Joint Injury Generates Damage Associated Molecular Patterns that Activate Synovial Fibroblasts Towards a Fibrotic Phenotype

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DISCLOSURES: RLM (5 – 4WEB Medical)

INTRODUCTION: An estimated 4 out of 10 adults in the US over the age of 70 suffer from osteoarthritis[1]. While symptom management and surgical procedures exist for the treatment of post-traumatic osteoarthritis (PTOA), there are no disease-modifying therapeutics. Current research implicates changes in the synovium, particularly inflammation and fibrosis, as potential drivers of PTOA.[2,3] Activated fibroblast-like synoviocytes (FLS) play a key role in driving both fibrosis and inflammation,[4,5] making them a promising therapeutic target. However, the initial triggers of FLS activation and the pathways that sustain activation remain unclear. Prior work suggests that inflammation driven by innate immune receptors called Toll-Like Receptors (TLRs), especially TLR2 and TLR4, which detect molecules from pathogens (PAMPs) or damaged tissues (DAMPs), may play a role in FLS activation, synovial inflammation, and fibrosis.[6] Here, using an *in vivo* porcine animal model of PTOA, we evaluated acute changes in the synovium and synovial fluid, and applied factors from identified pathways to assess their role in *in vitro* FLS activation. We hypothesized that pathologic FLS activation is initiated and maintained in part by DAMPs present within the joint following injury.

METHODS: *In Vivo Joint Injury Model and Synovial Analysis:* All animal studies were approved the UPenn IACUC. Adult male Yucatan minipigs (n=3) underwent bilateral arthroscopic destabilization of the medial meniscus (DMM), with or without an additional arthroscopic anterior cruciate ligament transection (ACLT). Synovial fluid and synovial tissue biopsies were acquired at the time of surgery and at euthanasia (day 7). Synovial tissue was fixed, processed into paraffin sections, and stained with H&E, followed by semi-quantitative scoring.[7] Synovial fluid was processed by mass spectrometry, and differentially expressed proteins (DEPs) were identified and subjected to GO pathway analysis.[8] *In Vitro Studies:* Porcine fibroblast-like synoviocytes (FLS) were isolated from healthy synovium (n=5 donors from unrelated studies) and expanded through passage 3.[4] FLS from each donor were seeded onto glass slides (5000/cm^2) and incubated for three days in basal media or with media containing LPS (0.3 ug/mL, TLR4 agonist PAMP), Pam3CSK4 (0.25 ug/mL, TLR2 agonist DAMP), or S100A8/9 (1.0 ug/mL, TLR4 agonist DAMP). Cells were then fixed, permeabilized, and stained for paxillin, αSMA, and actin (phalloidin) and counterstained with DAPI. Samples were imaged at 20X using a Zeiss Axio Scan Z1 slide scanner. Cell morphology (area, compactness, eccentricity) and fluorescent staining intensity was quantified in CellProfiler (n>20 cells/donor/condition). For one donor, cell-mediated matrix contraction was assessed by in FLS-embedded collagen I gels (30,000 cells/gel, n=4) cultured in basal media or with TLR agonists, with images taken at regular intervals over one week. Gel area was measured using ImageJ. Differences were assessed using one-way ANOVA with significance set at multiple comparison-corrected α=0.05.

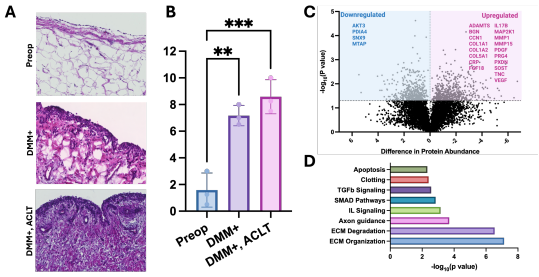

***Figure 1**: A) Synovial H&E staining. B) Total synovial histopathology score (summing hyperplasia, vascularity, inflammation, and fibrosis sub-scores). **p<0.01, ***p<0.005. C) Volcano plot showing differentially expressed proteins (DEPs) and D) significant pathways identified.*

RESULTS: Synovial tissue staining revealed marked changes; both DMM and DMM+ACLT groups showing significant increases in total histopathologic scores compared to control (**Fig 1A**), with the DMM+ACLT group also showing increases in the fibrosis sub-score (not shown). Proteomic analysis identified numerous proteins elevated in post-surgical synovial fluid, with no significant differences between surgical groups (**Fig 1B**). Combining upregulated proteins and performing pathway analysis identified several pathways increased after injury, including apoptosis, IL signaling, fibrosis (TGF/Smad) and ECM degradation (production of DAMPs). Based on these findings, and the evidence of matrix fragments in the injury synovial fluid, we next assessed how individual PAMPs and DAMPs regulated fibroblast activation. IF staining showed that both Pam3CSK and S100A8/9 decreased cell area (**Fig 2B**) and dramatically altered cytoskeletal organization, with pronounced actin stress fibers apparent in all stimulated groups (**Fig 2A**). Quantification showed an increase in actin (**Fig 2C**, Pam3CSK and S100A8/9) and αSMA (**Fig 2C**, all agonists) signal intensity. When these PAMPs/DAMPs were applied to FLS in collagen gels (**Fig 3A**), a decrease in gel contraction was observed for LPS and Pam3CSK (**Fig 3B**), with differences more apparent at later time points.

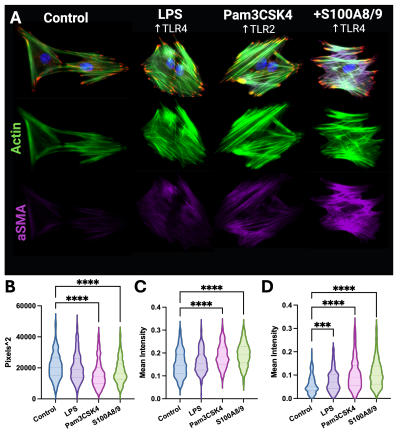

***Figure 2**: A) IF of FLS on day 3 with PAMP/DAMP treatment. Green: actin, purple: αSMA, red: paxillin. Violin plots of B) cell area, C) actin, D) αSMA intensity. ***p<0.005, ****p<0.0001. N>20 cells from 5 donors.*

DISCUSSION: Our large animal data show that, following injury (DMM and DMM+ACLT), the synovium undergoes rapid changes, including increases in inflammation and fibrosis. Proteomic analysis of the synovial fluid revealed upregulation of various processes that produce damage associate molecular patterns (DAMPs), including apoptosis and ECM degradation. When these DAMPs were presented to FLS in vitro, we noted a clear change in cellular state, with an increase in actin stress fibers and expression of fibrotic markers (αSMA). In 3D contraction assays, LPS and to a greater extent Pam3CSK reduced gel compaction. This finding was counter to our expectation but may relate to the stability and turnover of the cytoskeleton; future studies are needed to fully explicate this finding. Taken together, this data supports that destabilizing joint injuries instigate a rapid change in the synovium, and that DAMPs present in this injury environment activate FLS towards a fibrotic phenotype.

SIGNIFICANCE/CLINICAL RELEVANCE: Our large animal PTOA model identified key pathways upregulated following injury and indicate that TLR-agonizing DAMPs present in the synovial fluid can activate synovial fibroblasts. These data provide new insights for the development of TLR-targeting disease modifying therapeutics for the treatment of PTOA.

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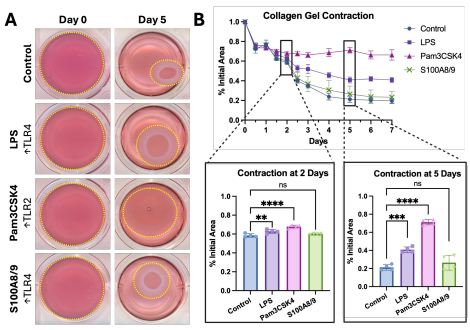

***Figure 3**: A) FLS-mediated gel contraction with PAMP/DAMP treatment. B) Quantification of contraction with time/treatment. N=4 replicates for one donor FLS. **p<0.01, ****p<0.0001.*

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