Inhibition of the NF-κB pathway suppresses macrophage-induced inflammatory response in the subacromial bursa *in vitro*

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INTRODUCTION: The subacromial bursa is a tissue located between the acromion and the tendons of the rotator cuff. It has previously been described as a synovial-like tissue, although it extends medially beyond the acromion to become a primarily adipose tissue which sits on top of the supraspinatus tendon and muscle. The bursa becomes biologically activated and inflamed in response to rotator cuff tendon injury, and it has a potential role in regulating its paracrine environment to affect the underlying tendon [1]. Because of its response to tendon injury, location directly above the rotator cuff, and its fatty nature, the bursa holds great promise as a therapeutic target to modulate rotator cuff healing. A proof-of-concept experiment using dexamethasone delivery to the bursa showed the potential for the bursa to so serve an immunomodulatory role in the rotator cuff [1]. However, despite these prior studies, several factors need to be clarified before the bursa can be effectively targeted, including, e.g., determining mechanisms of activation and whether bursa activation is due to phenotypic shifts in native cells or due to infiltrating immune cells. Therefore, the current study expands on our prior *in vitro* model, which was limited to the use of a single inflammatory cytokine (IL-1β) for bursa activation and a broadly acting anti-inflammatory drug (dexamethasone), by activating the bursa with M1 macrophage conditioned media (mimicking the milieu of cytokines present after tendon injury) and modulating the NF-κB-dependent inflammatory response. Inflammation in rotator cuff pathology is mediated by NF-κB [2], and systemic inhibition of NF-κB can enhance rotator cuff tendon healing [3], making this pathway an attractive therapeutic target. The goals of this *in vitro* study were to: *(i)* investigate the activation of the bursa using physiologically relevant M1 macrophage conditioned media (M1CM) and *(ii)* investigate the effects of NF-κB inhibition on the activated bursa.

METHODS: *Study design:* To investigate activation of the bursa via inflammatory factors, bursas were cultured in M1CM (Bursa + M1CM) or normal media (Bursa alone) for 72 hours (**Figures 1 and 2**). To investigate the role of NF-κB, bursas were cultured with M1CM for 72 hours before being supplemented with the IKKB inhibitor ACHP (Bursa + M1CM + ACHP) or replaced with non-inflammatory media (Bursa + M1CM removed) for 24 hours. Control groups consisted of bursa cultured in normal media for 96 hours (Bursa alone), bursa in normal media with ACHP added for the last 24 hours (Bursa + ACHP), and bursa in M1CM for the full 96 hours (Bursa + M1CM) (**Figure 3**). Cells and tissues were isolated from 13-14 week old Sprague-Dawley rats (equal numbers of male and female). *M1CM collection:* Macrophages were isolated from rats (n=3-5) and polarized to a pro-inflammatory M1 macrophage phenotype by LPS and IFN-γ stimulation. After 24 hours, the stimulation media was removed and replaced with fresh media, which was harvested after 24 hours. *Bursa explant culture:* Bursa explants were dissected from freshly euthanized rats and cultured according to previous methods [1]. *In vitro activation and ACHP delivery:* Bursa explants were activated by adding M1CM 1:1 with the culture media for 72 hours (n=5 paired). Additional bursa explants were activated with M1CM for 72 hours before adding 1 μM solubilized ACHP hydrochloride (MedChemExpress) to the M1CM, or removing the M1CM and replacing with normal media for 24 hours (n=4-8). *Immunohistochemistry:* Bursa tissue was fixed and cryosectioned, then stained with CD68 and imaged with a fluorescent microscope. *Gene expression:* Bursa tissue was homogenized in TRIzol and RNA was isolated. RT-qPCR was performed for genes related to inflammation to confirm activation of bursas in response to M1CM and to determine the effects of ACHP treatment after M1CM activation. ΔCt was normalized to *Gapdh* and results were plotted as fold change relative to bursa alone. *Statistics:* Statistical significance ($p<0.05$) for the 72-hour bursa activation was determined using paired t-tests. Statistical significance ($p<0.05$) for activation and ACHP delivery was determined using one-way ANOVA with Fisher's LSD test.

RESULTS SECTION: CD68+ resident macrophages were observed in bursas cultured in normal media or in M1CM for 72 hours (**Figure 1**). Activation of the bursa via M1CM led to upregulation of *Il6* (3.3x increase), *Il1b* (9.8x increase), *Cox2* (12.1x increase), and *Inos* (25.5x increase) (**Figure 2**). Inhibition of IKKB via ACHP or replacing the M1CM with non-inflammatory media led expression of *Il6*, *Il1b*, *Cox2*, and *Inos* to return to baseline (**Figure 3**). No differences were seen in *Tnfa* or *Cd68* gene expression with the addition of ACHP compared to the M1CM group (**Figure 3**).

DISCUSSION: We previously validated an *in vitro* model that can be used to activate and study the bursa in an inflammatory environment [1]. The current study expands on that model by providing a more physiologically relevant inflammatory stimulus to simulate the robust M1-driven inflammatory response seen after tendon injury. M1CM may induce broader or synergistic transcriptional changes in the bursa compared to more targeted effects of using a single inflammatory cytokine. The bursa was activated in this new model, as demonstrated by upregulation of inflammatory genes. The return to baseline expression of *Il6*, *Il1b*, *Cox2*, and *Inos* after inhibiting IKKB indicates a key role for the NF-κB pathway in bursa activation, and its inhibition can mitigate the inflammatory response of the bursa. Similar results were seen when M1CM was replaced with normal bursa media, which suggests that NF-κB inhibition in an inflammatory environment has a similar effect on the bursa as shifting to a non-inflammatory environment. CD68 staining revealed resident macrophages in the bursa explants, which could have a role in responding to the inflammatory activation and inhibition. Future work will use sequencing techniques to comprehensively investigate changes in gene expression and as well as cell phenotype shifts in response to activation. In the longer term, the therapeutic effects of targeting the bursa with ACHP will be tested in an *in vivo* rat rotator cuff injury and repair model.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates activation of the subacromial bursa *in vitro* via M1CM. It also demonstrates the ability to mitigate this inflammatory activation via ACHP delivery to suppress the NF-κB pathway.

REFERENCES: [1] Marshall B. et al., Sci Transl Med., 16(744):eadd8273, 2024, [2] Abraham, A. et al., Sci Transl Med, 11(481):eeav4319, 2019, [3] Golman, M. et al., Am J Sports Med, 49(3):780-789, 2021.

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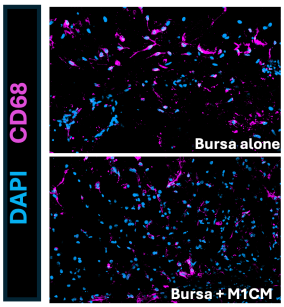

Figure 1. CD68 expression in Bursa alone or Bursa + M1CM.

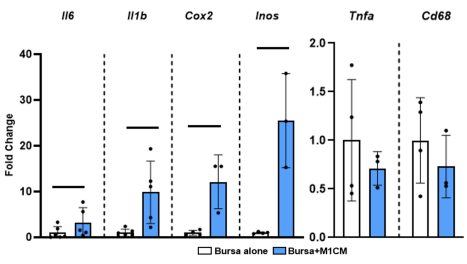

Figure 2. Gene expression of *Il6*, *Il1b, Cox2, Inos, Tnfa, and Cd68* in the bursa activation group. Solid bars denote significance of $p<0.05$.

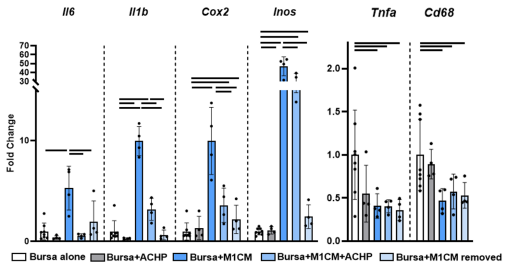

Figure 3. Gene expression of *Il6*, *Il1b, Cox2, Inos, Tnfa, and Cd68* in the bursa activation/ACHP supplementation groups. Solid bars denote significance of $p<0.05$.