Determining the Mechanisms of Blood-Derived Mononuclear Cell-Mediated Meniscus Catabolism

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Introduction: There is a high rate of post-traumatic osteoarthritis (PTOA) development following meniscus injury; however, the mechanisms that lead to PTOA are currently unknown[1,2]. Meniscal tears located in the inner zone have poorer clinical outcomes than tears in the outer zone and this may be related to vascularity differences and/or the unique zone-specific cellular and extracellular matrix (ECM) composition[3,4]. Hemarthrosis, or bleeding into the joint, is induced during surgical procedures and occurs in traumatic knee injuries, including outer zone meniscus tears and ACL injuries, which occur concurrently with meniscus tears in 70% of cases[5]. Our lab has previously demonstrated that blood exposure induces catabolism of meniscus explants, which is mediated by mononuclear cells (MNCs)[6]. However, there is a gap in knowledge regarding the mechanism(s) of this catabolism and how zonal differences affect this pathophysiology. The goal of this study was to identify zone specific transcriptomic consequences of blood and blood-derived MNC exposure to meniscus tissue and to identify the mediators that drive MNC-induced meniscus catabolism. We hypothesize that soluble factors, such as inflammatory cytokines secreted by MNCs, suppress ECM genes and upregulate degradative enzymes to result in catabolism of meniscus tissue.

Methods: Female (due to local tissue availability) porcine (2-3 years old) knee joints were obtained from a local abattoir. Explants (4mm) were isolated from the inner and outer one-third of the medial meniscus for zonal studies or from the midline for the IL1Ra study. Porcine blood was purchased or collected via an IACUC-approved protocol. MNCs were separated from whole blood using a Ficoll-Hypaque gradient (1.077 g/ml). Meniscus explants were incubated with media alone (control), 10% whole blood (v/v), or MNCs at 10% of their numbers present in whole blood (based on cell counts before and after isolation) resuspended in media. For all experiments, meniscus explants were cultured with blood components for 3 days at 37ºC and 5% CO_2, then media was collected (Day 3), and explants were washed 3x in PBS, simulating hemarthrosis and blood clearance after joint trauma[7]. Explants for RNA extraction were snap-frozen, while explants for biochemical content were cultured for an additional 3 days (Day 6) in media. *Transcriptomic changes:* Explants were pulverized in TRIzol using a freezer mill, and RNA was extracted by chloroform separation and column purification (Zymo Direct-zol). RNA-sequencing was performed by the Duke Sequencing and Genomic Technologies Core. Differences in gene expression between treatment and control were considered significant with an adjusted p-value < 0.05 and log-2 fold-change > |2|. Selected targets were validated by RT-qPCR. *Role of soluble mediators:* To assess the effects of soluble factors from blood or mononuclear cells, inner and outer zone meniscus explants were cultured in polycarbonate inserts (0.4µm pore size) and blood/MNCs were added to the surrounding well. *Role of IL-1*: To determine the role of IL-1, 300ng/mL porcine IL-1 receptor antagonist (IL1Ra) was added every 3 days throughout culture. *Biochemical assessment:* Media was collected again on day 6. Total matrix metalloproteinase (MMP) activity and sGAG release (DMMB assay) were assessed in media. Explants were lyophilized, weighed, and papain digested. Tissue digests were assessed for DNA (Quant-iT PicoGreen™ assay) and sGAG content (DMMB assay). Biochemical outcomes were normalized to the dry weight of the explant or total DNA content. *Statistics:* Fig 1B-E were log2 transformed to normalize the data prior to running two-way ANOVAs and Tukey post-hoc test. All other data were analyzed using the Kruskal-Wallis test and Dunn's multiple comparison tests and Fig 2A-D were analyzed within zone. p<0.05 was considered statistically significant.

Results: Exposure to blood and blood-derived MNCs resulted in significant alterations in the meniscus transcriptome with MNC exposure relative to control causing the largest number of shared differentially regulated genes between zones (**Fig 1A**, 423 downregulated, 162 upregulated). The inner zone had the largest number of unique differentially regulated genes when comparing MNC to control (874 downregulated, 262 upregulated). There was a decrease in key ECM genes (*ACAN* and *COL1A1*) and an increase in catabolic enzymes (*ADAMTS5* and *MMP3*) due to blood and MNCs (**Fig 1B-E,** p<0.05). Indirect culture of blood and MNCs with meniscus tissue caused a decrease in sGAG content in the inner zone (**Fig 2A**, p<0.01). In the media of both inner and outer zone explants, sGAG release was increased due to MNC exposure (**Fig 2B**, p<0.05). MMP activity was elevated during blood and MNC exposure (**Fig 2C**, p<0.01) and explants continued to upregulate MMP activity post-MNC exposure compared to blood and control samples (**Fig 2D**, p<0.05). Inhibition of IL-1 signaling during MNC exposure trended towards preventing proteoglycan loss both as measured by tissue content (**Fig 3A**, p=0.05) and release (**Fig3B**, p=0.07). There was a significant decrease in MNC-induced MMP activity due to IL-1Ra both during (data not shown) and after MNC exposure (**Fig 3C**, p<0.05). Whole blood-induced meniscus catabolism was not prevented by IL1Ra (**Fig 3D-F**).

Discussion: Due to the greater number of differentially regulated genes and greater loss of sGAGs due to the MNC exposure, the inner zone tissue appears to be more sensitive to blood-derived immune cell-mediated catabolism than the outer zone. sGAG and MMP changes were detected both when the blood and MNCs were cocultured directly with the meniscus (**Fig 3**) and when they were separated by a permeable insert (**Fig 2**), which allowed for sharing of soluble factors without direct cell-tissue contact. MMP activity remained upregulated after blood and MNC removal, suggesting prolonged catabolic signaling after hemarthrosis, which could contribute to PTOA development. In fact, MMP activity is elevated in the synovial fluid of meniscus-injured knees at the time of surgery[8]. We show a potential role for IL-1 signaling in MNC-mediated meniscus catabolism. However, this was not observed with whole blood exposure, potentially indicating a role for additional soluble mediators in whole blood-induced meniscus catabolism. In the future, we will perform unbiased mass-spectrometry based proteomics to identify additional factors that are secreted by these immune cells, which may be mediating meniscus catabolism.

Significance: This study identified potential therapeutic targets to prevent the catabolic effects of blood-derived immune cells on meniscus tissue.

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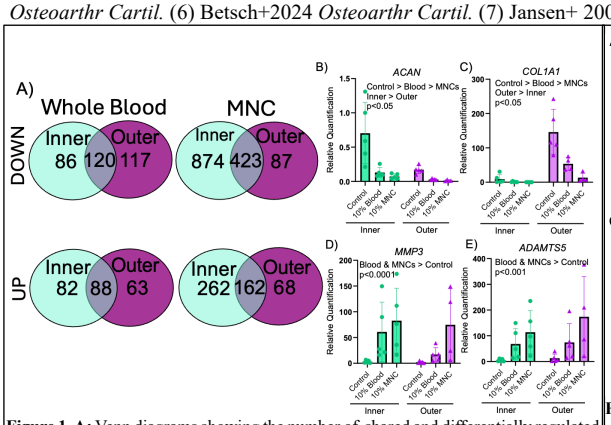

Figure 1. A: Venn diagrams showing the number of shared and differentially regulated genes with blood and mononuclear cell (MNC) exposure in inner and outer zone tissue explants based on RNA Sequencing (N=4/group). **B-E:** RT-qPCR validation of selected targets. Relative expression calibrated to an inner control sample. N=4-5.

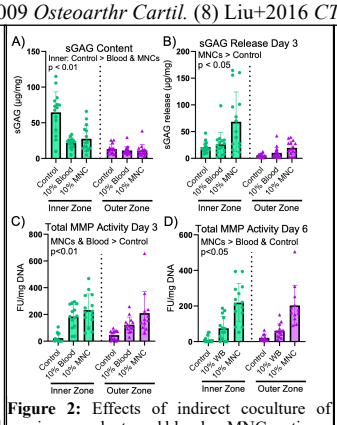

Figure 2: Effects of indirect coculture of meniscus explants and blood or MNC on tissue sGAG content (**A**) and sGAG release into the media (**B**). Total MMP activity during (**C**) and after (**D**) blood and MNC exposure. n=15-17/group.

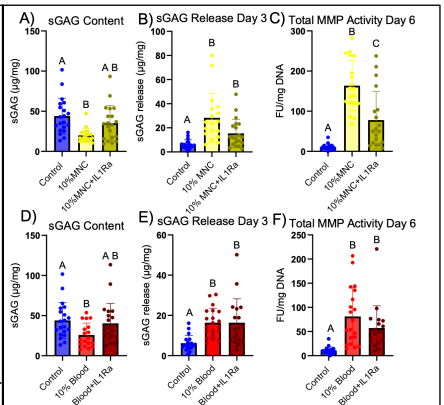

Figure 3: IL-1Ra effects on MNC and blood-mediated sGAG content (**A,D**), sGAG release (**B,E**), and total MMP activity after culture with MNCs and blood (**C,F**). n=18-21/group. All groups not sharing the same letter have p < 0.05.