Biologic and Transport Functions for the ACL Sheath Under Inflammatory Conditions

James Rogot1, Jennifer A. Kunes2, Minkyu Kim3, Roshan P. Shah4, Christopher S. Ahmad2, Nadeen O. Chahine1,5, Clark T. Hung1,2, Stavros Thomopoulos1,2, Beth G. Ashinsky3

1Department of Biomedical Engineering, 2Department of Orthopedic Surgery, Columbia University, New York, NY 543774@alumni.columbia.edu

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.1-3 While ACL reconstruction is effective for restoring athletic function to patients, donor site morbidity4 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 (SF), 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 and may serve as a distinct cell-surface barrier to the ACL.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 the 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; NDRR). 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 confluence, cells were cultured in 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 5 µm pore-size filter as a barrier between core and sheath cells. The inflammatory cytokine, IL-1β, was added to the core layer, while the viable sheath cells were added to the sheath layer. IL-1β treatment was conducted using PowerUP SYBR Green Mastermix (Applied Biosystems) to analyze the expression of IL6, MMP3, COX2, TNC, COLA1, COLA4, CXCR1, CLDN5, and αSMA. Fig. 1B: In the OA sheath, a significant downregulation of the inflammatory panel of genes was observed, however no significant differential expression was observed between control and treatment cells. Cells 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 the expression of IL6, MMP3, COX2, TNC, COLA1, COLA4, CXCR1, 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 in 500 µL of 1,5 MDa FITC-HA ± synovial fluid containing HA and PRG4 (n=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 post-hoc test for multiple comparisons.

RESULTS: Monolayer Gene Expression: The inflammatory marker IL6 was significantly upregulated in all subgroups (p<0.0001) in treated groups, with no difference observed between the core and sheath. A similar trend in IL6 was observed in control 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). COLA1 was downregulated following IL-1β treatment in the OA sheath (p=0.04), but neither cell type showed significant changes in COLA1 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 CXCR1 expression was significantly upregulated in the OA sheath (p=0.01). No significant differential expression of αSMA was observed. (p>0.05). Both cell types were downregulated compared to respective controls (p<0.05). Total RNA did not show an apparent expression between subgroups in the core (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.

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 distinct from the ACL core 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 markers. The study was limited by the availability and diversity of healthy ACL donors as well as 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.