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

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Disclosures

None

Introduction

While the majority of cells within the growing tendon are tendon fibroblasts that express the common tendon reporters Scx-GFP and Col1a1-CFP, we recently 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[1]. It is unknown at what stage these resident macrophages begin to populate the tendon and what their role is in tendon growth and development. Therefore, 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.

Methods

Transgenic mice

All procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Col1a1(3.6kb)CFP (Col1CFP) mice containing 3.6kb of the Col1a1 promoter driving CFP expression were used in this study[2].

Experimental design

Knees from Col1CFP mice at E15.5, P4, P28, and P56 were used for patellar tendon (PT) immunofluorescence (n = 2-3/time point). Tail tendons (TTs) were isolated from 4-6-week-old Col1CFP mice for explant culture (n = 4). TTs from P28 mice were used for cell sorting and gene expression analysis (2 mice per biological replicate; n = 3). Knee sections were stained with rat anti-F4/80, stained with Hoechst, and imaged.

TT explant and protease substrate culture

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 qPCR

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.* qPCR results were compared via Kruskal-Wallis followed by Mann Whitney U tests adjusted for multiple comparisons. Publicly available single-cell RNA sequencing (scRNA-seq) datasets were obtained from the NCBI GEO (GSE139558[3] and PRJNA506218[4]). Count matrices were filtered, normalized, scaled, cell cycle regressed, reduced, and clustered using Seurat v3.1[5].

Results

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 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 (Figure 1; magenta).

Tendon resident macrophages internalize cleaved DQ Collagen and MMPSense

Because macrophages are present throughout tendon growth and development, we hypothesized that they may play a role in ECM assembly. Upon culturing P28 TT explants with media supplemented with DQ Collagen and MMPSense, we found that the unquenched cleaved fluorescent substrates were localized almost exclusively within the Col1CFP(−) cells (Figure 2; yellow arrows). Given this finding and the fact that virtually all Col1CFP(−);MMPSense(+) are F4/80(+) [1], we concluded that resident macrophages are capable of internalizing excess proteolytically cleaved collagen

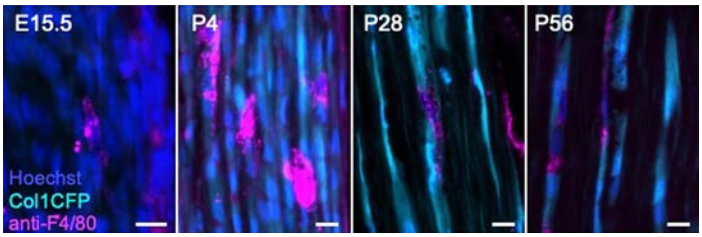

Figure 1. F4/80 immunofluorescence on Col1CFP patellar tendons (scale = 10μm).

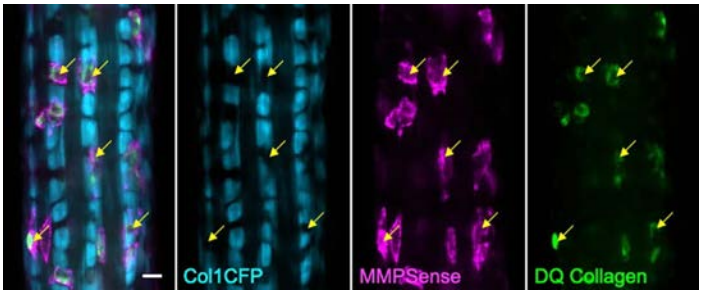

Figure 2. Col1CFP tendons cultured in medium containing MMPSense and DQ Collagen (scale = 10μm).

Potential cell signaling circuit between F4/80-enriched and F4/80-depleted cell populations

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* (Figure 3). Because TGFβ signaling plays a major role in tendon development [3] and macrophages have been shown to signal to fibroblasts via TGFβ [6], we analyzed expression levels of key TGFβ ligands and their receptor. All surveyed genes were detected in both populations. We found the macrophage-enriched population to have 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.

scRNA-seq datasets confirm potential cell-cell communication between fibroblasts and macrophage

Analysis of two previously published scRNA-seq data sets showed that, among clusters enriched for *Adgre1* (F4/80), 78.0% (P7 forelimb and hindlimb tendons[3]) and 64.6% (3-month-old PTs[4]) of cells express *Csf1r*; of these *Csf1r*(+) cells, 44.4%[3] and 36.5%[4] express *Tgfb1*. Furthermore, among clusters enriched for *Tnmd* (tenocyte marker), 11.0%[3] and 18.3%[4] of cells express detectable levels of *Csf1* and 12.5%[3] and 14.4%[4] express *Tgfbr2*.

Discussion

In this study, we demonstrated that resident macrophages are present alongside fibroblasts during embryonic tendon development and throughout postnatal growth (Figure 1).

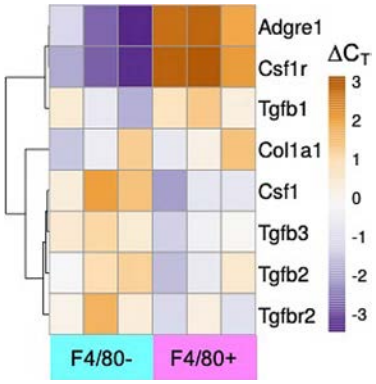

Figure 3. Gene expression of F4/80-sorted cells.

This macrophage population is capable of internalizing proteolytically cleaved DQ Collagen and MMPSense within their native environment (Figure 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* (Figure 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.

Significance

An improved understanding of the cells and signaling pathways that define and regulate the tendon lineage will be crucial to developing new therapies to attenuate the progression of pathologies and improve repair outcomes following injury. This study gives new insight into potential roles of resident macrophages during tendon development and growth and their interaction with *Col1a1*-expressing tendon fibroblasts.

Acknowledgements

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References

1. **Bautista *et al.,*** ORS 2020
2. **Kalajzic *et al.,*** *JBMR* 2001
3. **Tan *et al.,*** *eLife* 2020
4. **Harvey *et al.,*** *NCB* 2019
5. **Stuart *et al.,*** *Cell* 2019
6. **Wynn and Barron**, *Semin Liv Dis* 2020.