**Interaction With Macrophages Attenuates Fibroblast-like Synoviocyte Adamts5 (aggrecanase-2) Gene Expression Following Inflammatory Stimulation**

Rhiannon E. Morgan, MRCVS¹, Peter D. Clegg¹, John A. Hunt², Simon R. Tew³.

¹The University of Liverpool, Neston, Wirral, United Kingdom, ²The University of Liverpool, Liverpool, United Kingdom.


**Introduction:** Synovitis is a key mediator of osteoarthritis (OA), and in the perpetuation of cartilage degradation. Synovial inflammation is characterised by increases in catabolic cytokines, which are predominantly produced by synovial macrophages. These cytokines cause changes in fibroblast like synoviocyte (FLS) gene expression, including up regulation of damaging cartilage matrix degrading proteinases and further cytokines. An important enzyme activity in the progression of OA is the aggrecanase; the two most potent enzymes are ADAMTS4 (aggrecanase-1) and ADAMTS5 (aggrecanase-2). ADAMTS4 gene expression is influenced by IL-1β whilst ADAMTS5 is thought to be unaffected, and studies in chondrocytes indicate that it is constitutively expressed. Previous reports suggest ADAMTS5 is the central aggrecanase responsible for cartilage degradation in the murine model; ADAMTS5 knockout mice, and normal mice treated with a potent anti-ADAMTS5 monoclonal antibody, subjected to destabilisation of the medial meniscus (DMM) experienced significantly less cartilage destruction than wild type mice. The synovium expresses both ADAMTS4 and 5; synovium co-cultured with injured cartilage was found to express significantly lower levels of ADAMTS5 compared to synovium in coculture with normal cartilage. These factors suggest a central role for this aggrecanase in cartilage degeneration, with a possible synovial protective component. ADAMTS5 presents a potential disease-modifying target for OA, however further insight into the regulation of this aggrecanase is needed. This study investigates the cellular interactions between macrophages and fibroblast-like synoviocytes (FLS) in the inflammatory response, to help determine the influence synovitis has in OA and cartilage degeneration, and the potential use synovium could offer as a disease-modifying target.

**Methods:** FLS were isolated from the synovium of healthy equine metacarpophalangeal joints using 0.2% trypsin and 0.2% collagenase type II. These cells were then labelled with the Cell Proliferation dye eFluor 670 (eBioscience, San Diego, USA). Canine macrophages (DH82 cell line) were transfected with green fluorescent protein (GFP) expressing lentivirus and selected with puromycin. Proliferation characteristics of both populations in co-culture over 10 days were analysed using flow cytometry, to determine the optimum co-culture model. Cytokine and aggrecanase gene sequences gathered via Genebank were analysed for similarity through NCBI BLAST search, and species-specific primers designed using Primer Express 2.0 (Applied Biosystems, UK). To investigate inter-cellular signalling in the inflammatory response, three conditions were examined: (1) An optimised co-culture model using equine FLS (EFLS) and canine macrophages (DH82 cells). siRNA-mediated gene knockdown in DH82 cells prior to co-culture was also employed in this system. (2) EFLS exposed to conditioned medium from DH82 cells. (3) EFLS and DH82 cells co-cultured without contact using well-inserts. All conditions were exposed to 10µg/ml lipopolysaccharide (LPS) for up to 24hrs. Cell specific expression of IL-1β, IL-6, ADAMTS4 and ADAMTS5 was determined via qRT-PCR using species-specific primers, gene expression
was normalised to GAPDH and compared to synoviocytes cultured independently. Experiments were repeated in triplicate.

**Results:** Flow cytometric analysis of cell proliferation characteristics, revealed a comparable rate of proliferation between EFLS and DH82 cells after 24h culture, when seeded at a 1:1 ratio. This model was therefore used for 24h time course experiments. LPS stimulation of EFLS caused rapid increases in IL-1β, IL6, ADAMTS4 and ADAMTS5 mRNAs. Stimulation was not consistently different when co-cultured with DH82 cells, except for ADAMTS5, where the response was repeatedly dampened (Figure 1). When EFLS were cultured with DH82 cells in transwells, or when exposed to DH82 conditioned media, the ADAMTS5 response to LPS stimulation was similarly reduced indicating that a soluble mediator is likely to be responsible. DH82 cells increased IL-1β expression substantially following LPS stimulation. However, knockdown of IL-1β in DH82 cells prior to co-culture did not change the inhibitory effect on FLS ADAMTS5 gene expression.

**Discussion:** We conclude that LPS stimulation, presumably mediated through toll-like receptors, leads to rapid increases in gene expression of inflammatory cytokines and matrix degrading proteases in FLS. Interestingly macrophages can influence FLS gene expression through a soluble mediator, and this intercellular signalling led to the suppression of FLS ADAMTS5 gene expression during LPS stimulation. Knockdown of IL-1β in DH82 cells prior to co-culture did not affect the suppression of ADAMTS5 making it unlikely that it is the soluble mediator responsible. This supports previous reports suggesting that IL-1β, predominantly produced by macrophages, does not influence ADAMTS5 expression, unlike ADAMTS4, which is upregulated2. Responses of FLS ADAMTS4 gene expression in co-culture varied, however knockdown of IL-1β in DH82 cells dampened ADAMTS4 gene expression.

Previous reports suggest that nuclear factor-κB family member RelA/p65 is a strong transcriptional activator of ADAMTS5 in chondrocytes6. Work is now underway to determine whether this signalling pathway also mediates ADAMTS5 mRNA regulation in FLS. FLS ADAMTS5 suppression has also been associated with injured cartilage5; it is hypothesised that surrounding cells exert a negative feedback response to FLS during an inflammatory event. The co-culture model described in this study will be developed to incorporate a cartilage component, which will further allow investigation of the relationship between cartilage and synovium during OA.

This co-culture model offers a unique opportunity to dissect the cellular responses of different cell types in co-culture. This is especially important when investigating disease-modifying targets for potential beneficial use, and to understand possible off-target effects. However, cell populations from two different species may elicit less efficient signalling processes than those from either a solely canine or equine model, which must be taken into consideration.

**Significance:** This study has identified a new novel mechanism of FLS ADAMTS5 mRNA regulation by macrophages. Due to the critical role that ADAMTS5 (aggrecanase-2) plays in murine arthritis studies, understanding how this occurs could lead to new methods of disease intervention.
Figure 1. Fold changes in EFLS gene expression; when cultured independently or in co-culture with DH82 cells. Cells were stimulated with 10μg/ml LPS over 24 hours. mRNA levels of IL-1β, IL6, ADAMTS4 and ADAMTS5 were normalised to GAPDH and compared to untreated EFLS at 0h. (Solid black line = EFLS cultured independently, Dotted black line = EFLS in co-culture).