1 **Interaction with macrophages attenuates Equine Fibroblast-Like Synoviocyte**
2 **ADAMTS5 (aggrecanase-2) gene expression following inflammatory stimulation.**

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22 **Running title:** Macrophages attenuate synovial fibroblast response

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27 submitted manuscript.
28
29 **Abstract**
30 The joint synovium consists of a heterogeneous cell population, chiefly comprised of
31 macrophages and fibroblast-like synoviocytes (FLS). An inter-species co-culture
32 model was developed to examine interactions between these cells. Equine FLS and
33 the canine macrophage line DH82 were differentially labelled using fluorescent
34 markers and results from direct co-culture compared with those from both indirect co-
35 culture, and conditioned media experiments. The transcript expression of IL-1β, IL-6,
36 ADAMTS4 and ADAMTS5 in each cell type were determined using species-specific
37 qPCR assays. Lipopolysaccharide stimulation of EFLS rapidly increased IL-1β, IL-6,
38 ADAMTS4 and ADAMTS5 mRNAs. The induction of ADAMTS5 was significantly
39 reduced when equine FLS were cultured with DH82 cells directly or indirectly.
40 Exposure of equine FLS to denatured conditioned media also significantly reduced
41 ADAMTS5 induction. DH82 cells increased interleukin-1β expression substantially
42 following LPS stimulation. However, knockdown of interleukin-1β in DH82 cells, or
43 inhibition of NF-κB in equine FLS prior to co-culture did not change the inhibitory
44 effect on equine FLS ADAMTS5 gene expression. This work indicates that
45 macrophages can influence FLS gene expression through a soluble mediator, and
46 modulate the expression of an enzyme critical in osteoarthritis pathology during
47 inflammatory stimulation.
48
49 **Key words:** Synovium, macrophage, aggrecanase, ADAMTS5
50

51 **Introduction**

52 The synovial membrane is a specialized tissue that lines non-articulating surfaces of
53 diarthrodial joints. It contains a population of fibroblast-like synoviocytes in addition
54 to monocytic cell populations, predominantly macrophages. Synovitis is a key
55 mediator of osteoarthritis (1), and is involved in the perpetuation of cartilage
56 degradation (2). Synovial inflammation is characterised by mononuclear cell
57 infiltration (3) and increases in catabolic cytokines, especially IL-1β and TNF-α (4),
58 which are predominantly produced by the synovial macrophages. These cytokines
59 regulate fibroblast-like synoviocyte gene expression, including those encoding
60 cartilage matrix degrading proteinases (5). Examples of these include the ADAMTS
61 (A Disintegrin And Metalloproteinase with ThromboSpondin motif) family of
62 enzymes, particularly ADAMTS4 and ADAMTS5, which can cleave the major
63 cartilage proteoglycan aggrecan with the latter being heavily implicated in the
64 development of cartilage degeneration during osteoarthritis (6, 7) Although
65 constitutively expressed in human chondrocytes and osteoarthritic synovial cells,
66 there is evidence that murine and bovine chondrocyte ADAMTS5 mRNA levels
67 respond to treatment with IL-1β (8). This may be mediated through NF-κB signaling,
68 with a recent study identifying the transcription factor RelA/p65 as a potent
69 transcriptional activator of ADAMTS5 in chondrocytes during osteoarthritis (9). In
70 addition to transcriptional regulation, ADAMTS5 can be controlled at the post-
71 translational level (8) and recent work has identified LRP-1-mediated endocytic
72 clearance of active ADAMTS5 as a key regulatory process (10).

73

74 Like humans, the horse is susceptible to chronic joint diseases such as osteoarthritis
75 with associated synovial inflammation (11) and equine synovium expresses both

ADAMTS4 and 5 (12). Interestingly, normal equine fibroblast-like synoviocytes (EFLS) expressed significantly lower levels of ADAMTS5 mRNA when co-cultured with injured cartilage in comparison to co-culture with normal cartilage (13). These factors suggest that EFLS may have a protective influence during joint inflammation, although how fibroblasts and macrophages in the synovium interact is poorly understood.

In this study, an *in vitro* co-culture system was created to allow interactions between EFLS and macrophages to be identified. EFLS proliferative and gene regulation responses to inflammatory stimulation and to contact with macrophages was analysed.

Methods

Isolation of equine fibroblast-like synoviocytes (EFLS)

Forelimbs from three horses were collected from a local abattoir. The metacarpophalangeal joint was cleaned and skinned before opening. The joint surface was scored macroscopically to ensure absence of orthopaedic disease (3). Synovium was dissected, finely diced and cells isolated using 0.25% (w/v) trypsin / 0.1% (w/v) EDTA digestion followed by incubation with 2mg/ml collagenase type 2 (Worthington Biochemicals; Berkshire, UK) Cells were seeded at 6×10^4 cells/cm^2 and expanded for up to 3 passages using split ratios of 1:3. Unless stated otherwise, all cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies; UK) containing 10% foetal bovine serum (FBS), 0.2% amphotericin B (2µg/ml), and 1% penicillin (100U/ml) streptomycin (100µg/ml) at 37°C in 5% CO_2.

101

102 **Western Blot Analysis**

103 EFLS (n=3) cells were cultured with and without 10µg/ml LPS (E. Coli 026:B6,
104 Sigma-Aldrich, MO, USA) for 6 or 16 hours. Culture media was collected, the cells
105 washed using PBS, and lysed in 1x SDS sample-loading buffer (62.5mM Tris-HCl,
106 pH 6.8 at 25°C, 2% w/v SDS, 10% glycerol, 50mM DDT, 0.01% w/v bromophenol
107 blue or phenol red). Cell lysate and culture media protein concentrations were
108 measured using the Pierce™ 660 Protein Assay (Life Technologies; Carlsbad, USA).
109 Protein (40µg) was extracted from each culture media sample using StrataClean^{Tm}
110 Resin according to manufacturer protocol and eluted by boiling in 15µl 1x SDS
111 sample buffer. Cell lysate and culture media protein samples were run on SDS-PAGE
112 gels and blotted onto nitrocellulose membranes, which were then probed using a
113 sheep anti-mouse ADAMTS5 antibody (a kind gift from Amanda Fosang, Melbourne
114 Australia) (14) at a dilution of 1:500 followed by an anti-goat/sheep IgG-peroxidase
115 secondary antibody at a dilution of 1:1000 (A9452; Sigma-Aldrich, MO USA).
116 Antibody localisation was visualised using Western Lightening-Plus
117 chemiluminescence reagent (Perkin Elmer, MA, USA), and imaged on a UVP
118 ChemiDoc-it imaging system. Intensity of bands was quantified using imageJ
119 software.

120

121 **Multi species fibroblast-like synoviocyte /macrophage co-culture model**

122 The canine macrophage-derived cell line DH82 was obtained from American Tissue
123 Culture Collection (ATCC, VA USA) and cultured under the same conditions as the
124 EFLS. Fluorescently labelled DH82 cells were produced by infecting them with a
125 lentivirus expressing green fluorescent protein (GFP) (Sigma) and subsequent

126 selection with 2μg/ml puromycin (Life Technologies; Carlsbad, USA). EFLS were
127 labelled with 8μM Cell Proliferation Dye eFluor® 670 (eBioscience Inc., San Diego,
128 USA) for 10 minutes using the manufacturers protocol. Cells were mixed and seeded
129 in 6-well dishes at 3.3 x 10^4cells/cm^2 at the following EFLS:DH82 ratios: (1) 1:0 (2)
130 0:1 (3) 1:2; (4) 2:1; (5) 1:1. Co-cultures were analysed using an Accuri™ C6 flow
131 cytometer (BD Biosciences, California, USA).
132

133 **Design, validation and testing of species-specific qPCR assays**

134 Equine and canine mRNA sequences for the following genes; GAPDH, IL-1β, IL-6,
135 ADAMTS4 and ADAMTS5, were obtained from the NCBI GenBank database (Table
136 1). Equine and canine mRNA sequences were directly compared using NCBI BLAST
137 to determine dissimilar regions, to which primers were designed. All primers were
138 designed using Primer Express 2.0 software (Applied Biosystems, UK) and
139 synthesized by Eurogentec (Seraing, Belgium). PCR efficiencies of primer pairs were
140 determined using serial 2-fold dilutions of species-appropriate cDNA.
141

142 **EFLS and DH82 Co-cultured in direct contact**

143 EFLS (n=3) and DH82 cells were seeded independently or mixed 1:1 at 1.5 x
144 10^5/cm^2. Cells were allowed to attach overnight and then exposed to 10μg/ml LPS,
145 and harvested 1, 3, 6, 12, and 24 hours later. Where necessary, DH82 cells at 150,000
146 cells/well of a 6-well plate, were transfected with 25pmol canine anti-IL-1β siRNA or
147 silencer Cy3 labelled negative control siRNA (csiRNA) (Life Technologies,
148 California, USA) using the lipofectamine 2000 (Life Technologies, California, USA)
149 prior to co-culture, or EFLS were pre-treated for 1-hour with either 0.8μM Bay-11-

150 7082 (B5556; Sigma-Aldrich, Missouri, USA) or 200µM Pyrrolidinedithiocarbamic
151 acid (PDTC; Enzo Life Sciences, New York, USA).
152
153 **EFLS and DH82 Co-cultured without direct contact**
154 DH82 cells were stimulated with 10µg/ml LPS for 24h and the conditioned media
155 collected and added to EFLS cells seeded at 5 x 10^4cell/well in 24 well plates. To
156 examine the effect of protein denaturation, conditioned media was heated to 100°C
157 for 10 mins prior to use. Compartmentalised culture was performed by seeding 2 x
158 10^4 DH82 cells into 0.45µm cell culture inserts (Merck Millipore, Darmstadt,
159 Germany) for 24 hours, before transferring the insert to a well containing a monolayer
160 of EFLS before stimulation with 10µg/ml LPS for 6 and 12 hours.
161
162 **Reverse Transcription and Quantitative RT-PCR**
163 Total RNA (1µg), purified using the Guanidinium-thiocyanate-phenol-chloroform
164 technique (15), was reverse transcribed using M-MLV reverse transcriptase
165 (Promega, WI, USA) using random primers. qRT-PCR was performed with 160ng
166 cDNA using SYBR Green master mix (GoTaq qPCR Master Mix, Promega, WI,
167 USA) and 300nM individual primer concentrations on an ABI 7300 instrument. The
168 relative quantification of each gene normalised to GAPDH was calculated using the 2^{-}
169 $^{\Delta Ct}$ method (16).
170
171 **Statistical Analysis**
172 Two tailed t-tests were used to compare fold changes in EFLS mRNA expression
173 when cultured independently in standard culture media, compared to those cultured
174 with DH82, in conditioned media, in denatured conditioned media, and with DH82

175 cells in well-inserts, and also to compare the gene expression of DH82 cells cultured
176 alone and in co-culture with EFLS. Student t-tests with bonferroni post hoc
177 corrections were used to compare DH82 gene expression when treated with control
178 siRNA compared to anti-IL-1β siRNA, and EFLS ADAMTS5 gene expression when
179 cultured with DH82 cells either treated with control siRNA or anti-IL-1β siRNA, or
180 when pre-treated with NF-κB inhibitors.
181
182
183 **Results**
184
185 **LPS stimulated EFLS secreted increased levels of ADAMTS5 protein**
186 We were interested in the levels of the aggrecanase ADAMTS5 in the cell layer and
187 culture media of EFLS exposed to inflammatory simulation. Western blot analysis of
188 cell lysates identified bands at approximately 90 and 70kDa (Figure 1A). The
189 predicted molecular weight of the pro form of equine ADAMTS5 is 101kDa. There
190 was no LPS mediated induction of ADAMTS5 protein in the cell layer fraction after 6
191 hours of treatment, although levels were slightly increased in both control, and LPS
192 treated cells after 16 hours (1.3x and 1.4x increase respectively, compared to 6-hour
193 control conditions). We detected an approximately 50kDa band, in western blot
194 analysis of EFLS media (Figure 1B), which potentially corresponds to ADAMTS5
195 which has undergone auto-catalytic removal of the C-terminal domains (14). After 6
196 hours, levels of the 50kDa ADAMTS5 were 2.9 times higher than controls.
197 Examination of media after 16 hours of culture again revealed higher levels of 50kDa
198 ADAMTS5 in LPS treated cells (2x higher than 16-hour control conditions). Upon
199 repeating, using cells from a second horse, we again observed more intense 50kDa

200 ADAMTS5 immunopositive bands in LPS treated cells at both time points (Figure
201 1B).
202
203 **Macrophages stimulate EFLS mitosis**
204 Given that EFLS could upregulate ADAMTS5 in response to an inflammatory
205 stimulation, we wanted to determine how EFLS ADAMTS5 response was affected by
206 interactions with the inflammatory cells that they would encounter *in vivo*. To do this
207 we generated a multi-species co-culture model which could allow investigation of cell
208 interactions whilst permitting specific measurement of EFLS response. We
209 fluorescently labelled EFLS with the Cell Proliferation Dye eFluor® 670 and
210 generated non-clonal, canine DH82 macrophage cell cultures that expressed GFP,
211 which enabled discrimination of the cells when cultured together (Figure 2A). Using
212 microscopy and flow cytometric analysis we observed a slightly higher rate of EFLS
213 proliferation compared to DH82 cells, when co-cultured over 24 hours. Side scatter
214 (SSC) versus forward scatter (FSC) density plots identified two groups of cells with
215 differing morphological characteristics within the mixed culture. After analysis of
216 individual cultures, EFLS were identified to be smaller and less granular than DH82
217 cells (Figure 2B(i) and (ii)).
218
219 When the two populations within the mixed culture were gated on the SSC vs FSC
220 density plot (G1 = EFLS, G2 = DH82) (Figure 2B(iii)), the majority of cells within
221 the G1 gated area were consistent with efluor- labelled EFLS (Figure 2B(iv)), whilst
222 those within the G2 gated area were consistent with GFP-expressing DH82 cells
223 (Figure 2B(iv)), demonstrating that the two populations could be independently
224 assessed in the mixed cultures.

225

226 Proliferation rates of each population in co-culture were analysed. A slightly higher
227 rate of EFLS proliferation vs. DH82 proliferation, during 24 hours co-culture was
228 observed, and EFLS mitosis was stimulated by co-culture with DH82 cells (Figure
229 2B(v) & (vi)). When EFLS were cultured independently, 67% of the cells were
230 labelled with the efluor dye (Figure 2B(v)). As cells undergo mitosis, the dye is
231 distributed equally between the two daughter cells, and this can be measured by the
232 fluorescence intensity halving. Within mixed co-cultures, fluorescence intensity of the
233 eFluor dye identified two sub-populations, emitting fluorescence intensity at $10^{5.3}$-
234 $10^{6.7}$ or $10^{3.3}$-$10^{5.3}$, consistent with primary and secondary generations of EFLS,
235 (Figure 2B(v)). This secondary EFLS population was not identified over the same
236 time scale when EFLS were cultured alone, indicating that the presence of DH82 cells
237 stimulates EFLS mitosis.
238 The number of labelled DH82 and EFLS cells within each co-culture was analysed to
239 calculate the proportion of each population after 24 hours (Figure 2B(v) & (vi)). The
240 initial seeding ratio of 50% EFLS and 50% DH82 produced a culture of 60% EFLS
241 and 40% DH82 after 24 hours. An infiltration of cells into the synovial membrane,
242 resulting in 25-50% of the synovial cell population consisting of mononuclear cells,
243 has been classified as a moderate (grade 3 out of 4) synovitis (McIlwraith, Frisbie et
244 al. 2010). This proportion of cells was therefore deemed appropriate to model a
245 moderate synovitis after 24 hours in culture.

246

247 **Production of species-specific qRT-PCR assay**

248 To allow us to specifically measure cell-specific gene expression in co-cultures, we
249 designed species-specific qRT-PCR assays. Primers deemed to be species-specific

250 when they measured robust expression, reflected by low Ct values when used to
251 analyse expression cultures of the same species (alone or in co-culture), but exhibited
252 high or undetectable Ct values when used to analyse expression in cultures just
253 containing the other species' cells (Figure 2C).
254
255 We also ensured that dissociation curves of PCR products displayed a single peak
256 when analysing cultures that contained cells of the target species as the primers, but
257 were either not evident or negligible when primers were used on cultures solely
258 containing cells of the other species. Discriminatory assays were not always possible
259 to generate however, meaning that specific analysis of ADAMTS4 and ADAMTS5 in
260 DH82 cells was not carried out as repeated iterations of primer design failed to
261 identify a specific assay.
262
263 **Macrophages attenuate EFLS ADAMTS5 gene expression.**
264 EFLS cultured independently demonstrated rapid increases in IL-1β, IL-6,
265 ADAMTS4 and ADAMTS5 mRNAs in response to 10μg/ml LPS (Figure 3A). Peak
266 expression of each mRNA occurred 3 to 6 hours post-LPS exposure. Maximum fold
267 changes in mRNA levels of EFLS cultured independently and with DH82 cells
268 respectively, were 10 x (at 6 hours) and 14 x (at 3 hours) for IL-1β mRNA, 50 x (at 6
269 hours) and 27 x (at 6 hours) for IL-6 mRNA, 18 x (at 3 hours) and 16 x (at 3 hours)
270 for ADAMTS4 mRNA, and 4 x (at 6 hours) and 1.4 x (at 6 hours) for ADAMTS5
271 mRNA. Interestingly, the increased expression of ADAMTS5 mRNA over this time
272 period was significantly ($p < 0.05$) lower in EFLS that were co-cultured with DH82
273 cells. Although ADAMTS4 and IL-6 mRNA levels tended to be lower when EFLS
274 were in co-culture, these differences were not statistically significant.

275

276 DH82 Macrophage Gene Expression when Co-Cultured with EFLS

277 DH82 cells cultured independently or in co-culture with EFLS, demonstrated a
278 reproducible, rapid increase in the expression of the cytokines IL-1β and IL-6 mRNA
279 following exposure to LPS (Figure 3B). Peak fold change in IL-6 gene expression
280 occurred earlier than IL-1β (3 hours and 12 hours, respectively), and the mean
281 expression of both cytokines was higher when co-cultured with EFLS, although this
282 was not statistically significant.

283

284 Macrophage Attenuation of EFLS ADAMTS5 is Not Driven by IL-1β Expression

285 When IL-1β binds to the IL-1 membrane bound receptor, it activates several pathways
286 including the NF-κB pathway. RelA/p65, an NF-κB family member, is a strong
287 transcriptional activator of ADAMTS5 in chondrocytes (17). It was hypothesised that
288 increased DH82 IL-1β gene expression may influence EFLS ADAMTS5 gene
289 expression. Statistically significant down-regulation of macrophage IL-1β gene
290 expression was achieved at 6h ($p<0.05$) and 12h ($p<0.01$) post-LPS exposure using
291 siRNA designed to target canine IL-1β compared to a non-specific control siRNA
292 (Figure 4A). Co-culture of IL-1β knockdown DH82 cells EFLS did not change the
293 inhibitory effect of the macrophages on the induction of ADAMTS5 mRNA
294 following LPS stimulation, indicating that macrophage-derived IL-1β expression is
295 not responsible for controlling this inhibition (Figure 4A).

296

297 A Soluble Mediator Produced by Macrophages is Responsible for the 298 Attenuation of ELFS ADAMTS5 Gene Expression

299 EFLS cultured in LPS-containing DH82 conditioned media (CM) expressed lower
300 levels of ADAMTS5 mRNA (Figure 4B) compared with those cultured in LPS-
301 containing unconditioned media (NM). This suggests that the attenuation of
302 ADAMTS5 in the co-culture system is mediated by a soluble factor. Interestingly,
303 EFLS expression of ADAMTS5 mRNA was also lower when the cells were cultured
304 in denatured conditioned media (DCM) ($p < 0.01$), indicating that this soluble factor
305 is thermally stable (Figure 4B). Consistent with these findings, indirect co-culture
306 culture of EFLS with DH82 using well inserts, also led to repressed ADAMTS5
307 mRNAs ($p < 0.01$) (Figure 4B).
308

309 **Co-Culture Does Not Influence EFLS Through NF-κB Signalling**

310 To investigate the influence of the NF-κB signalling pathway on EFLS ADAMTS5
311 gene expression, EFLS in co-culture with DH82 cells were pre-treated with NF-κB
312 inhibitors before being exposed to LPS (Figure 4C). Neither inhibitor prevented the
313 suppression of ADAMTS5 induction in co-cultures. The presence of the inhibitor
314 PDTC led to an overall suppression of EFLS ADAMTS5 gene expression levels,
315 whilst the inhibitor Bay 11-7082 led to similar levels of ADAMTS5 mRNA compared
316 to non-treated EFLS in co-culture. None of these effects were statistically significant.
317

318 **Discussion**

319 Synovial fibroblasts are a potential source of the catabolic factors that contribute to
320 joint tissue breakdown during diseases such as osteoarthritis. Previous studies have
321 identified that inflammatory mediators can control the expression of a range of
322 proteinases by these cells. This study aimed to determine whether there was cross talk
323 between synovial fibroblasts and macrophages, using a novel, multi-species culture

324 model. The role of macrophages within the inflammatory response to joint injury has
325 been investigated using murine macrophage depleted experimental OA models. A
326 reduction in osteophyte formation, fibrosis, synovial activation, synovial lining
327 growth factor production, MMP-induced neoepitope formation, and synovial MMP3
328 and -9 mRNA expression, were observed in macrophage-depleted subjects,
329 suggesting that synovial macrophages are moderators of OA joint pathology (18).
330 However, macrophage-depletion in OA models, has also been linked to increased
331 acute joint inflammation involving significantly higher synovitis scores, increased
332 cellular density, and a reduction in bone mineral density (19, 20). Macrophages are
333 obviously pivotal in the synovial response to joint injury, and provide essential
334 moderation of joint inflammation. Understanding how macrophages interact with
335 other cells within the synovium should be valuable, given the emerging role of
336 synovitis in osteoarthritis pathology.
337
338 We initially observed that ADAMTS5 protein secretion was increased by EFLS
339 following stimulation with LPS. Various catalytically active isoforms of the
340 ADAMTS5 protein have been observed by other groups. We observed a variety of
341 immunopositive bands present in the cell layers of EFLS but only one major band in
342 the media fraction, which migrated at 50kDa. This could be analogous to a similarly
343 sized form missing the C-terminal cysteine rich and spacer domains but still retaining
344 the pro-domain which was observed by Kosasih et al (2016) (14). This would suggest
345 that the most abundant isoform of ADAMTS5 secreted into the media by EFLS is not
346 active. We should not currently rule out the possibility of other isoforms being present
347 in the EFLS culture media however, as we used a precipitation method to concentrate
348 the secreted protein which may have led to non-uniform isoform enrichment.

349

350 Having observed that LPS stimulation of EFLS led to an increase in their secretion of
351 ADAMTS5 protein, we decided to further examine the regulation of ADAMTS5 in
352 our co-culture system. Although ADAMTS5 has multiple tiers of control, many of
353 which occur post-translationally, we found that mRNA was induced over a similar
354 time scale to its protein secretion. This supported the use of mRNA expression to
355 determine ADAMTS5 response in a cell-specific manner in our co-cultures, taking
356 advantage of species-specific PCR primers for transcript quantification. We used the
357 canine DH82 cell line in this study. These cells were originally derived from a
358 histiocytic sarcoma and share many properties with canine macrophages (21),
359 although they don't appear to be strongly polarized toward either M1 or M2
360 macrophage subtypes (22).

361

362 One major finding of this study is that co-culture with macrophages promotes
363 proliferation of EFLS. When EFLS were co-cultured with DH82 cells, loss of
364 fluorescence intensity of the eFluor dye occurred in proportion to the number of
365 DH82 cells present, indicating that EFLS mitosis was stimulated by the co-culture of
366 DH82 cells. Macrophage migration inhibitory factor (MIF) has been shown to
367 significantly stimulate the proliferation of rheumatoid arthritis FLS, with indirect IL-
368 1β and TNF-α involvement (26). IL-1 is also known to stimulate FLS proliferation
369 (27). As macrophages are major producers of both IL-1 and MIF, these factors may
370 explain the influence that DH82 cells have on EFLS proliferation and this may be
371 relevant to the synovial hyperplasia observed during osteoarthritis. EFLS ADAMTS5
372 gene expression was significantly attenuated when cells were co-cultured with DH82
373 macrophages ($p<0.05$).

A further finding of the study was that co-culture of EFLS with macrophages inhibits inflammatory-mediated stimulation of ADAMTS5 expression in the same cells. Induction of ADAMTS5 gene expression by LPS was also suppressed when EFLS were exposed to macrophage-conditioned medium, and even more strongly when EFLS cells were cultured with macrophages indirectly using well inserts. The suppressive effect of DH82 conditioned medium was also evident when it had been heat denatured. A soluble factor released by macrophages, which is either thermally stable or can reversibly denature, is likely to be responsible for these findings. We had wanted to determine whether IL-1β mediated the process as its expression by macrophages was higher when they were co-cultured with EFLS. However, siRNA-mediated knockdown of IL-1β in DH82 cells did not affect their ability to attenuate EFLS ADAMTS5 expression in co-culture. This observation is consistent with a previous study which reported that EFLS ADAMTS5 gene expression did not respond to IL-1β stimulation alone (28). We could also not demonstrate a specific role for NFkB signaling in the modulation of EFLS response by macrophage conditioned media. Equine synoviocyte ADAMTS5 mRNA was reduced when normal synoviocytes were cultured with injured cartilage, compared to normal cartilage (13), a process that was proposed to be a mechanism to protect injured cartilage. Therefore, crosstalk clearly exists between fibroblast-like synoviocytes and other cells within joint tissues and further work is needed to determine the mechanisms that underpin this.

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401

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403

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487

Table 1. Species-specific primer sequences and gene accession numbers for sequences used in their design. F: forward sequence, R: reverse sequence, all sequences are shown 5' to 3'.

Gene	Gene Accession No.	Primer Sequence
Equus Caballus GAPDH	NM_001163856.1	F: TGACCCCCTAACATATTGAGAGTCT R: GCCCCTCCCCTTCTTCCTG
Equus Caballus IL-1β	NM_001082526.1	F: GAGCCCAATCTTCAACATCTATGG R: ATACCAAGTCCTTTTACCAAGCCTG
Equus Caballus IL-6	NM_001082496.1	F: CCTGGTGATGGCTACTGCTTTC R: GGATGTACTTAATGTGCTGTTTGGTT
Equus Caballus ADAMTS4	NM_001111299.1	F: CAGCCTGGCTCCTTCAAAAA R: ATGTGGTCACTATTCCTGCGG
Equus Caballus ADAMTS5	XM_003364218.2	F: ACCGATCCTGCAGTGTCACA R: AAATCTTTTCGCCATGAGCAG
Canis Lupus GAPDH	NM_001003142.1	F: AACTGCTTGGCTCCTCTAGCC R: CCACGATGCCGAAGTGGT
Canis Lupus IL-1β	NM_001037971.1	F: CTATCATCTGCAAAACAGATGCG R: GCATGGCTGCATCACTCATAAA
Canis Lupus IL-6	NM_001003301.1	F: CCTGGTGATGGCTACTGCTTTC R: TGGCATCATCCTTGGAATCTC

488

489

490 **Figure Legends**

491

492 **Figure 1. Expression of ADAMTS5 in EFLS cell layer and culture media.** EFLS
493 were cultured in the presence or absence of 10μg/ml LPS. Western blotting was
494 performed on **(A)** cell layer harvested from one horse and **(B)** culture media harvested
495 at 6 and 16 hours post-LPS exposure from two different horses, using an antibody
496 which recognises ADAMTS5. GAPDH was used as a loading control for the cell
497 layers **(A)**. Forms of ADAMTS5 with molecular weights of 75kDa, 100kDa and 55-
498 60kDa were detected within the cell layers, whilst a 55kDa form was detected in
499 EFLS culture media (indicated by arrowheads in A and B).

500

501 **Figure 2. Characterisation of EFLS/DH82 co-culture system. (A)** Confocal
502 microscopy of GFP-expressing DH82 and Efluor-labelled EFLS cells in 1:1 co-
503 culture. Efluor-labelled EFLS (purple) were excited using a far red (633nm) laser line
504 and detected with a 660/20nm band pass filter. GFP-expressing DH82 cells (green)
505 were excited using a green (488/532nm) laser line and detected with a FITC band
506 pass filter. An overlay image of both channels is shown as well as a phase contrast
507 image of DH82 (arrow) and EFLS (arrowhead) cells in co-culture. **(B)** Flow
508 cytometry analysis of GFP-expressing DH82 and efluor-labelled EFLS cells in co-
509 culture. Density plot diagrams of DH82 (i) and EFLS (ii) cultured individually and in
510 co-culture (iii); EFLS (G1) and DH82 (G2) populations were gated. (iv) Fluorescence
511 intensity emitted from cells within the gated areas (G1 and G2). (v and vi).
512 Fluorescence intensity emitted by labelled DH82 and EFLS after 24 hours in co-
513 culture at different proportions, detected by two different interference filters
514 675/25nm (v, red) and 533/30nm (vi, green) respectively. Gates corresponding to

515 EFLS primary (G3) and secondary (G4) generations, and DH82 cells (G5) were
516 applied to enable cell numbers within each co-culture to be determined. (C) Average
517 qRT-PCR cycle threshold (Ct) values for each equine and canine sequence primer
518 pair, when used to measure expression in DH82, EFLS or co-cultures (n=3). Ct values
519 were higher, reflecting low expression of genes, when primers of one species were
520 applied to cDNA from the second species. Error bars represent SEM.
521
522 **Figure 3. Fold changes of EFLS gene expression when cultured independently or**
523 **in co-culture with DH82 macrophages. (A) Gene expression of** IL-1β, IL-6,
524 ADAMTS4 and ADAMTS5 measured by qRT-PCR in EFLS when cultured
525 independently or in co-culture with DH82. (B) Gene expression of IL-1β and IL-6
526 gene measured by qRT-PCR in DH82 cells when cultured independently or in co-
527 culture with EFLS (n=3). Data are displayed as fold changes (n=3) compared to the
528 gene expression of independently cultured EFLS at 0 hours with no LPS exposure.
529 Gene expression was measured at 1, 3, 6, 12 and 24 hours post-LPS exposure. Error
530 bars represent SEM. Increased expression of ADAMTS5 mRNA over the 24-hour
531 period was significantly (* p<0.05) lower when EFLS were cultured with DH82.
532
533 **Figure 4. Mechanistic investigation of EFLS ADAMTS5 mRNA attenuation by**
534 **DH82 cells. (A)** DH82 were transfected with either csiRNA or with canine anti-IL-1β
535 siRNA and co-cultured with EFLS (n=3) in the presence of 10µg/ml LPS. Histograms
536 display DH82 IL-1β gene expression and EFLS ADAMST5 gene expression. (B)
537 EFLS ADAMTS5 gene expression when cultured in DH82 conditioned media, or
538 with DH82 cells within well-inserts. Fold changes in EFLS ADAMTS5 gene
539 expression are compared to EFLS cells cultured in LPS-containing normal media

540 (NM). EFLS expression when cultured with DH82 conditioned media (CM) and
541 DH82 denatured conditioned media (DCM) (** = $p < 0.01$), and with DH82 cultured
542 in well inserts (** = $p < 0.01$) is displayed. All conditions (n = 3) were harvested 12
543 hours after 10ug/ml LPS exposure. (C) Fold changes in EFLS ADAMTS5 gene
544 expression after pre-treatment with NF-κB inhibitors. EFLS (n =3) in direct co-culture
545 with DH82 cells were pre-treated with the NF-κB inhibitors; Bay 11-7082 and PDTC
546 before exposure to 10μg/ml LPS. Cells were harvested at 0, 6 and 12 hours post-LPS
547 exposure. Data is presented as fold changes in EFLS ADAMTS5 gene expression
548 compared to the gene expression of EFLS cells in co-culture at 0h. All data in this
549 figure is presented as mean +/- SEM, * = $p<0.05$, ** = $p<0.01$

550

551 **Conflict of Interest Disclosure**

552 There are no conflicting interests to declare.

Figure 1

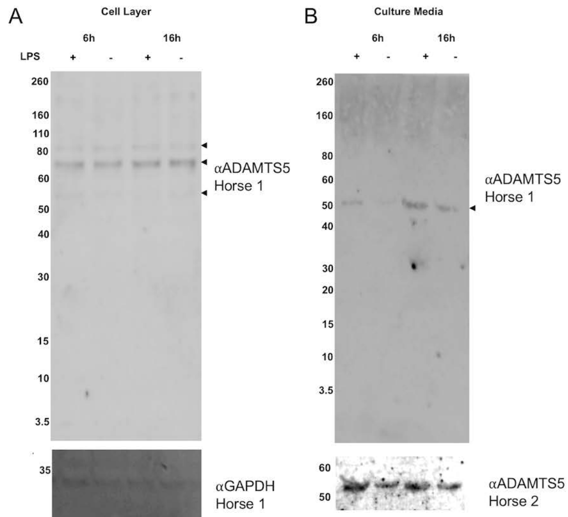


Figure 2

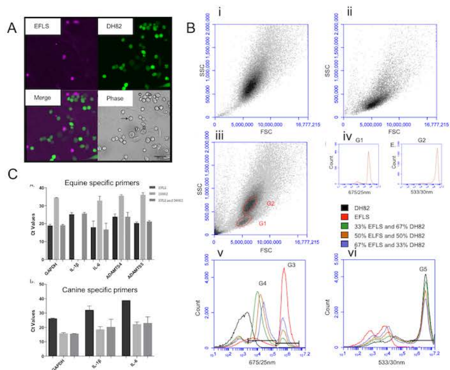
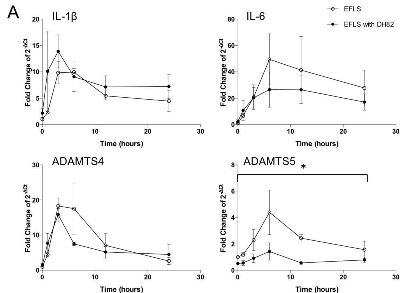


Figure 3



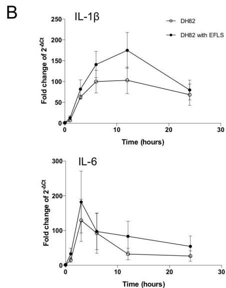


Figure 4