Senescent Phenotype of an ACL Injury and Recovery

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INTRODUCTION: Military service members and elite athletes both have a high risk for an anterior cruciate ligament (ACL) injury and can end up missing active-duty days and important sporting events due to a long rehabilitation time. Of even greater concern, about 50% demonstrate signs of post-traumatic osteoarthritis (PTOA) within 10 years of an ACL injury leading to long-term pain and disability. Synovial fluid profiles in the acute knee injury setting have remained largely undefined but have shown evidence of cartilage extracellular matrix degradation and upregulation of inflammatory markers, which may play a role in the development of PTOA and knee fibrosis. Recently, cellular senescence, a characteristic of cellular aging that contributes to inflammation, has been shown to directly contribute to OA; however, the origin and role of senescent cells following acute injury has not been studied. Our goal is to prospectively identify, measure, and compare senescent profiles between synovial fluid and peripheral blood in patients that have sustained an acute knee injury within 72 hours and follow these patients longitudinally through their first 3 months of care.

METHODS: This clinical study received approval from our IRB. Nine subjects (2 males, 7 females) between 30-65 years of age who presented within 72 hours of an acute knee effusion with a confirmed ACL injury from magnetic resonance imaging were prospectively enrolled following informed consent. Synovial fluid (4-30 mL) and peripheral blood (~15 mL) were collected in anticoagulant at the time of initial evaluation, processed using red blood cell lysing buffer and/or separation gradient (Lympoprep^{TM}) following manufacturer instructions, and then the number of senescent cells were quantified using flow cytometry (Cytek NL-2000) and analyzed using SpectroFlo software. Cells were gated to singlets and live-dead (Sytox Blue viability stain) groups and then labeled with multiple epitopes of senescence – $C_{12}FDG$, SPiDER-Gal, CD87 – along with bafilomycin to reduces false positives. Senescence was further characterized within specific lymphoid and myeloid cells populations using antibodies for the following cell types: hematopoietic cells (CD45), T-cells (CD4, CD8), B-Cells (CD19), monocytes (CD14, CD16), NK cells (CD56) and macrophages (CD68) in both the peripheral blood and synovial fluid. Senescence was distinguished by a selective gating strategy that represented cellular populations most accurately based on reference groups, fluorescence minus one groups, and cell count. Inflammatory and regenerative biomarkers in the acellular fractions of synovial fluid and blood were measured using a multiplex immunoassay (Luminex). Resulting analyte concentrations were then calculated using the Belysa^{TM} Immunoassay Curve Fitting Software System.

RESULTS SECTION: Within the synovial fluid, CD45-positive red blood cells showed more senescence than the CD45-negative cell population at the time of injury (Fig. 1A, p=0.0135; Fig. 1C, p=0.0028). Therefore, the senescent hematopoietic cells were analyzed in the synovial fluid and peripheral blood. Of the selected senescent population, CD16-monocyte displayed the highest level of senescence in synovial fluid (Fig. 1B, p=0.0083: Fig. 1D, p=0.0006). In the peripheral blood, CD4 T-Cells (Fig. 1C, p=0.0135; Fig. 1D, p<0.0001) and CD14-monocytes (Fig. 1C, p<0.0001; Fig. 1D, p<0.0001) had significantly elevated senescence. Furthermore, analytes tested as regenerative biomarkers at the time of injury were not significantly elevated in the synovial fluid; however, inflammatory biomarkers MCP-1 (p=0.313), IP-10 (p=0.0313), IL-15 (p=0.0156), IL-8 (p=0.0156), and IL-6 (p=0.0313) were significantly elevated in synovial fluid compared to serum (Fig. 2). Senescent cells and regenerative/inflammatory biomarkers were then tested longitudinally. No significant differences were seen longitudinally from the biomarkers, and senescence in the peripheral blood showed decreasing trends with time, but this did not reach significance in our small patient cohort (Fig. 3A and B).

DISCUSSION: The most important finding of this study is that there is a significant number of senescent cells in synovial fluid within hours of an acute intraarticular ligamentous knee injury, but that these cells were identified as non-synovial myeloid cells rather than synovial cells. The invading myeloid cells in the synovial fluid had the same senescent signatures as the peripheral blood cells. Further, we found the importance of using highly specific, dual labeling to repeatably and reliably quantify cellular senescence. For instance, the dual labeling of using either of the senescence associated beta-galactosidase markers identifying senescent cell types display similar trends at varying degrees. Further investigation is needed with additional antibodies to identify specific cell types and additional biomarkers using an increased patient population. The inflammatory biomarkers elevated in synovial fluid play a central role in regulating the immune response by recruiting monocytes and T-Cells, which can be seen in certain senescent populations. Lastly, senescence appeared to decrease with time from acute injury, but more patients are needed with longer follow-up time to support this conclusion.

SIGNIFICANCE/CLINICAL RELEVANCE: Detection of senescent cells and their associated phenotypic factors has been shown to correlate with osteoarthritis through chondrocyte proteolysis and cartilage regeneration. This data may provide a blueprint for modification of the synovial environment in acute knee injuries within the potential to decrease inflammation, promote tissue restoration, and prevent the progression of osteoarthritis.
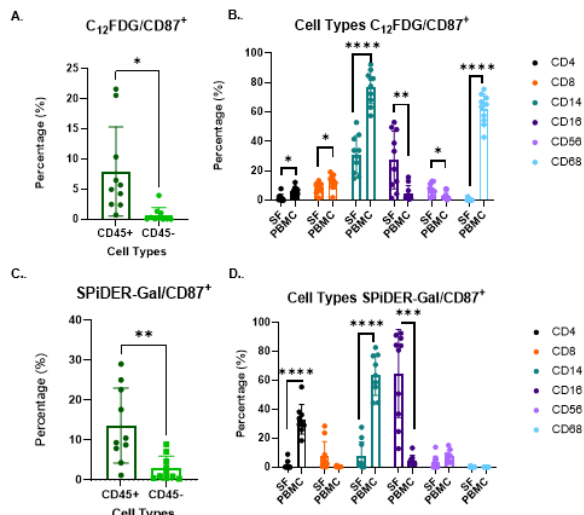

Figure 1: Senescent cell populations in synovial fluid and peripheral blood. (A) $C_{12}FDG/CD87^+$ displayed senescent hematopoietic cells in synovial fluid, (B) in which specific cell types were elevated in synovial fluid or blood. (C) SPiDER-Gal/CD87$^+$ displayed senescent hematopoietic cells in synovial fluid, (D) in which specific cell types were elevated in synovial fluid or blood.
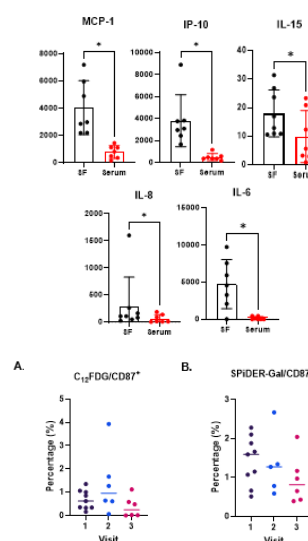
Figure 2: Inflammatory biomarkers significantly elevated in synovial fluid compared to blood at the time of injury.
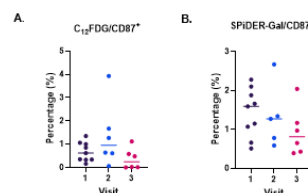
Figure 3: Longitudinal senescent profiles of patient blood over three visits using (A) $C_{12}FDG/CD87^+$ and (B) SPiDER-Gal/CD87$^+$.