Cited2 Mediates A Novel Chondroprotective Pathway Involving Cross-talk Between Mechanical Loading And IL-4 To Suppress Mmp-13

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Introduction: The biomechanical environment of the chondrocyte is critical in maintaining normal joint function [1]. Mechanical loading within a physiological range of intensities and frequencies is required to maintain cartilage homeostasis. Interleukin (IL)-4, mainly produced in T-lymphocytes in the thymus, is an anti-inflammatory cytokine that reduces pro-inflammatory cytokine production and activity [2]. Mechanical stimulation induces the secretion of IL-4 in chondrocytes and a chondroprotective role of IL-4 has been proposed [3]. CITED2 (CBP/P300-interacting transactivator 2, also known as MRG-1) is a multiple stimuli response transcriptional regulator, originally identified as an IL-4-inducible molecule in T-lymphocytes [4] and a critical mediator that down-regulates MMPs during moderate loading [5-7]. Collectively, these findings raised the following questions: (1) Is CITED2 inducible by IL-4 in chondrocytes? (2) Does CITED2 serve as a common mediator for both IL-4 and mechanical loading? (3) Could cross-talk between the anti-inflammatory and mechanical loading pathways result in synergy that would serve as a new intervention for chondroprotection in articular cartilage? Accordingly, we carried out the in vitro and in vivo studies to address these questions.

Methods: C28/I2 chondrocytes were treated with IL-4 (R&D) at various concentrations or uniaxial strain at (0 to 10%, 1Hz) in the presence of IL-1β, or inhibitors of Jak3, or STAT6 (Santa Cruz). IL-4 knockout mice or the wild-type littermates (5-6 mo., male, n=6/group) were treated with intra-articular injection of CITED2 siRNA, 24 hours before they were subjected to moderate treadmill running at 10 m/min for 45 min. Cited2 and Mmp13 mRNA levels were analyzed by real-time PCR with GAPDH as an internal control. Phosphorylation of Jak3 and Stat6 was analyzed by Western blotting using Jak3 and Stat6 phosphorylation-specific antibodies (Cell Signaling). Data were analyzed using Student t-test with p<0.05 as an indicating a significant difference between the treatment and control.

Results: Effect of IL-4 on CITED2 and MMP-13 mRNA. CITED2 gene expression is inducible by IL-4 in a dose-dependent manner (Fig. 1A) and with a time course of peak induction at 8 hrs, declining thereafter (Fig 1B), which was associated with downregulated MMP-13 gene expression. A previous study demonstrated that Jak and Stat play a critical role in mediating IL-4 signaling [8]. Targeted screening using a signaling inhibiting approach revealed that the induction of CITED2 by IL-4 was abolished by JAK3- or STAT6-specific inhibitors (Fig 1C). Western blotting further demonstrate that upregulation of CITED2 is mediated by phosphorylation of Jak3 and STAT6 in C28/I2 chondrocytes (Fig 1D).

Effects of loading on CITED2 and MMP-13 mRNA, alone or in combination with IL-4. Expression of CITED2 was induced by moderate loading at 2.5% uniaxial strain at 1Hz for 1hr (Fig 2A). Moderate
loading (2.5% uniaxial strain, 1Hz, 1 hr), when combined with IL-4 (1ng/ml), elevated CITED2 mRNA to a level significantly higher than that induced by loading or IL-4 alone, but to a level similar to that achieved with a high dose of IL-4 (10ng/ml). Interestingly, IL-4 treatment alone at 1 ng/ml exerted no significant effect on CITED2 induction. However, the level of CITED2 expression increased from 1.75 to 2.89-fold when IL-4 treatment was combined with moderate loading. MMP-13 expression, which correlated inversely with CITED2 expression, was downregulated from 0.58 to 0.25 compared to untreated control (Fig 2A). Similar results were observed with the combination treatment of IL-4 at 0.1ng/ml and moderate fluid shear (5dyn/cm2) and the time course studies show a prolonged effect on both CITED2 induction and MMP-13 downregulation (data not shown). This downregulation was abolished in chondrocytes when CITED2 was knocked down by siRNA prior to the relevant treatment (Fig 2B).

Effects of loss of function of IL-4 and Cited2 on Mmp13 downregulation induced by moderate loading. Moderate treadmill running (10 m/min, 45 min) reduced Mmp13 mRNA levels in chondrocytes in the knee articular cartilage of wild type mice. This reduction was partially abolished in IL-4 knockout mice and completely abolished in the chondrocytes when CITED2 expression was knocked down by intra-articular injection of Cited2 siRNA in knee joints (Fig 3).

Discussion: Our studies suggest that two stimuli, IL-4 and mechanical loading, independently and collaboratively, to activate a common mediator CITED2, and induce anti-catabolic cellular responses leading to the down-regulation of proteolytic enzymes such as MMP-13. IL-4 has been used for clinical trials for cancer, but has not been well received due to its side effects [9, 10]. Our study provides evidence that the effects of IL-4 at low doses can be significantly amplified by mechanical loading, at least on the anti-catabolic effects such as suppressing MMP-13, and may provide a new intervention strategy to prevent cartilage degradation and serve as an OA therapeutic.

Significance: We identified a novel cross-talk between the IL-4 and moderate mechanical loading, in which the transcriptional regulator CITED2 is the common mediator. This study demonstrated a proof-of-concept for a potential osteoarthritis intervention with moderate loading, through exploiting the ability of CITED2 to amplify the anti-catabolic effect of IL-4 (i.e., inhibition of MMP-13) to avoid side effects.
**Fig 3.** Moderate treadmill running suppresses Mmp-13 expression in the articular cartilage. This effect is partially abolished in IL4KO mice and completely abolished in IL4KO mice with Cited2 knocked down.