Type III Collagen Reduction Has Minimal Immunomodulatory Impact in Young Adult Mouse Tendon Healing

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INTRODUCTION: Tendon injury initiates the canonical phases of healing: inflammation, proliferation, and remodeling. A critical component of tendon healing is regulation of inflammatory processes, including the polarization of macrophages (Mφs) from an M1 (pro-inflammatory) to an M2 (anti-inflammatory) phenotype.[1–3] Anti-inflammatory Mφ polarization supports tissue remodeling and coincides with the transition from a disorganized, type III collagen (COL3)-rich matrix,[4,5] to a more functional, aligned, type I collagen (COL1)-rich matrix in late healing. Although COL3 is highly expressed in healing tendon[6] and it supports Mφ polarization in other contexts like endometrium,[4] the role of COL3 in immunomodulation during tendon healing remains unknown. Therefore, the objective of this study was to define COL3's regulation of Mφ polarization in each phase of tendon repair. We hypothesized that COL3 is a critical immunoregulator that increases the polarization of Mφs from M1 to M2 in the tendon healing niche.

METHODS: Postnatal day 90 (p90) mixed sex mice with inducible, global *Col3a1* knockdown (Rosa26-CreER^{T2}; *Col3a1*$^{f/f}$) and Cre-negative littermate controls (wildtype, WT) received a single tamoxifen injection (200mg/kg) one day before undergoing bilateral patellar tendon biopsy punch surgery (IACUC approved). Mice were euthanized at 1-week, 3-weeks, or 6-weeks post-injury (wpi) to isolate *Col3a1* knockdown (KD) effects in the inflammatory, proliferative, and remodeling phases, respectively. Uninjured Cre-positive and -negative controls received a tamoxifen injection (200mg/kg) at p90 and were euthanized at the 3-week timepoint. Gene Expression (n ≥ 8/group): Following euthanasia, whole tendons were frozen in RNA*later*, thawed, homogenized in a phenol lysis agent, and processed (Direct-zol RNA Microprep, Zymo). RNA was converted to cDNA and used for qPCR (Standard BioTools). ΔCt values were calculated as follows: ΔCt = average Ct of housekeeping genes (*18S*, *Abl1*, and *Rps17*) – Ct gene of interest. Immunostaining (n ≥ 7/group): Lower limbs were harvested, fixed, and optically cleared (30% sucrose, w/v). Patella-patellar tendon-tibial insertion complexes were embedded in optimal cutting temperature medium, sectioned (anterior-posterior plane, 8 µm), adhered to glass slides (1% chitosan in acetic acid), post-fixed (4% paraformaldehyde), and underwent antigen retrieval (1:1000 Proteinase K in TE buffer). Sections were stained for general Mφ marker CD68 (rat anti-mouse CD68, donkey anti-rat Alexa Fluor™ 488), M1-like Mφ marker inducible nitric oxide synthase (iNOS, rabbit anti-mouse iNOS, donkey anti-rabbit Alexa Fluor™ 555), M2-like Mφ marker CD206 (goat anti-mouse CD206, donkey anti-goat Alexa Fluor™ 647), and nuclei (SYTOX™) and imaged with a Zeiss Axioscan (20X). A custom MATLAB code quantified CD68+, iNOS+. and CD206+ cells. Statistical Analysis: After outlier removal (2.2 IQR), results were compared with two-way ANOVAs to assess the effects of genotype, timepoint (wpi), and their interaction. Significance was set at $p \leq 0.05$.

RESULTS: Expression of general (*Adgre*), M1 (*CD80*, *CD86*), and M2 (*CD163*, *CD206/Mrc1*) Mφ markers (Fig.1) displayed no differences distinct to genotype but changed significantly over time ($p \leq 0.0052$ for all listed genes). Mφ markers were also assessed via immunostaining. CD68+ cell density (Fig.2A-B) in KD and WT tendons was significantly different across timepoints ($p<0.0001$) but not genotype. The percent of CD68+/iNOS+/CD206- (M1-like) cells (Fig.2C) changed over time ($p=0.0221$) and was significantly increased in KD groups ($p=0.0301$), although the effect of KD did not depend on healing timepoint. The percent of CD68+/iNOS-/CD206+ (M2-like) cells (Fig.2D), CD68+/iNOS+/CD206+ cells (Fig.2E), and CD68+/iNOS-/CD206- cells (Fig.2F) displayed no differences across timepoint or genotype. These minor changes indicate a subtle role for COL3 in immunomodulation of tendon healing.

DISCUSSION: We defined the role of COL3 in regulating macrophage polarization during tendon healing via *Col3a1* KD at the time of mouse patellar tendon injury. Results of this study contrast our hypothesis, displaying minimal impact of *Col3a1* expression reduction on immunomodulation of macrophages in healing young adult tendon. Collectively, these findings reveal tissue-specificity in the immunoregulatory capacity of COL3 due to its different roles in endometrium[4] and tendon, as shown here. This corroborates our previous paradigm-challenging finding of minimal influence of COL3 loss on healing tendon matrix structure, function, and composition in young adult mice.[6] Further evaluation of *Col3a1* reduction effects in injury, including transcriptomic or proteomic immune profiling of tendon healing and wholistic investigation of the inflammatory profile of the healing tendon, may provide further insight into the role of COL3 in young adult murine tendon healing and immunomodulation.

SIGNIFICANCE: Understanding immunoregulators of tendon healing will provide insight into the design of therapeutics and injury prevention strategies.

REFERENCES: [1] de la Durantaye. *JOR*. 2014. [2] Sugg. *JOR*. 2014. [3] Chamberlain. *Stem Cells*. 2019. [4] You. *Regenerative Biomaterials*. 2023. [5] Spörlein. *Plastic and Reconstructive Surgery - Global Open*. 2024. [6] Tamburro. *ORS Abstract*. 2025.

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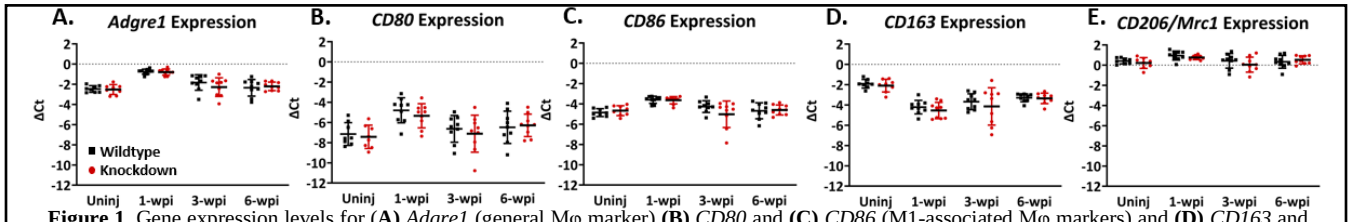

Figure 1. Gene expression levels for **(A)** *Adgre1* (general Mφ marker) **(B)** *CD80* and **(C)** *CD86* (M1-associated Mφ markers) and **(D)** *CD163* and **(E)** *CD206/Mrc1* (M2-associated Mφ markers), displayed significant changes over time without genotype-specific influences.

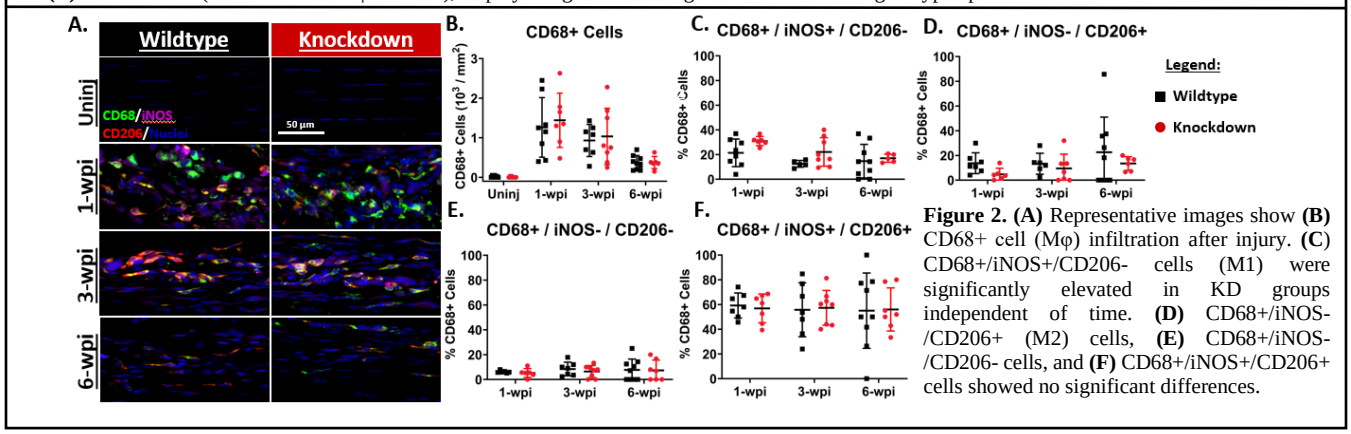

Figure 2. (A) Representative images show **(B)** CD68+ cell (Mφ) infiltration after injury. **(C)** CD68+/iNOS+/CD206- cells (M1) were significantly elevated in KD groups independent of time. **(D)** CD68+/iNOS-/CD206+ (M2) cells, **(E)** CD68+/iNOS-/CD206- cells, and **(F)** CD68+/iNOS+/CD206+ cells showed no significant differences.