Time-Dependent Crosstalk between Tendon Stem/Progenitor Cells and Macrophages in Tendon Healing

Md Sarker, Chen Zong, Chang H Lee
Center for Dental and Craniofacial Research, Columbia University, New York, NY

DISCLOSURES: Md Sarker (N), Chen Zong (N), Chang H. Lee (N)

INTRODUCTION: The immune system plays essential roles in tendon pathology, repair, and regeneration. Macrophages are actively involved in tendon healing from the early inflammatory phase to the late remodeling phase through their polarization, respectively. We previously reported a novel combination of pharmacokinetic small molecules (SMs), Oxo-M and 4-PPBP, which leads to tendon regeneration by promoting endogenous tendon stem/progenitor cells (TSCs). Recently, we have found that Oxo-M attenuates M1 (pro-inflammatory) polarization and 4-PPBP promotes M2 (anti-inflammatory/remodeling) polarization. To guide regenerative healing of tendons by regulating the timely polarization of macrophages, we developed a precise, sequential delivery system for Oxo-M and 4-PPBP by implementing layer-by-layer (LbL) fabrication. Our LbL tendon patch, sequentially releasing Oxo-M and 4-PPBP (OP), successfully attenuated M1 polarization in the early healing phase and promoted M2 polarization in the later healing phase, consequently promoting tendon healing. In this study, we aimed to delineate the crosstalk between macrophages and tenocytes/TSCs at different time points in tendon healing, with and without OP treatment via LbL tendon patch.

METHODS: This study was approved by IACUC. LbL tendon patches were prepared, per our established methods, on a 3D-printed polycaprolactone (PCL) patch. Briefly, cationic polymer poly-l-lysine (PLL) and anionic polymer methacrylated hyaluronic acid (HAMA) were sequentially applied at a concentration of 0.5 mg/mL to build the $(PLL/HAMA)_{40}$ nanolayers. Oxo-M or 4-PPBP was loaded between the PLL (positively charged) and HAMA (negatively charged). 4-PPBP was loaded in the first half of the layers, and Oxo-M was loaded in the other half of the layers for the release of Oxo-M, followed by 4-PPBP. PCL barriers with 4 µm pores were added in between the 4-PPBP-loaded and Oxo-M-loaded layers to achieve a prolonged, sequential release of Oxo-M and 4-PPBP. The SM-loaded LbL PCL patch was then applied to our well-established patellar tendon repair model (n = 8 per group/sex). Briefly, after creating a full-thickness rat patellar tendon (PT) incision, a cerclage suture was applied, and then the LbL PCL patch was attached on top of the repaired tendons. PCL patch without SM served as a control. At 1 wk and 2 wks post-op, cells were isolated from the harvested tendons for single-cell RNA sequencing (scRNA-seq) with Next Generation Sequencing (NGS). We utilized 10X Genomics analysis software to yield >1,000 unique molecular identifiers (UMI) and genes per cell, followed by producing the t-SNE graphs. Differentially expressed genes (DEG) analysis was performed per cell type. CellChat-embedded social network analysis tool, integrated with pattern recognition methods and manifold learning approaches, was used to characterize and compare the inferred intercellular communications quantitatively. In vitro experiments with siRNA knockdown (KD) were performed to confirm the roles of Tn-C and Postn in macrophage-TSC crosstalk.

RESULTS: The LbL tendon patch was prepared in a micro-thin membrane showing 6,000 nm-thick 40 layers of PLL/HAMA with 75 nm of each polymer layer (not shown). The $(PLL/HAMA)_{40}$ layered PCL patch with sequential loading of 4-PPBP and Oxo-M showed a fast release of Oxo-M by 7 - 10 days, followed by slow release of 4-PPBP up to 42 days (not shown). Application of SM-releasing LbL tendon patches in full-transected rat patellar tendons resulted in notable improvements in tendon healing with significantly enhanced collagen reorganization and functional properties (data not shown) compared to controls with PCL patches without SMs. Immunofluorescence showed attenuated M1-like macrophages at 1 wk and promoted M2 polarization at 2 wks (not shown). scRNA-seq data revealed multiple cell populations in the tendons, including but not limited to tenocytes, macrophages, T cells, B cells, and endothelial cells (data not shown). At both times, LbL tendon patch-treated tendons showed higher numbers of anti-inflammatory/remodeling macrophages and TSCs as compared to control (not shown). In addition, genes associated with tendon matrix synthesis and remodeling were significantly increased in TSCs and tenocytes in LbL patch-treated tendons (not shown). DEG in TSC, tenocytes, and macrophages with detailed phenotyping indicating increased TnC and Postn in TSC & tenocytes and reduced pro-inflammatory genes and increased anti-inflammation/remodeling-associated genes in macrophages (**Fig. 1A**). Robust Tenascin and Postn communication from TSC to macrophages was identified in the OP treated group, in comparison with controls (**Fig. 1C, D**). Quantitatively, Tnc-sdc4 and Postn-Itav axes in TSC-to-macrophages were significantly increased in the O-P treated group by 1 wks ($p<0.0001$; not shown). By 2 wks post-op, pro-inflammatory genes were significantly reduced, and anti-inflammation/remodeling-associated genes (M2a polarization markers) were dramatically elevated in macrophages per DEGs (not shown). In TSC/tenocytes, tendon matrix synthesis and remodeling markers were significantly elevated in the OP group (not shown). In terms of cell-cell communication, robust Tnc-sdc4 signals persisted in TSC/tenocyte-to-macrophage by 2 wks (not shown). The overall tenascin signal was identified in communication between TSC/tenocytes and macrophages (**Fig. 1E**). At 2 wks, interestingly, Postn signal was robust in communication between TSC and tenocytes (**Fig. 1F**), suggesting its roles in tendon matrix remodeling at 2 wks in contrast to its involvement in macrophage polarization at 1 wk (**Fig. 1D**). From macrophage to TSC/tenocytes, TGFβ signals were robustly increased at 2 wks with OP treatment in comparison with control (not shown), suggesting macrophages' role in promoting tendon remodeling via TSC/tenocytes given the roles of TGFβ in tendon remodeling. Our *in vitro* siRNA KD study confirmed the function of Tn-C and Postn in tenocyte-induced macrophage polarization.

DISCUSSION: Our data suggest critical signaling axes regulating crosstalk between macrophages and TSC/tenocytes, leading to regenerative healing of the tendon. The distinct cell-cell communications in different tendon healing phases may support the importance of temporally regulated bioactivities for tendon regeneration. Our study with scRNA-seq/CellChat analysis can be expanded to understand crosstalk between other types of cells during pathological repair and regenerative healing of tendons, potentially deepening our knowledge in tendon biology and healing.

SIGNIFICANCE: This study elucidated the essential cell-cell communication signaling between macrophages and TSC that may have significant implications in the biology of tendon healing and the development of regenerative therapy.

IMAGES AND TABLES:

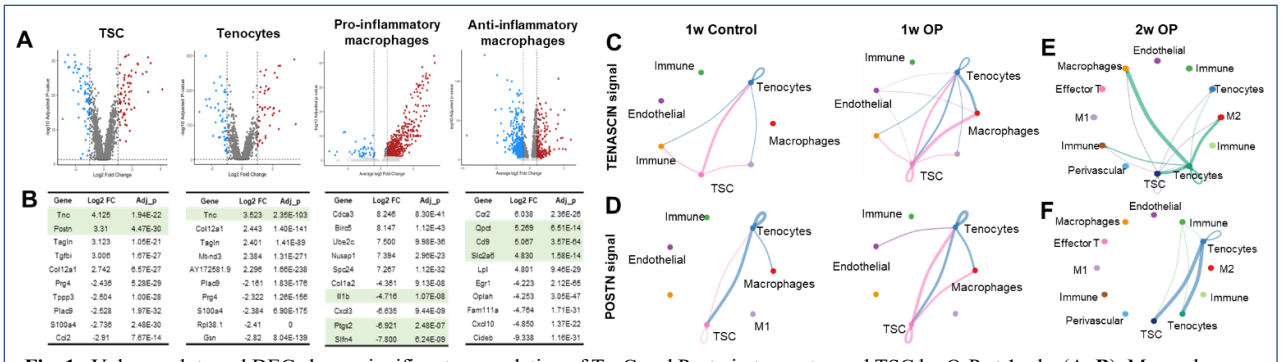

Fig. 1. Volcano plots and DEG shows significant upregulation of Tn-C and Postn in tenocytes and TSC by O-P at 1 wks (**A, B**). Macrophages showed reduced expression of pro-inflammatory signals and increased anti-inflammation/matrix remodeling-associated genes (**A, B**). CellChat analysis confirmed robust Tenascin and Postn signals from TSC to macrophage in the OP treated group compared to controls (**C, D**). At 2 wks, tenascin signaling persisted in communication between tenocytes and macrophages (**E**). Postn signals were dominant between tenocytes and TSCs (**F**), suggesting its different roles in 2 wks.