Chemokine receptor CX3CR1 mediates tendon macrophage responses to loading

Lee Song[1], Stavros Thomopoulos[1,2]
[1]Department of Orthopedic Surgery, [2]Department of Biomedical Engineering, Columbia University, New York, NY
sl705@columbia.edu

Disclosures: Lee Song (N), Stavros Thomopoulos (N).

INTRODUCTION: Tendons function to transfer load from muscle to bone to produce joint motion. Tendons are under high cyclic loads during movement and are prone to damage and injury [1]. Resident tendon and enthesis cells remodel the matrix and continually repair minor damage due to pathology or overloading. However, accumulation of damage can result in degeneration and eventual rupture. As sentinel cells, tissue resident macrophages detect damage and invasion from pathogens. Macrophages phagocytose the damaged cells and tissue, engulf and destroy any pathogens, and/or initiate inflammatory responses [2]. Previously, we demonstrated the presence CX3CR1-expressing (CX3CR1+) resident macrophages in mouse tendons and entheses; these cells were roughly divided into CX3CR1+ resident and CCR2+ migratory cells [3]. Macrophages play important roles in tendon responses to major tendon and enthesis injuries; however, their function in tendon development and under overuse conditions remains unclear [4,5]. Here, we hypothesize that as sentinel cells, tendon resident macrophages detect homeostatic alternations under overloading conditions and modify their behavior in an effort to repair microdamage to the extra-cellular matrix (ECM). To test this hypothesis, we used our previously described Cx3cr1$^{GFP/KO}$ reporter mice, in which the resident macrophages are marked with eGFP and the chemokine receptor CX3CR1 is knocked out. These mice were crossed with Ccr2$^{RFP/KO}$ reporter mice, in which migratory macrophages are marked with RFP and the chemokine receptor Ccr2 is knocked out. The cross produced Ccr2$^{RFP/KO}$; Cx3cr1$^{GFP/KO}$ double homozygous mice that have resident macrophages labeled with eGFP, migratory macrophages labeled with RFP, and lack the CX3CR1 and CCR2 receptors. These mice were compared to Cx3cr1$^{GFP/WT}$;Ccr2$^{RFP/WT}$ double heterozygous mice, in which resident macrophages were labeled with eGFP and migratory macrophages were labeled with RFP, but the expression of the receptors was maintained.

METHODS: Animal work was approved by Columbia University's IACUC. Cx3cr1$^{GFP/KO}$ and Ccr2$^{RFP/KO}$;Cx3cr1$^{GFP/KO}$ mice were obtained from Jackson Laboratory. Ccr2$^{RFP/KO}$;Cx3cr1$^{GFP/KO}$ mice were mated with C57Bl/6J mice to generate Ccr2$^{RFP/WT}$;Cx3cr1$^{GFP/WT}$ heterozygous mice. To load the tendon, the mice were subjected to -15° downhill running at 25 cm/sec, 40 min/day for 1-4 weeks after 1 week of training [6]. At the end of the final run, the Achilles tendon and calcaneus bone were dissected and briefly fixed in 4% paraformaldehyde for 1 hour and inspected under a Nikon microscope as a whole mount. The number of CX3CR1+ and CCR2+ cells were counted using ImageJ software. Statistical significance was determined using student t-tests. Flow cytometry was used to confirm the microscopic data: after 1 week of running, the Achilles tendons from 3 mice were pooled and digested with Liberase (1.25 mg/ml, Roche) and the cells were stained with Live/Dead Fixable Dead Cell Stain Kit (Invitrogen), CD45-BV711, and CD11b-BV510 (BioLegend). Gates were determined using spleen and bone marrow cells. Littermates allowed cage activity were used as controls. To measure MMP levels and cell proliferation, Achilles tendons were fixed in 4% paraformaldehyde at 4 °C, decalcified in Buffered Versenate (pH 7.4, StatLab), embedded in OCT, and cut to 10 µm thick cryosections. The sections were then stained with rabbit anti-MMP 3, 9, or 13 (Abcam and Sigma-Aldrich, respectively), or Ki67 (Abcam).

RESULTS: To investigate the role of resident macrophages in tendon responses to overuse conditions, we subjected Cx3cr1$^{GFP/KO}$ mice to treadmill running [6]. Overuse activity significantly increased the number of resident macrophages in the tendon by ~3-fold (Fig. 1). Ccr2$^{RFP/WT}$;Cx3cr1$^{GFP/WT}$ heterozygous mice, which had restored CCR2 and CX3CR1 expression, showed the opposite trend, with CX3CR1+ resident macrophages decreased by ~2-fold (Fig. 2). Notably, the number of CCR2+ migratory macrophages was not changed. It was expected that the CX3CR1+ resident macrophages would expand and actively respond to the damage caused by overuse conditions, as we observed in the Cx3cr1$^{GFP/KO}$ mice. However, the proliferation marker Ki67 and MMPs were only marginally detected in these macrophages (Fig. 3).

DISCUSSION: The number of CX3CR1+ resident macrophages in tendon was dependent on overuse loading conditions and on the expression of CX3CR1. In the absence of CX3CR1, resident macrophage numbers increased; in the presence of CX3CR1, resident macrophages decreased. As a chemokine receptor, CX3CR1 receives its signal from the CX3CL family of chemo-attractants, e.g., fractalkine. When overloaded, tendon fibroblasts and resident macrophages may down regulate their CX3CL chemokines to release CX3CR1+ resident macrophages. CX3CR1^{hi} resident macrophages may take residence in tendon during development and maintain their numbers by self-renewal; in contrast, CX3CR1^{lo} cells may migrate to the tendon from the circulation. The migratory CX3CR1^{lo} cells may only infiltrate the tendon under more severe conditions such as tendon rupture an/or exuberant inflammation [7]. Current approaches, however, cannot distinguish between CX3CR1^{hi} from CX3CR1^{lo} cells, and it remains to be seen if tendon resident macrophages will share functional similarities with these macrophage phenotypes observed in other tissues. The lack of robust expression of MMPs and cell proliferation (based on the marker Ki67) by tendon CX3CR1+ resident macrophages implies that these cells serve more of a sentinel role than an active tendon remodeling role.

SIGNIFICANCE: We characterized tendon resident and migratory macrophages under overuse loading conditions using KO/reporter mice. These mouse models provide novel tools to study these cells under physiological and pathophysiological conditions.

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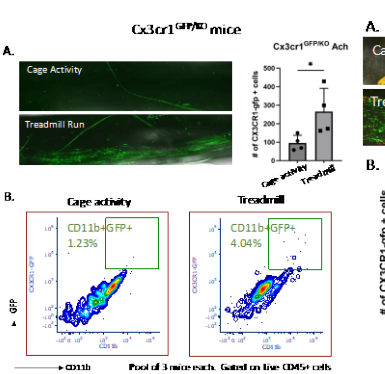

Fig 1. A) Whole mount Achilles tendons from CX3CR1$^{GFP/KO}$ mice (left) and cell counts from whole mount images (right). B) CX3CR1+ cells from cage and treadmill in CX3CR1$^{GFP/KO}$ tendons. * p < 0.05.

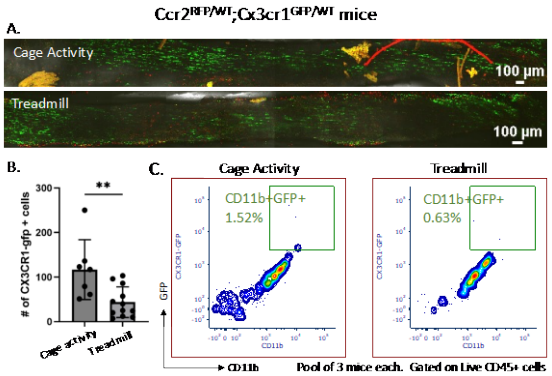

Fig 2. A) Whole mount Achilles tendon from Ccr2$^{RFP/WT}$;Cx3cr1$^{GFP/WT}$ mice. B) Cell counts from whole mount images (**:p<0.01). C) CX3CR1+ cells from cage and treadmill in Ccr2$^{RFP/WT}$;Cx3cr1$^{GFP/WT}$ tendons. * p <0.05.

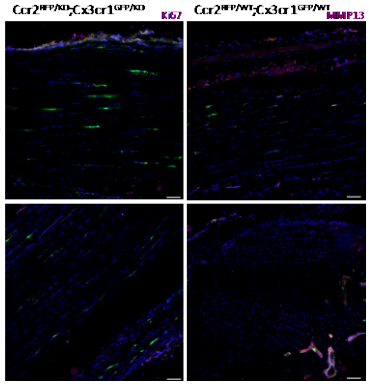

Fig 3. IF staining for Ki67 (left) and MMP13 (right) in heterozygous mice in cage (top) and treadmill (bottom) Achilles tendons.