

Interleukin Receptor Therapeutics Attenuate Synovial Inflammation in Canines following Cruciate Ligament Injury

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Introduction: Knee injuries create instability that perpetuates inflammation, resulting in irreversible damage to all joint structures across species.¹ In companion dogs, tears to the cranial cruciate ligament (CCL), the anatomic equivalent to the human anterior cruciate ligament (ACL), are a leading cause of joint disease.² While treatment in both species focuses on restoring knee mechanical stability via surgical correction, few therapeutics treat the synovial pathology that perpetuates joint-wide inflammation. Despite this, recent research shows that synovial inflammatory markers in human patients after ACL injuries may be predictive of cartilage damage severity and clinical outcomes after repair.³ We posited that canines with spontaneous CCL tears may be an ideal model to evaluate novel interventions following injury, given their similarities to human disease progression. To establish this model, we first evaluated the structural and micromechanical changes in canine knee synovium following spontaneous CCL injury in comparison to healthy donors. Next, we used RNA sequencing to identify transcriptional changes in the synovium of dogs presenting with spontaneous CCL tears relative to healthy controls. Using this information, we tested whether the IL1 receptor antagonist (Anakinra) or the IL6 receptor inhibitor (Tocilizumab) could attenuate inflammation in synovial organ cultures of dogs with CCL tears.

Methods: Synovium Histology and Scoring: Synovial samples were collected from healthy or CCL canine joints (n=10/group), stained with hematoxylin-eosin (H&E), and scored for synovitis per OARSI guidelines⁴ by 4 blinded reviewers (Fig 1A-B). **Atomic Force Microscopy (AFM):** Histology guided AFM (Fig 1A-B,D) was applied to 40 µm-thick cryo-sections (healthy or CCL synovium, n=5/group) in PBS using a microsphere tip ($R \approx 6 \mu\text{m}$, nominal $k \approx 0.6 \text{ N/m}$) and a Dimension Icon AFM. For each region (subintima and intima), the effective indentation modulus was calculated⁵ for 15-20 locations. **RNA Sequencing:** Illumina truSeq stranded poly-A libraries were generated and single-end reads were sequenced using an Illumina NovaSeq. Deseq2 was used for differential expression analysis (n=5/group). Gene ontology was carried out using DAVID (Fig 2B). **qPCR of Canine Synovium:** Healthy (n=10) or CCL tear (n=14) synovium was collected and processed for RNA extraction and cDNA synthesis prior to qRT-PCR. **Synovial Organ Culture:** CCL tear synovium (n=5/group) was collected and cultured for 3 days with either an IL1 receptor antagonist (100ng/mL) or IL6 receptor inhibitor (100ng/mL). qPCR was conducted as above (Fig 3A-B). **Statistical Analyses:** For synovium scoring, a Mann-Whitney test was used. For qPCR of healthy/diseased synovium, unpaired t-tests were used. For AFM and synovium organ culture and treatment, 1-way ANOVA was used.

Results: Synovium from dogs with CCL tears showed increased inflammation, vasculature, fibrosis, and hyperplasia compared to healthy synovium (Fig 1A-C). CCL synovium also had a higher indentation modulus in both the intimal (not shown) and subintimal (Fig 1D) regions compared to healthy controls. RNAseq revealed that CCL synovium had 766 genes upregulated and 365 genes downregulated ($p < 0.0001$) compared to healthy controls. Gene ontology of these DEGs identified immune response and cell adhesion as two of the most enriched pathways, with increased expression of interleukins (IL6 and IL1) as well as ACAN (aggrecan) and POSTN (perlestin) (1.5-fold cutoff, $p < 0.05$) (Fig 2A-E). In addition, genes associated with negative regulation of Rho protein signaling were reduced (Fig 2A-C,F). In CCL synovium explants, expression of PRG4 (lubricin) decreased while the inflammatory markers IL1 β , IL6, and PTGS2 (the gene encoding cyclooxygenase-2) increased (Fig 3B). When CCL synovium was treated with an IL1 receptor antagonist or an IL6 receptor inhibitor, both restored PRG4 expression to control levels while decreasing expression of IL1 β , IL6, and PTGS2 (Fig 3B).

Discussion: This study shows that structural and mechanical synovial disease progression in canine patients with spontaneous CCL tears mimics that of human patients with ACL tears.⁶ Notably, both RNAseq and AFM showed some heterogeneity across donors in terms of degree of fibrosis and activation of specific genes, with patients with a shorter history of disease anecdotally showing less changes. This suggests that a tailored approach, based on the stage of disease progression, may be necessary to increase clinical efficacy of treatments. Importantly, both the human formulation of an IL1 receptor antagonist (Anakinra) and an IL6 receptor inhibitor (Tocilizumab) successfully downregulated critical inflammatory mediators, IL1 β , IL6, and PTGS2, and restored expression of PRG4, a glycoprotein necessary for cartilage health.⁷ While interleukin receptor targets were effective at downregulating inflammatory pathways in diseased synovium, it has not yet been established if these molecules can reverse or halt the progression of synovial fibrosis. To address this, current studies are evaluating the efficacy of interleukin receptor therapeutics in a clinical trial in which these agents are applied intraarticularly to client-owned canines with CCL tears after tear diagnosis and prior to surgical intervention.

Significance/Clinical Relevance: Canines with spontaneous CCL tears have a knee joint environment that mimics the structural, mechanical, and molecular phenotypes of humans with ACL tears – providing a valuable model to evaluate novel therapeutics targeted at knee joint pain and inflammation after injury.

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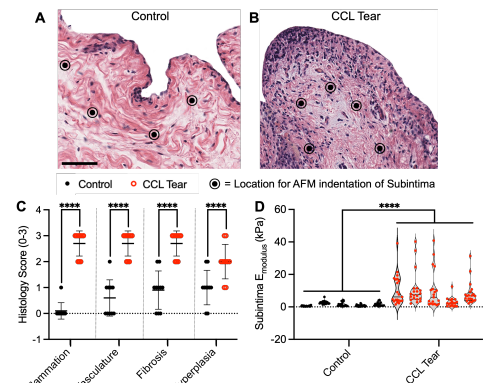


Fig 1. H&E images (20X) of (A) healthy control and (B) CCL tear synovium. Scale bar: 100µm. (C) Mean synovial histology score. (D) AFM modulus. Mean +/- SD, **** indicates ($p < 0.001$).

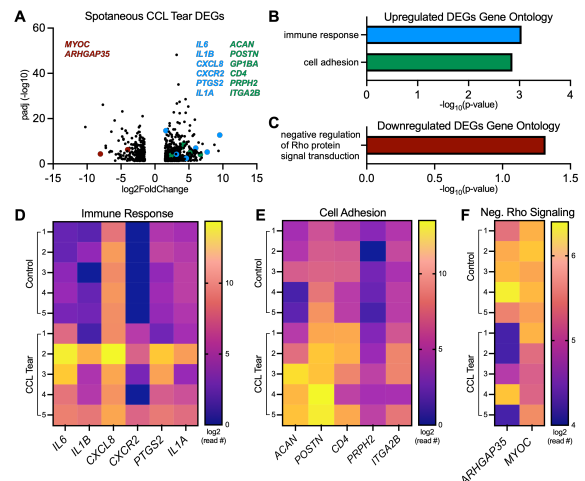


Fig 2. RNAseq results with (A) volcano plot of DEGs, (B) gene ontologies identified from DEGs, and (D-F) heat maps of individual genes in each pathway across the five donors.

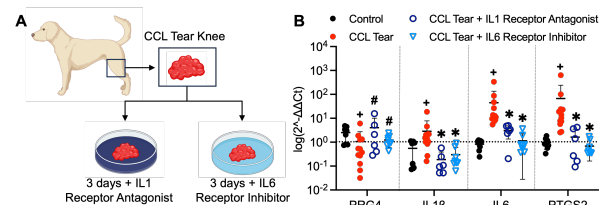


Fig 3. (A) Schema of diseased synovial cultures treated with IL1 or IL6 receptor therapeutics. (B) qPCR of inflammatory and anabolic genes in each condition. + indicates difference ($p < 0.05$) vs. control, * indicates difference ($p < 0.05$) or # ($p < 0.1$) vs. CCL tear synovium.