Biologic and Transport Functions for the ACL Sheath Under Inflammatory Conditions

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INTRODUCTION: Following rupture of the anterior cruciate ligament (ACL), most patients undergo reconstructive surgery because the ACL has shown to have a poor intrinsic healing capacity[1,2,3]. While ACL reconstruction is effective for restoring athletic function to patients, donor site morbidity[4] and the development of osteoarthritis (OA)[5] are common sequelae. Recent efforts have highlighted the healing capability of the ACL when supported by a bioactive scaffold in both animal and human subjects, allowing for primary repair without reconstruction[6]. Furthermore, it has been shown that the native healing ability of the ligament is suppressed by synovial fluid[1,2,3], which is mainly comprised of hyaluronic acid (HA) and lubricin (PRG4)[7]. It is likely that the synovial fluid of injured patients contains inhibitory factors that exacerbate the injury and attenuate remodeling and healing by the native ACL. *In situ*, healthy uninjured ACL is surrounded by a vascularized lining, or sheath, which has similarities to the synovium of the joint capsule[8]. Animal studies have shown that the sheath has a composition distinct from the ACL core, made up of primarily collagen type IV as well as distinct cell surface markers[9]. Moreover, our previous findings showed increased expression of HA-binding cell surface receptor, CD44, in ACL sheath compared to core. Clinical studies reported that patients with an intact synovial sheath showed improved recovery after primary repair compared to those without intact coverage of the tissue[10]. A barrier function of the synovium has been reported before, where synovial macrophages provide protection against inflammation in the knee[11]. We infer that the sheath can serve a comparable barrier function for the ACL while allowing key nutrients to freely diffuse into the ligament core. The goal of this study is to characterize the molecular profiles of healthy and OA sheath and core ACL, and investigate the sheath's protective potential of core cells against inflammatory insults in co-culture. Moreover, we investigated the core and sheath diffusivities to begin examining the potential of sheath to serve as a physical protective barrier to ACL core.

METHODS: *Human Model:* Human ACL tissues were collected from OA knee joints of donor patients undergoing total knee arthroplasty (n=5; Columbia University, IRB AAAQ2703) and healthy donors from the National Disease Research Interchange (n=3; NDRI). Tissues were divided into two categories: ACL core, from the ligament proper, and ACL sheath, from the thin membrane surrounding the ACL. The core and sheath tissues were prepared for histology or cell isolation. *Monolayer Cell Culture:* Viable cells were isolated using collagenase type II and expanded in αMEM containing 10% FBS and 1% penicillin-streptomycin. Upon reaching confluency, cells were separated into control and treatment groups, where the media was supplemented with 20 ng/mL IL-1β for 24hrs. *In vitro Co-culture:* To investigate the core-sheath crosstalk during inflammation, core and sheath cells from n=2 healthy donors were separately primed with IL-1β in a Transwell insert (Corning) for 24 hrs before being placed in culture with a naïve ACL core monolayer for another 24 hrs. *Gene Expression:* Total RNA was isolated from cell monolayers using the RNeasy Mini Kit (Qiagen) and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was conducted using PowerUP SYBR Green Mastermix (Applied Biosystems) to analyze expression of *IL6*, *MMP3*, *COX2*, *TNC*, *COL1A1*, *COL4A1*, *CX3CR1*, *CLDN5*, and *αSMA*. *Diffusion:* ACL tissues with intact sheath were dissected from whole juvenile bovine knees obtained from a local abattoir. The tissues were washed in sterile PBS and then incubated overnight in 50 µg/mL fluorescein isothiocyanate-tagged HA (FITC-HA) ± synovial fluid containing HA and PRG4 (n=4-5; Animal Technologies). 4% w/v agarose (ThermoFisher) gels were created as tissue phantoms (n=6). Following this, fluorescent recovery after photobleaching (FRAP) of the samples was conducted to determine diffusion coefficient of the ACL core vs. sheath. *Statistics:* Relative gene expression was calculated using the ΔΔCt method normalized to *GAPDH* and respective controls. All values were reported as mean ± standard deviation and analyzed using a two-way ANOVA with Fisher's LSD (qPCR) or Tukey's HSD (FRAP) post-hoc test where α=0.05.

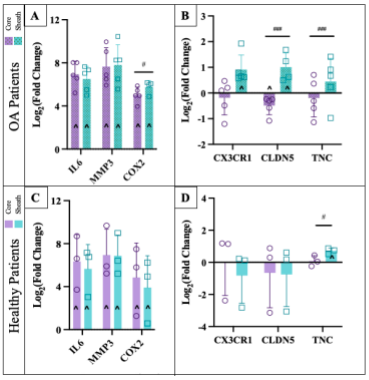

Figure 1. Gene expression data from OA and healthy patients. **A**. *IL6*, *MMP3*, and *COX2* expression following IL1β treatment of OA core and sheath cells. **B**. *CX3CR1*, *CLDN5*, and *TNC* expression of OA core and sheath cells. **C**. *IL6*, *MMP3*, and *COX2* expression following IL1β treatment of healthy core and sheath cells. **D**. *CX3CR1*, *CLDN5*, and *TNC* expression of healthy core and sheath cells. Two-way ANOVA with Fisher's LSD post-hoc test for multiple comparisons was run on each group for comparison of treated vs. controls (^ $p < 0.05$) and differential expression of core vs. sheath (# $p < 0.05$. ## $p < 0.01$).

RESULTS: *Monolayer Gene Expression:* The inflammatory marker *IL6* was significantly upregulated in OA core and sheath compared to controls ($p<0.0001$) in treated groups, with no difference observed between the core and the sheath. A similar trend in *IL6* was observed in core and sheath cells from healthy donors ($p = 0.002$ & 0.004, respectively). *MMP3* and *COX2* were significantly upregulated across all subgroups ($p<0.05$) (**Fig. 1A**). Both the core and sheath demonstrated measurable expression of the ligament marker *TNC* in OA and healthy samples, but expression was only significantly upregulated in the healthy sheath ($p=0.04$). *COL1A1* was downregulated following IL-1β treatment in the OA sheath ($p=0.04$), but neither cell types showed significant changes in *COL4A1* expression (data not shown). Significant upregulation of the tight-junction marker, *CLDN5*, was detected in the OA sheath compared to the core ($p=0.003$). Chemokine receptor *CX3CR1* expression was significantly upregulated in the OA sheath ($p=0.01$). No significant differential expression of *αSMA* was observed (**Fig. 1B**). Healthy cells were likewise significantly responsive to IL-1β treatment compared to respective controls ($p<0.05$) but no differential expression between subgroups was observed (**Fig. 1C**). Healthy human sheath cells significantly upregulated *TNC* expression in comparison to both controls and corresponding core ($p=0.03$) (**Fig. 1D**). *Protective Potential of the Sheath:* ACL core monolayers showed a baseline upregulation of *IL6*, *MMP3*, and *COX* in response to IL-1β treatment. When IL-1β-primed sheath cells were added to core monolayers, a downregulation of the inflammatory panel of *IL6*, *MMP3*, and *COX2* in the untreated core monolayer cells was observed, however no statistical significance was found. (**Fig. 2A**). FRAP was conducted on bovine ACLs to model diffusion characteristics of HA within the sheath versus the core. 1.5 MDa FITC-HA demonstrated significantly higher diffusion through the bovine ACL core than the outer sheath while in the presence of whole synovial fluid ($p<0.0001$). The presence of synovial fluid during overnight incubation did not significantly alter the diffusion of FITC-HA through agarose phantoms (**Fig. 2B**).

DISCUSSION: This study has characterized the molecular and diffusivity differences between the ACL core and sheath in the context of OA and healthy conditions in the knee. The vascularized sheath has characteristics of the ligament, but is a distinct and unique structure. Similar to the ACL core, the sheath is impacted by OA conditions: upon IL-1β insult, the expression of matrix remodeling genes was increased. The intact sheath can significantly impede the diffusion of high molecular weight HA from reaching the ligament and may also serve as a scaffold for *CX3CR1*-expressing cells to respond to chemical factors and further segregate the ACL from its surroundings. This process may be mediated by tight junctions, as *CLDN5* was differentially expressed in the sheath compared to the core. Cells derived from younger, healthier tissues appeared to be less sensitive to the IL-1β treatment, however these cells demonstrated a similar upregulation in key inflammatory makers. The study was limited by the availability and diversity of healthy ACL donors as well as short duration of cytokine insult. A treatment regimen modeling chronic inflammatory environment could reveal new differences in these cell types using the same outcome measures.

SIGNIFICANCE/CLINICAL RELEVANCE: This body of work will aid in the development of novel regenerative technologies for primary ACL repair.

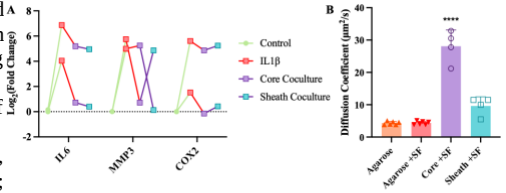

Figure 2. A. Expression ACL core monolayers to IL1β, primed core, or primed sheath cells. **B**. Diffusion coefficient of FITC-HA in bovine ACL compared to agarose phantoms. Two-way ANOVA with Tukey's HSD post-hoc test for multiple comparisons was run. **** $p < 0.0001$ compared to all other conditions.

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