. This decision was made considering that the research group have had previous problems were constructs behave considerably different in periods of time, and even during the same time that the affected constructs were formed. The reason behind this is unknown. Two batches of FBS was used during the experiments, one of which may have effected the contraction as it is established that FBS have batch-to-batch variations that may affect cell behavior [87], that in turn might affect how the cells behave in the fibrin gel.

A difference between the control and treatment groups was the culture conditions, which may have influenced the results. The control group was maintained only

in construct media, as no Transwell inserts or macrophage medium was required, whereas the co-culture groups were exposed to a mixed medium resulting from both the construct medium and the macrophage medium used for the M1 and M2 groups. It was observed that media diffused through the membrane of the inserts, meaning that soluble components such as growth factors and cytokines present in the macrophage media, could potentially affect tenocyte behavior independently of macrophage signaling. Therefore, some observed effects may not only originate from macrophage-tenocyte cross-talk, but also from differences in media composition between the control and co-culture groups. The base media used for the construct media (DMEM/F12) and the macrophage media (RPMI-60) differ in composition and which cells they are optimized for, for example in amino acid profile and nutrient composition. These differences are not known to directly upregulate MMP-1, MMP-13 or TIMP-1, or reduce construct stress or stiffness. The pattern observed in the co-culture constructs seem to be biologically coherent with macrophage cytokine signaling, and not with a medium composition artifact. However, this limitation should be taken into consideration when interpreting the results. It was considered not feasible to add Transwell inserts with macrophage media in the controls in this study.

There are several variables that may have induced variation between samples in the mechanical testing analysis. This including differences in fixed variables, such as number of replicates, donor and cell varieties, and the subsequent testing procedure. Although established testing protocols were used and samples were handled with care, it cannot be ignored that some variation may be induced in the preparation and mounting of the constructs. With concern to the fragility of the samples, constructs may have been altered prior to testing, either by accidental stretching or other handling causing small ruptures in the construct structures. This highlights the importance in having replicates to give a reflection of the biological differences rather than technical errors. Ideally, all donors and corresponding treatment would have had three replicates or more. Due to human and technical reasons, the number of replicate varies between two and four, however this was deemed acceptable and was taken into consideration in the interpretation of the results. While higher numbers are generally preferred, at least one previous study conducting mechanical testing on 3D tendon constructs demonstrated statistical interpretable results using two to three replicates [55]. Variation between donors is a natural consequence coming from the use of primary tenocytes, especially regarding that no factor such as age was considered when choosing donors. Although it has been proposed that age impacts biomechanical properties of tendons [88], this study focused on macrophage-related effects rather than donor-dependent variability.

CSA calculations may be inaccurate in terms of when, during the mechanical testing measurements, length and area were measured. As mentioned in Section 3.7, a length of 12 mm was selected as the reference length for measurements of the constructs. Optimally, the length and area would be measured at the same pre-load for all constructs to ensure that the dimensions reflects that the constructs are completely straight. In this study, one single pre-load that would work for all samples could not be choosen, as constructs differed considerably in maximum load. This

approach was tried but resulted in some constructs being slack while others already deformed at this point. 12 mm was chosen, as this was the clamp size where constructs had mostly straightened out without being deformed. Alternative lengths were considered, for example 8 mm, corresponding to the initial clamp length at which the constructs were mounted in the testing machine. However, this was not considered suitable since the constructs were still slack. Another alternative was 15 mm, corresponding to the theoretical construct length, but this was also unsuitable since several constructs appeared shorter, and thus may be deformed at that point. Nevertheless, using 12 mm may have resulted in a small over- or underestimation of the construct area and length, which may in turn have affected parameters that utilizes CSA, such as stress and strain. Ultimately, this was the best option for this study, and the possible deviations are regarded as minor.

Additionally, there have been differences in contraction times. The expected time for full contraction was 14 days, which was the case for most of the M2 and control constructs, where some of the M1-constructs contracted considerably faster, as early as in 7 days. Thus, three days were added to allow for complete construct formation, resulting in the mechanical testing to be performed at day 17 after seeding. However, a few constructs were not completely formed at that time point either. It is unknown how this affected the results, but one theory established in the research group is that M1-treated constructs might shrink if cultured for too long, as a result three days extra was sat as maximum.

Although effects were seen in the mechanical properties of macrophage-stimulated constructs, its challenging to interpret these findings. To the author's knowledge, no previous studies have investigated the effects of macrophages on the mechanical properties of *in vitro* 3D tendon constructs, which limits direct comparisons with existing research. It is also important to note that no direct statistical comparison between M1 and M2 was performed, meaning that no direct conclusions can be drawn regarding differences between the two groups.

The detection of CD163 expression in the experimental groups represents a limitation that should be considered when interpreting the results, as it suggests that macrophages were present within the tendon constructs. Consequently, the study may not have exclusively assessed paracrine signaling between macrophages and tenocytes, as direct cell-cell interactions could also have occurred. Macrophages present within the constructs may have produced cytokines and growth factors, thereby influencing the gene expression. This could have amplified or altered the responses attributed to the experimental treatments. Furthermore, potential effects on ECM turnover, collagen organization, and cellular phenotype may have contributed to changes in construct contraction and biomechanical properties. Therefore, the observed outcomes may reflect a combination of paracrine signaling and direct macrophage involvement within the constructs. Additionally, the thesis only assessed macrophage polarization on the gene expression level, the secreted cytokine protein levels in the conditioned media was not quantified, which further limits full characterization of the paracrine signaling environment. Another limitation of this study is that total cell number within the tendon constructs were not quantified. Al-

though gene expression were normalized to housekeeping genes, the total number of cells within the constructs were not assessed. Therefore, it cannot be excluded that differences in cellular content contributed to the observed gene expression patterns.

It was planned to evaluate the expression of COL1A1 in response to the treatments, but this was unsuccessful due to failed standard curves, despite repeating the experiment. Consequently, it was believed to be a technical problem with that specific primer. As collagen type I is the primary component of tendons, it is an important aspect in a study like this. Without COL1A1 expression, the study failed to quantify the COL1:COL3 ratio, which is an important metric to evaluate tissue maturation and fibrosis. Despite this limitation, the study managed to capture the expression of other important ECM components, giving an overview of the overall ECM remodeling. It was also planned to do immunohistochemistry in order to evaluate the collagen type I to collagen type III ratio. Although H&E staining provided valuable information regarding the overall morphology and cellular organization, immunohistochemistry would further enable the characterization of matrix composition and maturation within the constructs. These analyses could not be completed within the scope of the master's thesis due to time limitations.

5.5 Future Directions

Although this study obtained several significant results and interesting trends in biomechanical properties, gene expression, and histology in response to the macrophage phenotypes, future studies could further optimize the experimental workflow. First and foremost, the study would benefit from a larger sample size to improve statistical robustness. Including additional donors would strengthen the statistical analysis and increase the confidence in the observed trends. Furthermore, focus should lie in investigating why some constructs fail to contract and what factors is affecting it. Additionally, control and co-culture constructs should be maintained under identical experimental conditions. To minimize potential effects from differences in media composition, future studies should include empty Transwell inserts containing macrophage medium in the control groups to better capture and isolate the effect of the tenocyte-macrophage cross-talk. Related to this, working towards replacing FBS would be beneficial attributed to the potential affect it have on cells and thus the 3D tendon construct system used. Naturally, this would also reduce the ethical matter corresponding to using animal products in general.

Future studies should also confirm whether macrophages are only residing in the Transwell inserts, as preferred, or have migrated to the construct wells. This would make it easier to distinguish between paracrine effects and direct cell-cell interactions. It can be done by ensuring that Transwells are intact during the entirety of the co-culture period or by, for example, labeling the macrophages with fluorescent probes. Additionally, future work should include characterization of secretion profiles for M1 and M2 macrophages, for example by conducting ELISA, to better link macrophage secretion to tenocyte responses. It would also be advantageous if future studies include quantification of cell numbers as a complement to gene expression analysis, for example by construct digestion followed by cell counting or DNA content assays, to allow normalization to cellular quantity. There is also a need to optimize the construct culture period, further connecting to the need for investigation of what factors are influencing the contraction. Working upon this study, it would be advantageous to include direct statistical comparison between the treatment groups, as this study was limited to comparisons against the control group. This would improve the understanding on how M1- and M2-stimulation differ in their effects directly compared to each other. Another factor that would be desirable to include in the model is mechanical stimulation to better reflect the ECM deposition and collagen alignment in native tendons. As previously discussed, an important future inclusion should be the analysis of the expression of COL1A1 as well as doing immunohistochemistry to give further understanding on the ECM composition and remodeling in response to the stimuli groups.

6

Conclusion

This study aimed to evaluate how M1 and M2 macrophage phenotypes influence biomechanical and biological characteristics of *in vitro* 3D tendon constructs. The results demonstrate that macrophage stimulation alters both gene expression and mechanical properties. M1-induced pro-inflammatory environments were associated with reduced maximum load and stiffness, indicating impaired matrix organization and inferior mechanical properties. M2 macrophages displayed mechanical behavior similar to control, suggesting a more moderate effect on tendon construct mechanics. Furthermore, M1-stimulated constructs exhibited several significant changes in gene expression, characterized by an increase in MMP-1, MMP-13, TIMP-1, and IL-6, alongside a decrease in SCX expression. This indicates a shift toward enhanced matrix degradation and changed tenocyte-like phenotype. Conversely, M2-stimulated constructs demonstrated a significant upregulation only in TIMP-1 and IL-6, implying that the anti-inflammatory environment promotes matrix preservation rather than active tissue breakdown. As one of the first studies investigating macrophage-related alterations in 3D tendon constructs, this work offers a foundation for future research in the area. Further studies are needed to validate these findings and to better understand the underlying mechanisms of macrophage–tenocyte cross-talk in tendon repair.

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A

Appendix 1

A.1 Formulation of Media and Supplements

Various different media were required during the different experimental procedures. Culture-, construct- and macrophage media are prepared in advance and stored refrigerated until use. Monocyte- and tenocyte freezing medium is prepared when needed.

Table A.1: Composition of the media and supplements used for tenocyte and monocyte culture, construct preparation, and cryopreservation.

	Medium	Supplement
Culture medium	DMEM/F12	10% FBS + 1% Pen-Strep
Construct medium	DMEM/F12	10% FBS + 1% Pen-Strep + 1% Ascorbic acid + 1% L-Proline
Tenocyte freezing medium	DMEM/F12	20% FBS + 10% DMSO
Macrophage media	RPMI-1640	10% FBS + 1 % Pen-Strep
Monocyte freezing medium	RPMI-1640	20% FBS + 5% DMSO

A.2 Experimental Methods: Monocytes and Macrophages

A.2.1 Monocyte Expansion

The following protocol originate from the manufacturer's product sheet and can be found here [89].

1. Remove the cells from the dry ice packaging and immediately place the cells at a temperature below -130°C.
2. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
3. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations

from this point on should be carried out under strict aseptic conditions.

4. Transfer the vial contents to a centrifuge tube containing 9.0 mL of complete growth medium and spin at approximately 150 to 400 x g for 8 to 12 minutes.
5. Resuspend cell pellet with the recommended complete growth medium and dispense into the appropriate number of culture flasks. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the flask containing the complete growth medium to be placed in the incubator for at least 15 minutes to allow the medium to reach its optimal pH (7.0 to 7.6).
6. Incubate culture at 37 ° C in a suitable incubator and 5% CO₂.

A.2.2 Cell Maintenance and Subculturing

Cultures should be maintained by the addition of fresh medium. Full fluid changes should be performed by centrifuging and resuspending the cells in fresh media at least every 7 days. Subsequent resuspension at $1-4 \times 10^5$ viable cells/mL. Subculture when cell concentration reaches 8×10^5 cells/mL. Do not allow the cell concentration to exceed 1.5×10^6 cells/mL.

A.2.3 Macrophage Differentiation

1. Plate THP-1 monocytes at a density of 2×10^5 cells/mL in a tissue culture-treated 6-well plate or flask. Use monocyte culture media (RPMI-1640 media + 10% FBS + 1% penicillin-streptomycin).
2. Add PMA at a final concentration of 50 nM to the medium. Incubate for 24 hours at 37°C in a 5% CO₂ incubator. This induces differentiation into macrophage-like adherent cells.
3. After incubation, remove the PMA-containing medium. Wash cells gently 2-3 times with warm PBS to remove residual PMA and any floating, undifferentiated cells.
4. Replace the media with fresh monocyte culture media. Allow the cells to rest for 24 hours to recover and stabilize.

A.2.3.1 M1 Macrophage Polarization

1. Add LPS at 100 ng/mL and IFN- γ at 20 ng/mL to the medium.
2. Incubate cells for 24 hours at 37°C with 5% CO₂.

A.2.3.2 M2 Macrophage Polarization

1. Add IL-4 at 20 ng/mL and IL-13 at 20 ng/mL to the medium.
2. Incubate cells for 24 hours at 37°C with 5% CO₂.

A.3 Experimental Methods: Primary Tendon Cells

A.3.1 Sample Collection and Tendon Tissue Digestion

Sample collection

1. Collect samples from the operating theater in PBS containing 1% penicillin-streptomycin (approx. 20 mL in 50 mL Falcon tube).
2. Remove all visible non-tendon tissue and cut the whole tendon tissue into pieces in the size of about 2x2x2 mm.

Digestion

1. Transfer the pieces to a new petri dish with PBS to wash them. Repeat once more.
2. Digest in DMEM/F12 + 20% FBS + 1 mg/mL collagenase (type II) overnight in the incubator.
3. Shake the tube and pipette up and down the following day, clumps should be dissolved. If not properly digested, place it back in the incubator 2 for hours.
4. Centrifuge at 600 g for 6 minutes.
5. Retain the supernatant and transfer it to a new tube.
6. Wash the pellet in culture medium.
7. Centrifuge both tubes at 600 g for 6 minutes.
8. Aspirate the supernatant and add new culture medium.
9. Centrifuge at 600 g for 6 minutes.
10. Aspirate the supernatant and resuspend the pellets in culture medium.
11. Seed out in a cell culture flask.
12. Change medium the next day to remove debris.
13. Cultivate cells to confluency.

A.3.2 Cell Maintenance

A.3.2.1 Medium Change

Change medium every 4-5 days. It is important to use a new pipette every time you take medium from the media bottle.

- 5 ml per 25 cm² flask
- 12 ml per 75 cm² flask
- 25 ml per 175 cm² flask

A.3.2.2 Cell Passaging

1. Wash cells with PBS.
2. Trypsinate for 5-7 minutes in the incubator (1 mL per 25 cm² flask, 3 mL per 75 cm² flask and 5 ml per 175 cm² flask).
3. Inactivate Trypsin with appropriate amount of culture medium. 3 mL per mL Trypsin.
4. Transfer the cells to a Falcon tube and centrifuge at 600 g for 6 minutes.
5. Prepare new flasks with medium.
6. Discard supernatant and resuspend the pellet in appropriate amount of culture medium (1 mL/new flask).
7. Distribute in the new flasks.

A.4 Preparation of the 6-well Plates with Sutures

1. Mix 9 parts Dow SYLGARD 184 silicone base and 1 part silicone curing agent thoroughly and pour into each well in a 6-well plate to a thickness of approximately 3 mm.
2. Place the plate in an incubator at 55 °C overnight.
3. Make two markings 1 cm in from the edges of the wells to mark the placement of the suture ends. The distance between the sutures should be 1.5 cm.
4. Cut 8 mm long suture threads and use insect needles to fasten the sutures in the silicone.
5. Sterilize the plates in ethanol for 45 minutes before washing the wells with PBS twice.

A.5 Formation of Artificial Tendon Constructs

A.5.1 Sterilization of the 6-well Plates

1. Place the 6-well plates in an ethanol bath for 45 minutes.
2. Remove the plates from the ethanol bath and, in a LAF-bench, aspirate the ethanol and let the remaining ethanol evaporate with the lids semi-covering the wells.
3. Wash the wells twice with PBS (approximately 4mL/well)

A.5.2 Preparation of Cells

Trypsinization

1. Wash with PBS and aspirate
2. Add appropriate amount of trypsin and PBS, and incubate in 37°C for 5 minutes
3. Remove from incubator and hit on the sides of the flask, control that the cells have deattached from the surface of the flask, if not: incubate for another 5 minutes, hit with your palm, and control again. If the cells have deattached, stop the trypsination by adding culture media.

4. Pour into 50 mL Falcon tubes and centrifuge
5. Remove supernatant

Prepare cells for seeding

6. Resuspend pellet in 1 mL of construct medium until cells are in solution.
7. Add additional construct medium to a final volume of approximately 4 ml and mix.

A.5.3 Cell Counting

1. Add 10 μL of trypan blue and 10 μL of cells to an Eppendorf tube and mix.
2. Add 10 μL of the mixture to a cell counting chip.
3. Calculate the cells in 3 different squares and calculate the mean.
4. Dilute the cells to a concentration of 333 333 cells/mL.

A.5.4 Tendon Construct Formation

1. Label Eppendorf tubes (1/well).
2. Add 600 μL cell to each Eppendorf tube and 10 μL aprotinin and mix.
3. Add 200 μL fibrinogen and 4 μL thrombin to one tube, mix, and transfer the entire volume to the well quickly.
4. Ensure that the gel is evenly distributed in the well by swirling the plate against the bench.
5. Repeat steps 3 to 4 for each well, separately.
6. After all wells are finished, let the gels solidify in the incubator for approximately 1 hour before adding 3 mL of medium to each well and placing the plate back into the incubator for 48 hours.

A.5.4.1 Loosening of Edges

1. After the 48-hour incubation, aspirate the medium from the wells.
2. With a pipette tip, carefully loosen the fibrin gel from the walls of the wells to allow for contraction.
3. Add 3 mL of construct medium to each well and place the plate in the incubator.

A.5.5 Maintenance of Constructs

After loosening of edges, leave the constructs in the incubator to contract. Change medium every third day. It is important to use a new pipette every time you take medium from the media bottle. Aspirate the old medium from each well carefully without touching the gel (change aspiration pipette between donors), then add 3 mL of new culture medium.

A.6 Mechanical Testing

Preparation

1. Fill the water bath of the mechanical testing machine and allow it to heat up to 37 °C.
2. Cut fine sandpaper into small rectangles and place the two rectangles with a distance of about the length of a construct in between them on top of a piece of parafilm.
3. Place a small piece of gauze wetted with PBS in between the sandpaper rectangles, to avoid the constructs drying out during the mounting process.

Mounting the Constructs

1. Fully aspirate the media from the well.
2. Remove the needles keeping the suture thread in place.
3. Lift the construct carefully and place it on the two sandpaper rectangles so that the ends of the suture and a small part of the construct are on top of each rectangle.
4. Apply superglue to a third and fourth sandpaper rectangle and place them on top of the other rectangles and constructs.
5. Transfer the entire package and mount it in the mechanical testing machine.
6. Start the testing program (displacement of 4mm/min until failure).
7. Repeat for all constructs.

A.7 Gene Expression Analysis

1. Label and prepare one tube with a screw lid per well with 5 stainless steel beads, 5 pieces of silicone carbide beads and 1 mL of TRIzol and place on ice.
 2. Aspirate the medium and wash each well with 3 mL cold PBS.
 3. Remove the needles and scrape u half of the gel and suture and transfer it into the prepared tubes.
 4. Add some PBS on top of the remaining gel to prevent it from drying out in the preparation for histological analysis.
 5. Shake the tubes in a bead beater (MP, FastPrep-24 Classic) on the 5.5 setting for 20 seconds. Cool the tubes on ice for 2 minutes and shake them again before freezing them in liquid nitrogen.
- After this, the tubes can either be placed in a -80 °C freezer or analyzed directly.

RNA Extraction

1. Spin shortly to remove TRIzol from the lid.
2. Add 100 µL BCP per 1000 µL TRIzol.
3. Vortex for 15 seconds at max speed, do this step as fast as possible.
4. Leave in room temperature for 2-15 minutes.
5. Spin at 1200 g for 14 minutes at 4 °C. The mixture will now be separated into 3 phases: an aqueous phase containing RNA at the top, a middle phase containing DNA, and a lower phase containing proteins, including RNases.

6. Label Eppendorf tubes and add 4 μL of glycogen.
 7. Carefully transfer the top phase to the Eppendorf tube, be careful not to touch any of the other phases (the remaining phases can be saved for later).
 8. Add an exactly equal amount of isopropanol to the Eppendorf tube to precipitate the RNA, mix by inversion, and leave at room temperature for 5-10 minutes.
 9. Centrifuge at 12000 g at 4-25 $^{\circ}\text{C}$ for 8 minutes.
 10. Carefully remove the supernatant with a pipette.
 11. Add 1 mL 75% RNAse-free ethanol and wash sides by inversion.
 12. Centrifuge at 7500 g at 4-25 $^{\circ}\text{C}$ for 5 minutes.
 13. Carefully remove the supernatant with a pipette.
 14. Centrifuge at 7500 g at 4-25 $^{\circ}\text{C}$ for 1 minute.
 15. Remove the remaining supernatant.
 16. Add 100 μL RNAse-free water on top of the pellet, and leave for 5-10 minutes at room temperature.
 17. Dissolve the pellet by vortexing.
 18. Add 10 μL 3M NaAc (pH 5.5, RNAse-free) and mix by shaking vigorously.
 19. Add 200 μL 96% RNAse-free ethanol and wash sides by inversion, and leave at room temperature for 2-15 minutes.
 20. Centrifuge at 12000 g at 4-25 $^{\circ}\text{C}$ for 8 minutes.
 21. Carefully remove the supernatant with a pipette.
 22. Add 1 mL 75% RNAse-free ethanol and wash sides by inversion.
 23. Centrifuge at 7500 g at 4-25 $^{\circ}\text{C}$ for 5 minutes.
 24. Carefully remove the supernatant with a pipette.
 25. Centrifuge at 7500 g at 4-25 $^{\circ}\text{C}$ for 1 minute.
 26. Remove the remaining supernatant.
 27. Air-dry the pellet for 5 minutes by placing the tubes with the lids open. Be careful not to completely dry the pellet as it will be difficult to resuspend.
 28. Add 10 μL RNAse-free water on top of the pellet and leave at room temperature for 5 minutes.
 29. Vortex, and leave at room temperature for 1 minute.
 30. Vortex and spin
 31. Take out 3 μL to a new tube for quantification and quality measurement.
- The extracted RNA can then be stored in a -20 $^{\circ}\text{C}$ freezer until it is transcribed into cDNA.

Reverse Transcription

The following protocol are from the manufacturers user's manual, and can be found here [90].

1. Thaw components of Applied Biosystems High-Capacity cDNA Reverse Transcription Kit on ice.
2. To a tube on ice, add 2 μL 10X RT Buffer, 0.8 μL 25X dNTP Mix, 2 μL 10X Random Primers, 1 μL Multiscribe Reverse Transcriptase, and 4.2 μL nuclease-free water for each reaction to create a 2X RT Master Mix.
3. Mix 10 μL of the 2X Master Mix and 10 μL of the RNA sample. Also include samples with universal human reference RNA that can act as a standard curve.

4. Load the thermal cycler and run the program according to Table A.2.

Table A.2: Settings for the thermal cycler.

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

Samples containing cDNA can then be placed in the fridge short-term.

qPCR

1. Design the plate layout so that no replicates of the samples are included but the standard curve with duplicate samples and a 1:4 serial dilution in 5 steps are included along with a negative control.
2. In a tube, mix 7.5 μL TaqMan Mastermix, 0.75 μL TaqMan Primer Probe, 5.25 μL nuclease-free water for each reaction.
3. Transfer 13.5 μL of the mixture to each well and add the samples according to the previously designed plate layout.
4. Load the Real-Time PCR system and run the program according to Table A.3.

Table A.3: Program for the PCR system.

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	95 °C	95 °C	60 °C	Redo Step 2 40X
Time	10 minutes	15 seconds	1 minute	

A.8 H&E Staining

1. Air dry the glass slide samples at room temperature for 15 minutes.
2. Fix in 4% formaldehyde for 3 minutes.
3. Wash with running tap water for 5 minutes.
4. Stain with Harris hematoxylin for 5 minutes.
5. Wash with running tap water for 5 minutes.
6. Differentiate in acid alcohol, 3-5 dips.
7. Wash with running tap water for 10 minutes.
8. Stain with eosin for 5 minutes.
9. Rinse quickly with Milli-Q water 3 times.
10. Dehydrate rapidly to xylene:
 - I. 70% ethanol for 30 seconds
 - II. 95% ethanol for 60 seconds
 - III. Abs ethanol for 90 seconds
 - IV. Xylene for 2 minutes
 - V. Xylene for 2 minutes
11. Let dry and mount cover slips on the slides.

A.9 Cryopreservation

Monocytes

1. Wash cells with PBS.
2. Centrifuge in 1 mL cold monocyte freezing medium (RPMI-1640 + 20% FBS + 5% DMSO) per flask.
3. Resuspend in 1 mL cold freezing medium per flask.
4. Transfer to cryo tubes (1 mL per tube).
5. Put tubes immediately in Frostyman in -80°C.
6. After 2-5 days transfer to -140°C and note position in database.

Tenocytes

1. Wash cells with PBS.
2. Trypsinate for 5-7 minutes in the incubator (1 mL per 25 cm² flask, 3 mL per 75 cm² flask and 5 ml per 175 cm² flask).
3. Inactivate Trypsin with appropriate amount of culture medium. 3 mL per mL Trypsin.
4. Resuspend in 1 mL cold tenocyte freezing medium (DMEM/F12 + 20% FBS + 10% DMSO) per 175 cm² flask.
5. Transfer to cryo tubes, 1 mL per tube.
6. Put tubes in Frostyman in -80°C.
7. After 2-5 days, transfer to -140°C.

B

Appendix 2

B.1 Histological Scoring

The histological scoring was inspired by the Bonar Score, which is a histopathological grading system used to evaluate degenerative changes in tendons. Scoring was performed by comparing the samples to the reference board shown in Figure B.1. The board was provided to facilitate the identification and assessment of the different histological features and grading stages included in the scoring system. In Figure B.2 representative example images together with scoring is presented.

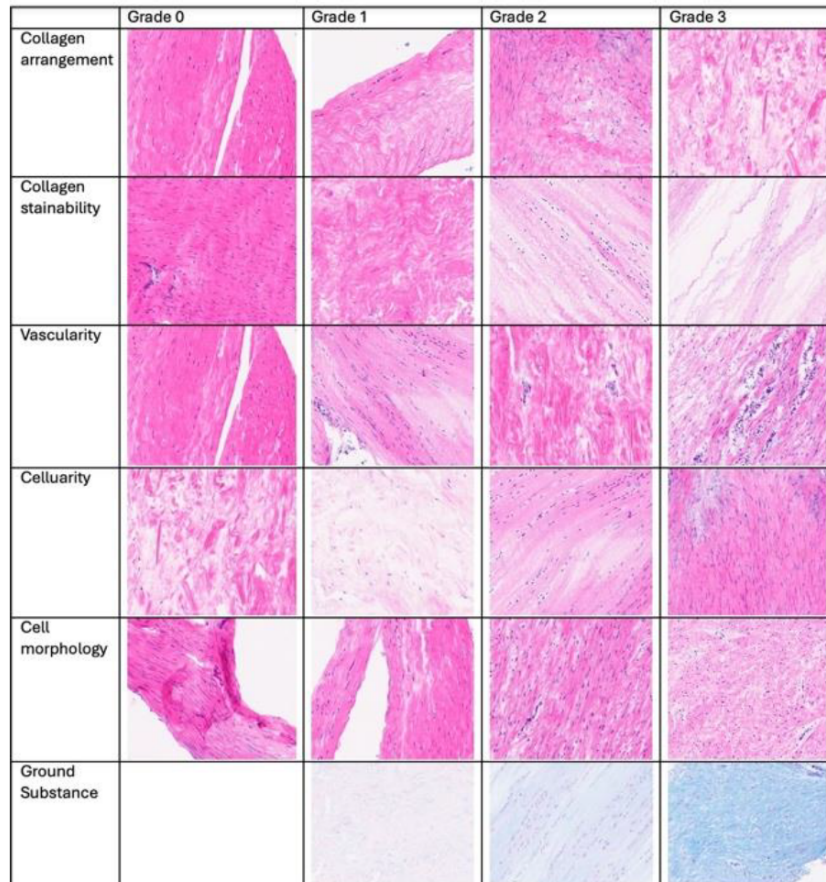


Figure B.1: Images that were used for comparison in the histological grading.

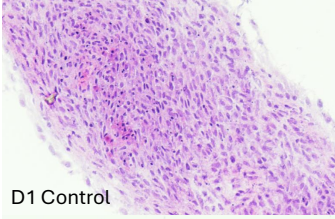
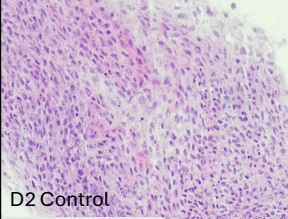
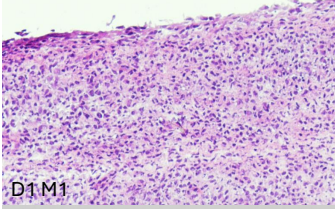
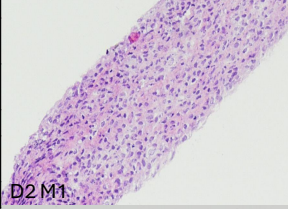
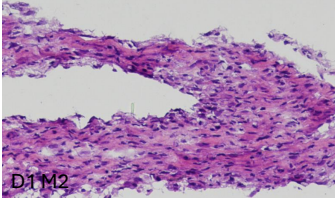
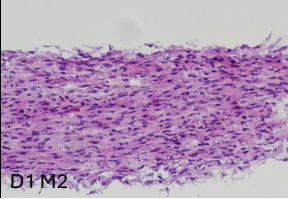
 D1 Control		Score	 D2 Control		Score
	Collagen arrangement	1		Collagen arrangement	2
	Collagen stainability	2		Collagen stainability	1.4
	Cellularity	3		Cellularity	3
	Cell morphology	2		Cell morphology	2
	Mean	8		Mean	8.4
 D1 M1		Score	 D2 M1		Score
	Collagen arrangement	2		Collagen arrangement	2
	Collagen stainability	1		Collagen stainability	2
	Cellularity	3		Cellularity	3
	Cell morphology	2		Cell morphology	2
	Mean	8		Mean	9
 D1 M2		Score	 D2 M2		Score
	Collagen arrangement	1.4		Collagen arrangement	2
	Collagen stainability	1		Collagen stainability	1.4
	Cellularity	3		Cellularity	3
	Cell morphology	1		Cell morphology	1.4
	Mean	6.4		Mean	6.4

Figure B.2: Examples of scores for two donors and the three experimental groups. Demonstrates the scores, representing a mean of the individual scoring that was set by each student, as well as the final mean score that was used in the downstream analysis.

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