Imaging Mass Cytometry Reveals a Dynamic Synovial Cellular Landscape in Canine Cruciate Ligament Disease

Lola Uliano[1,2,3], Kevin G. Burt[1,2], Elisabeth A. Lemmon[1,2,3], Janai Augustin[1,2], Carla R. Scanzello[2], Sarah Gullbrand[1,2], Kim Agnello[3], Robert L. Mauck[1,2]
[1]Dept. of Ortho. Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [2]Translational Musculoskeletal Research Center, Crescenz VA Medical Center, Philadelphia, PA; [3]University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA.

Disclosures: LLU (N) - ulianol@pennmedicine.upenn.edu, KGB (N), EAL (N), JA (N), CS (N), SEG (6), KA (N), RLM (5 – 4WEB Medical).

INTRODUCTION: Disease of the canine cranial cruciate ligament (CCL), the functional equivalent of the human anterior cruciate ligament (ACL), results in marked synovial inflammation and fibrosis, both of which are thought to drive progressive joint degeneration and pain in CCL disease (CCLD).[1,2] In a recent clinical case review, we found a strong correlation between the duration of clinical symptoms and arthroscopic scores of synovial inflammation.[3] Analysis of explanted synovial biopsies from CCL dogs also showed increased inflammation and fibrosis (evaluated on H&E-stained sections), greater stiffness (evaluated by atomic force microscopy), and clear inflammatory/immune cell activation (evaluated by bulk RNA sequencing).[4] However, these prior studies did not evaluate cellular phenotypes or spatial organization in the diseased canine synovial microenvironment. Prior work, using multiplex imaging mass cytometry (IMC) of mouse synovium, showed dynamic changes to the murine joint synovium following injury, inclusive of fibroblast activation and immune cell infiltration.[5] Here, we used IMC to evaluate changes in the cellular landscape of naturally occurring diseased CCL canine synovium, with a particular focus on fibrotic (COL1, α-SMA, fibronectin), inflammatory/immune (CD68, CD14, CD11B/C, CD20), and vascular (VEGF) markers. To our knowledge, this represents the first application of imaging mass cytometry to the analysis of canine tissue in a musculoskeletal context. We hypothesized that CCLD synovium from patients with advanced disease would show increases in cell populations expressing fibrotic, immune, and vascular markers.

METHODS: CCLD Samples: Synovium from canine patients with CCLD (n=8) and healthy canine patients (n=7) were collected from a cohort of dogs (3-6 years old), as previously described.[4] Synovial Scoring: H&E-stained synovial sections were scored for fibrosis and inflammation, with samples from each cohort spanning the range of disease severity within each category. Region of Interest (ROI) Selection: 400 x 400 µm ROIs were selected based on adjacent H&E sections, with two ROIs in the intima and two in the subintima for each sample. Biomarker Selection: Markers of interest were selected from a Hyperion-compatible human IMC panel based on confirmed cross-reactivity with canine targets. Cross-reactivity was further confirmed by the detection of markers within canine spleen (not shown). IMC (n=7-8): Paraffin-embedded synovial sections were subjected to heat-induced antigen retrieval and then incubated overnight with a 14-marker panel of metal-tagged antibodies. Nuclei were counterstained with Intercalator-IR, and imaging was subsequently carried out on a Hyperion Imaging System (Standard BioTools). Spatial Protein Expression and Cellular Phenotype Analysis: Single-cell masks were generated using the nuclear stain via DeepCell (deepcell.org). Data was normalized using arcsin transformation, and clustering was performed with t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction in IMACytE software.[6] Statistical Analysis: Welch's t-test or Pearson's correlation, with $p<0.05$ significant.

RESULTS: Histologic evaluation demonstrated structural features of synovitis, including synovial lining hyperplasia and expansion of the sub-synovial stroma with collagen deposition, all of which were more pronounced in sections from CCLD patients whose synovia had higher inflammation and fibrosis scores (**Fig. 1f).** IMC based cellular phenotyping of these samples revealed 8 distinct immune and non-immune cell clusters that were separated via unique levels of protein marker expression (**Fig. 1a, b**). Specifically, synovial fibroblast clusters (Clusters 1, 2, 3) were characterized by low expression of CD45 and other immune defining markers and higher expression of COL-1 and αSMA. The immune focused panel identified a possible CD20^{high} B cell cluster (Cluster 6) and multiple likely macrophage clusters (Clusters 7 & 8) expressing CD45, CD68, and CD163 (**Fig. 1a, c**). Comparing across groups showed an overall greater number of cells per ROI in CCLD samples compared to healthy controls (**Fig. 1c**). Quantification of subpopulations across groups revealed dynamic changes in likely fibroblast clusters (Clusters 1 & 2), where CCLD samples had a significantly greater number of cells (Cluster 2: p=0.0098) compared to healthy controls (**Fig. 1d**). When IMC results were correlated with synovial histopathologic scoring, a significant negative association was found for Cluster 1 (Undefined stromal cell, $r_p = -0.8335$, $p = 0.0296$) and a positive correlation for Cluster 4 (CD68+ Fibroblast, $r_p = 0.8335$, $p = 0.0496$) (**Fig. 1e**). It was not clear if this is a proper fibroblast population or if IMC is capturing fibroblasts and macrophages in close proximity to one another. Mapping these clusters back to the anatomic specimen showed an increase in Cluster 4 cells located both in the subintima and intima of the most diseased samples (**Fig. 1f).**

DISCUSSION: Simultaneous detection of multiple proteins in a spatially discretized manner with imaging mass cytometry can identify diverse cellular populations within a single tissue sample. Here, IMC revealed a significant increase in fibroblasts within the synovium of CCLD patients compared to healthy controls. These findings align with expectations of synovial remodeling under diseased conditions, where fibroblasts proliferate and deposit extracellular matrix — processes that are absent in healthy joints.[5] In addition, combining standard histopathologic metrics with IMC quantification revealed that a CD68+VEGF+ macrophage and/or fibroblast population (or the two cells in close proximity) positively correlated with histological measures of synovial inflammation. These results support the hypothesis that macrophage cell populations, possibly localized near VEGF+ endothelial cells and fibroblasts, are present in higher numbers within the inflamed synovium, and that their numbers scale with disease severity.[8] These findings are consistent with prior work describing the pro-inflammatory synovial landscape following human ACL injury, supporting the similarity in the post-injury synovial pathologic response across species. supporting the translational relevance of canine synovium as a model of human pathology and a testbed for therapeutic evaluation.[7]

SIGNIFICANCE: Our findings support the translational relevance of the canine CCLD model of spontaneously occurring joint injury as a testbed for evaluation of novel therapeutics, particularly those targeted to synovial inflammatory processes. This work demonstrates that imaging mass cytometry can serve as a novel tool to detect subtle changes in the synovial cell landscape during disease progression and treatment, while also establishing its application to canine tissue in a musculoskeletal context, thereby broadening the framework for analyzing joint disease and informing the development of targeted therapeutic strategies.

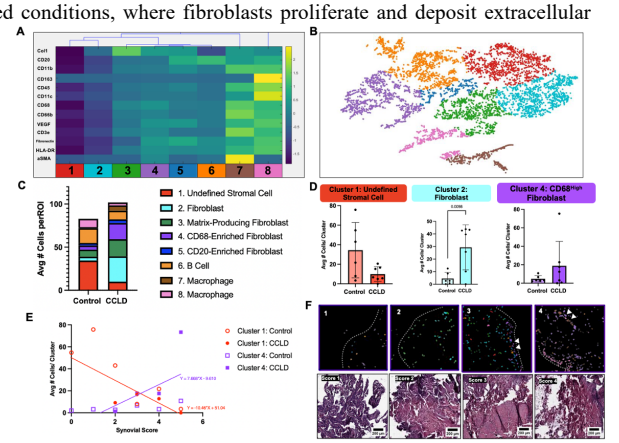

Figure 1: ***IMC analysis of synovial cell phenotypes with CCLD.*** *A) Marker expression heatmap identifying clusters and B) t-SNE plot showing all cells identified. (C) Average number of cells per ROI for each group (and distribution of subclusters). D) Comparisons within individual cell clusters showing change across disease state. E) Correlation of cells within clusters relative to H&E-assigned synovial score (Cluster 1: r = -0.8335, p = 0.0393; Cluster 4: r = 0.8305, p = 0.0406). F) Cell cluster assignment in synovium and corresponding H&E stained sections (1 = less pathology, 4 = more pathology). Synovial lining = white dashes.*

REFERENCES: [1]Wright+ J Small Anim Pract (2023), [2]Johnston+, Vet Clin (1974), [3]Lemmon+ Vet Surg (2025) [4]Lemmon+ OA&C (2024), [5]Burt+ Biorxiv (2025), [6]Somarakis+ IEEE Trans Vis (2020), [5]Magarian+ Knee (2011), [6]Cialdi+ Front Bioeng Biotechnol (2022), [7]South+ OA&C Open (2023), [8]Zhao+ Front Immunol (2023).

ACKNOWLEDGEMENTS: Funding – NIH (T35 OD010919) and the Penn Center for Musculoskeletal Disorders (P30 AR069619).