Tendon Resident Macrophages Internalize Type 1 Collagen and Express Trophic Signaling Factors

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Introduction

- Tendon cells oriented in linear arrays within tendon fascicles are known as tenocytes (i.e., internal tendon fibroblasts) and thought to be a homogeneous population.
- Our lab utilizes transgenic lineage tracing and fluorescent reporter mice to define markers of the tendon lineage and pathways that regulate tenogenic differentiation.
- Two such reporters include ScxGFP [1] and Col1a1(3.6kb)CFP, which display similar expression patterns within multiple tendons [2]. These reporters are expressed by most cells within the tendon fascicle.
- Recently, we identified a population of cells that are positive for the macrophage marker F4/80 and negative for the Col1a1-CFP and ScxCre;R26R-tdTomato fluorescent reporters in the mouse (**Fig. 1**) [3].
- It is unknown at what stage these resident macrophages begin to populate the tendon and what their role is in tendon growth and development.

The objective of this study was to determine the distribution of tendon resident macrophages throughout development and elucidate potential mechanisms by which these cells may support extracellular matrix (ECM) regulation and tenogenic differentiation.

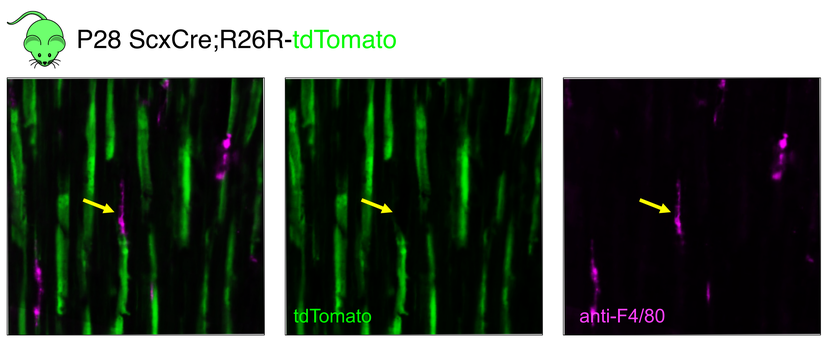

Fig. 1: F4/80 immunofluorescence of patellar tendon from ScxCre;R26R-tdTomato lineage trace.

Methods

All animals and procedures were approved by UPenn's IACUC.

Transgenic Mice. Col1a1(3.6kb)CFP mice containing 3.6kb of the Col1a1 promoter driving CFP expression [4].

Immunofluorescence. Patellar tendons (**PTs**) from E15.5, P4, P28, and P56 Col1CFP mice were fixed, embedded, sectioned, stained with rat anti-F4/80 primary and anti-rat Alexa Fluor 555 secondary antibodies, stained with Hoechst, and imaged (n=3/time point).

TT explant culture and F4/80 immunolabeling. Tail tendons (**TTs**) were cultured in individual channels of 6-channel slides (Ibidi μ-Slide VI) in media supplemented with 200 nM MMPSense 645 FAST MMP-activated fluorescent dye and 10 μg/ml DQ Collagen (Type 1, fluorescein conjugate) collagenase-activated fluorescent substrate (5 TTs/mouse); explants were imaged after 2 days in culture.

Cell isolation and gene expression analysis. TTs were serially digested to discard surface cells and obtain internal cells. Isolated cells were labeled with anti-F4/80 magnetic particles and sorted to obtain F4/80-enriched and F4/80-depleted populations. RNA was isolated and expression was measured via qPCR for *18S, Col1a1, Adgre1, Csf1, Csf1r, Tgfb1, Tgfb2, Tgfb3,* and *Tgfbr2.*

Single-cell RNA sequencing analysis. Publicly available single-cell RNA sequencing (scRNA-seq) datasets were obtained from the NCBI GEO (GSE139558 [5] and PRJNA506218 [6]). Count matrices were filtered, normalized, scaled, cell cycle regressed, reduced, and clustered using Seurat v3.1 [7].

Statistics. qPCR results were compared via Kruskal-Wallis followed by Mann Whitney U tests adjusted for multiple comparisons.

Results

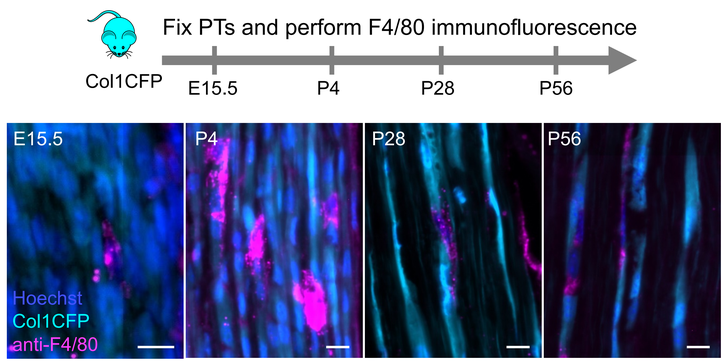

Fig. 2: Resident macrophages are present throughout tendon development.

- To investigate the presence of resident macrophages during tendon development, we performed immunofluorescence for the macrophage marker F4/80 on PT sections.
- We found that Col1CFP(-) F4/80(+) resident macrophages (magenta) were present in the linear arrays of the PT at E15.5, P4, P28 and P56, ranging from 4% to 9% of total cells within the midsubstance.

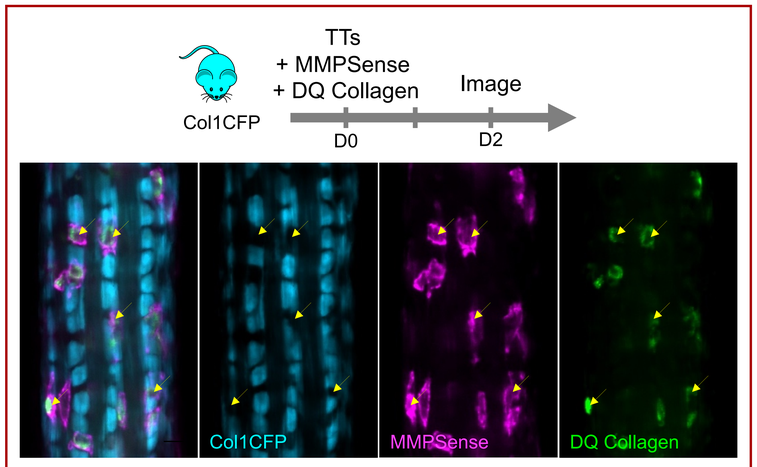

Fig. 3: Tendon resident macrophages internalize DQ Collagen and MMPSense.

- Because macrophages are present throughout tendon growth and development, we hypothesized that they may play a role in ECM assembly.
- The unquenched, cleaved fluorescent substrates were localized almost exclusively within the Col1CFP(-) cells, which are virtually all F4/80(+) (yellow arrows).

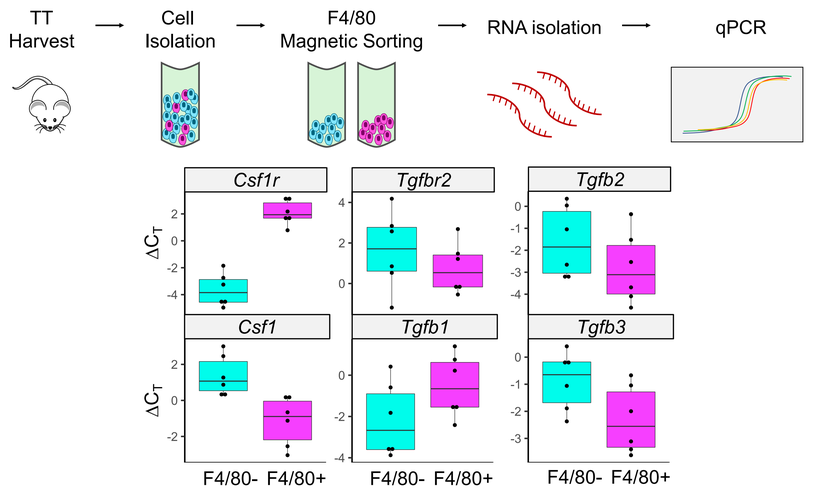

Fig. 4: F4/80-depleted cell population (fibroblasts) expresses *Csf1* and *Tgfbr2*; F4/80-enriched cell population (macrophages) expresses *Csf1r* and *Tgfb1*.

- Due to the observed proximity of fibroblasts and macrophages and previous studies demonstrating cell-cell communication between macrophages and stromal cells, we investigated potential signaling pathways in F4/80-sorted tendon cell populations.
- The F4/80-enriched (macrophage-enriched) population expressed 53.5-fold higher levels of *Csf1r* compared to the F4/80-depleted (fibroblast-enriched) population, which expressed 5.85-fold higher levels of *Csf1*.
- The macrophage-enriched population had a 2.75-fold higher expression of *Tgfb1*, while the fibroblast-enriched population exhibited 2.38, 2.36, and 2.65-fold higher levels of *Tgfb2*, *Tgfb3*, and *Tgfbr2*, respectively.

Table 1.

	Tan et al. (P7 hindlimb tendons)	Harvey et al. (3mo patellar tendons)
% Csf1+ in tenocyte clusters	10.5	18.3
% Csf1r+ in macrophage clusters	78.0	64.6
% Tgfb1+ in macrophage clusters	38.0	30.8

- We next examined this cross-talk further by analyzing recent scRNA-seq datasets [5,6].
- In both datasets, a subset of tenocytes expressed detectable levels of *Csf1*, while macrophages were enriched for the receptor *Csf1r.*
- The percentage of tenocytes expressing *Csf1* in these datasets is comparable to the number of macrophages within tendons (**Fig. 2**).
- In both datasets, the macrophage cluster was enriched for *Tgfb1.*

Discussion

- In this study, we demonstrated that resident macrophages are present alongside fibroblasts during embryonic tendon development and throughout postnatal growth (Fig. 1).
- This macrophage population is capable of internalizing proteolytically cleaved DQ Collagen and MMPSense within their native environment (Fig. 2), which suggests that these cells may be important in the degradation and/or clearance of ECM during development.
- We also established that fibroblasts express Csf1 (Fig. 3), a cytokine necessary for macrophage survival and function. scRNA-seq data showed that only a subset of fibroblasts expresses detectable levels of Csf1, suggesting that the spatial distribution of Csf1r(+) macrophages is dependent on Csf1 expression by stromal cells, as is the case in other tissues.
- Our data and others' showed that tendon resident macrophages express TGFβ ligands and are especially enriched for Tgfb1, which supports our working hypothesis that macrophages provide trophic signaling to Tgfbr2(+) fibroblasts.
- Resident macrophages in other tissues are necessary for their development and contribute to ECM regulation and cell signaling circuits with surrounding resident cells. Our future studies aim to determine if analogous phenomena occur in tendons.

References: 1. Pryce BA et al., Dev. Dyn., 2007; 2. Dyment N et al., ORS, 2016; 3. Bautista CA et al., ORS, 2020; 4. Kalajzic I, et al., JBMR, 2001; 5. Tan et al., eLife, 2020; 6. Harvey et al., NCB, 2019; 7. Stuart et al., Cell, 2019.
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