NF-κB Pathway Inhibition Mitigates Tendon Fibroblast Responses to Macrophage-Induced Inflammation *In Vitro*

McKenzie E. Sup[1], Min Kyu M. Kim[1], Lee Song[1], Jieon J. Kim[1], Stavros Thomopoulos[1,2]

[1]Department of Biomedical Engineering, Columbia University, New York, NY, [2]Department of Orthopedic Surgery, Columbia University, New York, NY

mes2293@columbia.edu

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INTRODUCTION: Increasing evidence is emerging that inflammation plays a critical role in the initiation and progression of tendinopathy, but a complete understanding of the inflammatory response in tendon pathophysiology is lacking [1]. *In vivo* models provide the most physiologically relevant platforms for studying tendon injury, but the inherent complexity of the inflammatory response *in vivo* presents a challenge for identifying the mechanisms involved. There is evidence that the NF-κB pathway plays an important role in tendon disease, but the extent to which it is necessary for the maintenance of the inflammatory response in tendon cells is unknown [2]. Furthermore, previous studies reported that mechanical loading drives healing outcomes, but how cells respond to loading in an inflammatory environment is not well understood. [3,4] We developed an *in vitro* model system that enables us to perform a controlled study to interrogate the relationship between tendon inflammation and mechanobiology. Inflammation is modeled using macrophages, as they are highly potent immune cells that are abundant and persistent in both chronic and acute tendon injury responses [5,6]. M1 macrophages are dominant in the first two weeks after injury, and help to propagate the acute inflammatory response [6]. They promote the degradation of extracellular matrix and secrete pro-inflammatory cytokines such as IL-1β and TNFα that activate surrounding cells [7]. We hypothesized that paracrine signaling from macrophages would induce a robust and widespread pro-inflammatory response in tendon fibroblasts, and that NF-κB inhibition would mitigate this response. We also hypothesized that macrophage-induced inflammation would alter tendon fibroblast responses to mechanical loading.

METHODS: *Cell Culture:* Tendon fibroblasts (TFs) and macrophages were isolated from 2-month-old C57BL/6 mice. TFs were isolated from tail tendon by collagenase digestion in a shaking incubator, while macrophages were isolated from femur and tibia bone marrow aspirate. Macrophages were polarized to a pro-inflammatory M1 phenotype by LPS and IFN-γ stimulation for 24 hours. Then, stimulation media was replaced with fresh cytokine-free media. 24 hours later, the media was harvested from the activated macrophages, to be added to TFs. For inhibition experiments, 2.5uM of IKK-2 inhibitor VIII was added to the TFs along with M1 conditioned media (M1-CM). *Mechanical Loading:* TFs were seeded on fibronectin-coated PDMS stretch chambers (STB-CH-04, STREX Co., Osaka, Japan). Chambers were cyclically stretched for 24 hours at a magnitude of 7% strain and a frequency of 0.5 Hz using the STB-1400 STREX cell stretch system. *Gene expression:* After 24 hours of treatment with M1-CM and/or 24 hours of tensile stretching, cells were lysed and total RNA was isolated (avg RIN = 8.7). Bulk RNA sequencing was performed using the Illumina NovaSeq Platform, and a DESeq2 Output was performed between untreated TFs and M1-CM-treated TFs (n=4). Enrichr was used to identify significantly upregulated pathways. For loading experiments, paired t-tests were performed to assess for differences between loaded TFs and unloaded TFs, with unloaded controls present in each group (M1-CM treated and untreated). Response to loading compared to respective controls for each group was plotted as $\log_2$(FoldChange/Control). * Indicates $p<0.05$ and ** indicates $p<0.01$. For inhibition experiments, RT-qPCR was performed for genes of interest related to tendon inflammation and remodeling, and results were plotted as relative gene expression to GAPDH ($2^{-\Delta CT}$). Differences between M1-CM-treated (M1 only), M1-CM-treated plus IKK-2 inhibitor (IKK Inh + M1), and untreated controls were assessed using ANOVA with Tukey correction for multiple comparisons (Figure 3). Statistical significance was set to $p<0.05$.

RESULTS: *Bulk RNA sequencing:* In response to treatment with M1-CM, 494 genes were significantly upregulated in TFs ($\log_2$(FoldChange) > 1.5, P_{adj} <0.05), while 126 genes were significantly downregulated ($\log_2$(FoldChange) < -1.5, P_{adj} <0.05). Figure 1A shows a volcano plot of the RNAseq results, with red, blue, and grey dots denoting upregulated, downregulated, and insignificantly changed, respectively. Pathway analysis via Enrichr revealed significant upregulation of several pro-inflammatory pathways, including cytokine- and chemokine-mediated signaling, responses to TNF and Interferon, as well as regulation of NIK/NF-kappaB signaling (Figure 1B). *Loading response:* TFs responded differently to mechanical loading after being stimulated with M1-CM. *Il6* and *Mmp13* expression both significantly decreased in response to loading in the inflammatory environment, while these genes showed significantly increased expression in response to loading in untreated TFs. *IKK-2 Inhibition:* When IKK-2, an essential mediator of the NF-κB pathway, was inhibited, the M1-CM response of some genes was suppressed, while others were not significantly affected (Figure 2). *Il6* and *Ccl2*, in particular, were significantly downregulated compared to M1-CM treated TFs, while *Mmp13* expression was unaffected.

DISCUSSION: Activated macrophages induced a complex and robust pro-inflammatory response in TFs through a variety of pathways. Our *in vitro* model system, which included macrophage-induced inflammation and tensile cyclic loading, is more physiologically relevant than typical *in vitro* approaches that rely on one cytokine (typically, IL-1β) to model inflammation. Changes in TF phenotype in response to M1-CM also selectively affected their responses to loading, reversing patterns of loading responses in multiple genes. Notably, the NF-κB pathway was shown to regulate TF inflammatory responses to M1 macrophages, as inhibition mitigated the inflammatory response, particularly for *Il6* and *Ccl2* genes. This suggests that NF-κB signaling is an important factor in TF response to inflammation, but that other pathways can sustain part of the inflammatory response independently of NF-κB. Future studies will investigate a more comprehensive list of gene expression responses, dose-responses, and inhibition of other key pathways using this model system.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates the effectiveness of inducing a widespread inflammatory response in TFs through the application of M1-CM. This has the advantage of creating a platform by which to study tendon inflammation, mechanobiology, and potential therapeutics in a highly controlled manner.

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Figure 1. A) Volcano plot and B) pathway analysis RNA-seq results of TF response to M1-CM.
Figure 2. Differential response to loading in M1-CM-treated TFs compared to untreated control TFs.
Figure 3. Genes upregulated by M1-CM-induced inflammation were suppressed with IKK-2 inhibition.