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Alpha2EQ downregulates proinflammatory cytokine and chemokine gene expression in cultured synovial fibroblasts

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Objective
To investigate the ability of the equine orthobiologic Alpha2EQ to control inflammation in cultured synovial fibroblasts.

Methods
Equine synovial fibroblasts (n = 16) were cultured in a monolayer, and a targeted transcriptomic analysis (NanoString nCounter) was performed to screen for upregulated inflammatory gene expression. Cells were classified according to their IL-6 expression level. In the first experiment, high IL-6 expression (n = 4) and low IL-6 expression (4) cells were treated with Alpha2EQ, and in the second, cells with basal IL-6 expression were stimulated with 10 ng/mL IL-1β (4) before treatment. Allogeneic Alpha2EQ was pooled from sound healthy horses (n = 6) at a dose of 25% vol/vol of cell culture media. Twenty-four hours later, RNA was isolated for NanoString gene expression analysis. The *t* tests assessed differences in mean gene expression fold changes between baseline and treatment, while one-way ANOVA or Wilcoxon signed-rank tests were used for multiple comparisons ($P < .05$).

Results
Alpha2EQ downregulated inflammatory genes in 2 cell culture models. Compared to baseline, Alpha2EQ treatment significantly reduced expression of IL-6, IL-15, and CCL2/MCP1 in IL-6^{HIGH} synovial fibroblasts by 1.88- to 4.21-fold, as well as expression of IL-1β, CCL5/RANTES, and PPBP/CXCL7 by 2.14- to 4.07-fold in a 10 ng/mL IL-1β model. In addition, CXCL6/GCP-2 and TNF-α were significantly downregulated by Alpha2EQ in both models (2.64- to 5.38-fold).

Conclusions
Alpha2EQ reduces inflammation by modulating the expression of cytokines and chemokines by synovial cells.

Clinical Relevance
This study provides early insights into Alpha2EQ's anti-inflammatory mechanisms in vitro and evidence to support its clinical use in the treatment of equine osteoarthritis.

Keywords: alpha-2-macroglobulin, osteoarthritis, synovial fibroblast, orthobiologic, equine

It has been well established that osteoarthritis (OA) is a complex disease of the joint as an organ, with the synovium driving a cascade of inflammation that ultimately results in cartilage degeneration and associated pain.[1–3] The rigorous work of others has shown that the majority of key OA cytokines are produced by synoviocytes.[1] In fact, examination of matched synovium and cartilage samples of human OA patients has revealed numerous inflammatory cytokines expressed exclusively by synoviocytes, including tumor necrosis factor (TNF), IL-6, IL-1β, and IL-15.[1] Specifically, IL-6 is used as a serum and synovial fluid biomarker in humans with OA.[4–6] In both naturally occurring and experimental models, the synovium, rather than the cartilage itself, plays a critical role in the early upregulation of matrix metalloproteinase (MMP) expression, effectors that can both degrade the extracellular matrix of cartilage and activate other MMPs.[7,8] Cross talk between the synovium and chondrocytes of the articular cartilage is therefore critical to disease progression.[1] No single in vitro model recapitulates the complex mechanisms of naturally occurring OA; however, cultured cells

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stimulated with IL-1β are routinely used to study the integral inflammatory pathways.[9–13]

In the equine athlete, OA is manifested as lameness, loss of use, and reduced quality of life. Currently, there is no disease-modifying treatment for OA. Intra-articular corticosteroids are used clinically to control inflammation and ameliorate pain; however, these medications have been shown to exert deleterious effects on chondrocyte extracellular matrix production[14] and proteoglycan breakdown, as evidenced by increased synovial glycosaminoglycan concentration.[15] Moreover, the high prevalence of equine metabolic syndrome further limits corticosteroid use due to concern for laminitis.[16] Orthobiologic therapies, derived from and delivered back to the patient, have emerged as a widely used alternative OA therapy in equine practice.[17,18] Alpha-2-macroglobulin (A2M) is a ubiquitous blood glycoprotein with broad anti-inflammatory activity via 3 main mechanisms: baiting and trapping of proteases to prevent substrate binding, direct cytokine interactions, and downregulation of proinflammatory gene expression.[19–21] Consequently, A2M has been concentrated from serum and plasma as an IA OA treatment in both humans[22] and horses. To date, the cellular effects of the equine commercial orthobiologic kit (Alpha2EQ (Creative Science [formerly Astaria Global, LLC])), which is purported to concentrate A2M in plasma, have remained unexplored.

The objective of this study was to investigate the ability of Alpha2EQ to control inflammation in cultured synovial fibroblasts. Specifically, our aims were to examine gene expression changes resulting from Alpha2EQ treatment in synovial fibroblasts classified by their high or low IL-6 expression (IL-6^{HIGH} vs IL-6^{LOW}) and in synovial fibroblasts with basal IL-6 expression (IL-6^{BASAL}) in an IL-1β stimulation model. We hypothesized that 24-hour Alpha2EQ treatment in vitro would reduce the expression of key proinflammatory genes implicated in the pathogenesis of OA (metalloproteinases, cytokines, and chemokines) in IL-6^{HIGH} synovial fibroblasts and in synovial fibroblasts stimulated with 10 ng/mL IL-1β.

Methods

A broad overview of the study design is provided in **Figure 1**. Briefly, synovial fibroblasts harvested from grossly normal equine joints were first screened for the expression of key regulatory genes involved in OA pathogenesis. Inclusion in each experiment of the study was dictated by IL-6 expression. Ultimately, horses with either IL-6^{HIGH} vs IL-6^{LOW} gene expression compared to background gene expression were included in the first experiment of the study (experiment 1), while horses with IL-6^{BASAL} expression were included in the second experiment using an IL-1β stimulation model (experiment 2). No horses were used in both experiments.

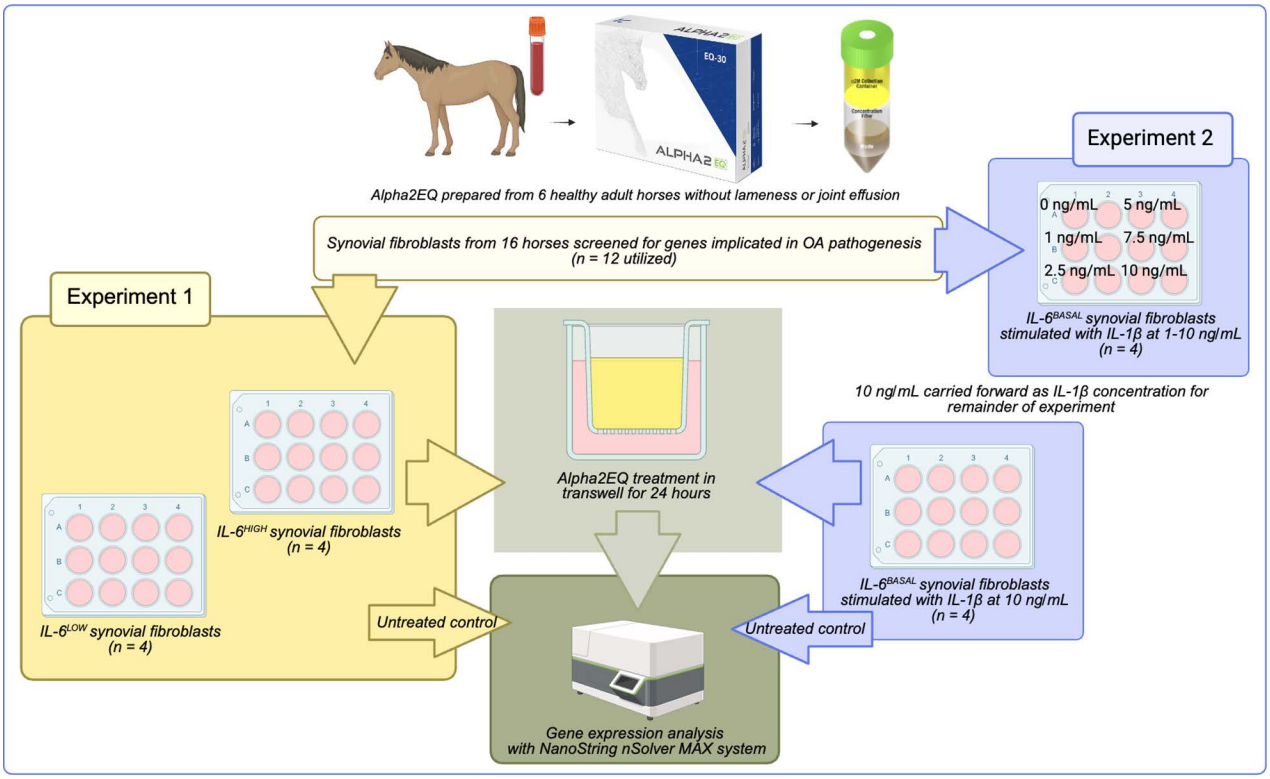

Figure 1—Schematic illustration of the experimental design of the 2 experiments in the study, first examining the effect of Alpha2EQ on synovial fibroblasts with endogenously high IL-6 gene expression (IL-6^{HIGH}; experiment 1) and then on synovial fibroblasts in an IL-1β model of osteoarthritis (experiment 2). Created with Biorender.com. IL-6^{BASAL} = Basal IL-6 expression. IL-6^{LOW} = Low IL-6 expression. OA = Osteoarthritis.

Preparation of Alpha2EQ

Alpha2EQ was prepared from 6 healthy adult horses free of lameness or joint effusion (Thoroughbreds; 3 castrated males, 3 intact females; mean age, 16 years; range, 12 to 23 years) using Alpha2EQ-30 kits, per the manufacturer's instructions, and with approval by North Carolina State University College of Veterinary Medicine's IACUC (protocol No. 22-300 renewed as No. 25-256). Briefly, two 60-mL syringes were prepared containing a 5-mL acid citrate dextrose solution A anticoagulant solution. Following aseptic preparation of the jugular vein, using an extension set with a 3-way stopcock and 16-gauge needle, 35 mL of jugular venous blood was drawn into each syringe to a total volume of 40 mL. After transfer to the kit-supplied vacutainer tubes, blood was centrifuged at 3,400 rpm for 10 minutes. Subsequently, 20 mL of plasma was transferred to each provided conical tube with filter (40 mL total plasma volume), and the conical tubes were centrifuged at 3,400 rpm for 10 minutes to collect the Alpha2EQ injectate (final volume, 30 mL). Aliquots (1 mL) were stored for each individual horse at −80 °C for no more than 7 months before experimentation. At the time of treatment, aliquots were thawed at 37 °C until no ice remained (samples cool). An equal volume of Alpha2EQ (900 µL) was then pooled from each of the 6 horses to create a pooled allogeneic Alpha2EQ treatment for immediate application in cell culture. A novel batch of Alpha2EQ was prepared (blood drawn, processed, and frozen in aliquots) for use in the first experiment of the study (prepared and stored December 2024) and for the second experiment (prepared and stored May 2025), with the intention of using a single batch of Alpha2EQ with sufficient volume for each experiment.

Postmortem tissue collection

Synovium was aseptically collected from the femoropatellar joints of healthy adult horses (n = 16) euthanized for reasons unrelated to this study, with approval by North Carolina State University College of Veterinary Medicine's IACUC (protocol No. 23-236). Horses were free of hind limb lameness, had no history of OA, and were not administered any systemic medications for at least 2 weeks before euthanasia. At the time of postmortem joint dissection, all femoropatellar joints were devoid of gross pathology.

Synovial fibroblast isolation and culture

Synovium was collected from the femoropatellar joint using sharp dissection with a No. 10 scalpel blade and processed for synoviocyte isolation using previously described techniques.[23] Briefly, during harvest and transport, synovium was submerged in PBS with 2% penicillin/streptomycin and stored on ice. Synovium was then digested using collagenase type II in the laboratory for 2 hours at 37 °C using constant rotation with a magnetic stir bar. Digest was filtered through a stainless-steel strainer and centrifuged at 800 *g* for 20 minutes. The resultant cell pellet was resuspended in synoviocyte growth media containing Dulbecco modified Eagle medium with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were then filtered through a 100-µm nylon cell strainer before centrifugation at 500 *g* for 5 minutes, which was repeated twice. The resultant isolated synoviocytes were stained with acridine orange/propidium iodide, and a live cell count was performed using an automated cell counter. Cells were resuspended in aliquots of 10 X 10^6 cells/mL in freeze media containing 80% growth media with 10% FBS and 10% dimethyl sulfoxide, first at −80 °C for 24 hours, and then they were banked in liquid nitrogen until experimentation.

Synoviocytes were expanded to the second passage to obtain a homogenous population of synovial fibroblasts for experimentation.[23,24] Second passage cryopreserved synovial fibroblasts were plated from frozen stocks in 100-mm plates at a density of 500,000 cells/plate. After passaging to the third passage, synovial fibroblasts were plated in 12-well tissue culture plates at a density of 30,000 cells/well. In all instances, 2 wells per horse per experimental group were plated (ie, 2 technical replicates). Culture conditions were maintained at 5% CO_2, 90% humidity, and 37 °C throughout. Cells were maintained in synoviocyte growth media during a 96-hour period of adherence, with a change of media at 24 hours after plating, before any treatment or stimulation.

Synovial fibroblast screening

Synovial fibroblasts from 16 horses were screened for endogenous expression of proinflammatory genes implicated in OA pathogenesis at baseline (see "Gene expression analysis" for details). Interleukin-6, a well-established gene in OA pathogenesis, was used to identify cells for inclusion in the 2 experiments of the study. Synovial fibroblasts with endogenous IL-6 expression 10-fold higher than background (≥ 200 normalized RNA counts) were classified as IL-6^{HIGH}, while cells with expression within background (≤ 20 counts) were classified as IL-6^{LOW} synovial fibroblasts, and cells with IL-6 expression ≤ 30 normalized RNA counts were classified as IL-6^{BASAL}. Cells with IL-6 expression between 31 and 199 normalized RNA counts were excluded from further analysis. Ultimately, synovial fibroblasts from 12 horses were utilized for this study and distributed throughout the 2 experiments, with no horse included in both experiments (9 Thoroughbreds, 1 Saddlebred, 1 warmblood, 1 Paint Horse; 7 castrated males, 1 intact male, 4 intact females; mean age, 12 years; range, 2 to 22 years).

Synovial fibroblast culture for experiments 1 and 2

Twenty-six hours before Alpha2EQ treatment, all cells (IL-6^{HIGH}, IL-6^{LOW}, and IL-6^{BASAL}) were observed for morphology and confluence using light microscopy at 40X magnification. Wells that were not 100% confluent or exhibited abnormal morphological characteristics were excluded from experimentation.

In the majority of instances, both technical replicates were included, and cells from these 2 wells were pooled at the time of cell lysis and RNA collection. Cells were then washed with PBS, and growth media were changed to OPTI-MEM (Gibco) serum-free media. After a 2-hour incubation, IL-6^{HIGH} and IL-6^{LOW} cells were again washed with PBS and fed with OPTI-MEM to remove remaining endogenous A2M from FBS. The IL-6^{BASAL} synovial fibroblasts were stimulated with IL-1β.

Synovial fibroblast stimulation with IL-1β

Following the 2-hour incubation in OPTI-MEM, IL-6^{BASAL} cells were washed with PBS and then stimulated with recombinant human IL-1β with carrier (R&D Systems) at doses of 1, 2.5, 5, 7.5, and 10 ng/mL of OPTI-MEM, in a total volume of 1.5 mL/well, for 24 hours. Control cells were left unstimulated (0 ng/mL IL-1β). Interleukin-1β was reconstituted per the manufacturer's instructions. Briefly, 100 µL PBS with 0.1% bovine serum albumin was added directly to 5 µg IL-1β to create a 50-ng/µL working solution, which was stored in aliquots at −80 °C for no more than 3 months before use. At the time of experimentation, aliquots were thawed at room temperature, and media solutions were immediately prepared and applied in cell culture.

Synovial fibroblast treatment with Alpha2EQ

Following 24 hours of incubation in OPTI-MEM (experiment 1) or in OPTI-MEM with IL-1β (experiment 2), all cells were washed with PBS, and OPTI-MEM (1.5 mL) was replaced in all wells. Transwell inserts seated within each culture well were utilized to apply the Alpha2EQ treatment to cells to avoid clotting of the blood-based orthobiologic. Alpha2EQ was applied at a dose of 25% vol/vol of cell culture media (0.5 mL/1.5 mL), approximately the dose delivered to the average equine joint in clinical practice. The mean ± SD medial femorotibial joint volume is reported to be 41.67 ± 5.77 mL.[25] Considering this reported SD and that the dose of Alpha2EQ for the equine stifle is 8 mL[26] ([8 mL/42 mL] X 100 = 19%), we selected a 25% vol/vol dose for our experiments. Cells were incubated with Alpha2EQ treatment for 24 hours. Untreated synovial fibroblasts served as controls in both experiments and had OPTI-MEM (0.5 mL) applied in transwells.

RNA extraction, quantification, and quality assessment

After 24 hours of Alpha2EQ treatment, total RNA was extracted from all synovial fibroblasts in both experiments using the RNeasy Mini Kit (Qiagen Inc) according to the manufacturer's instructions. To increase RNA yield, RNAse-free water was warmed to 37 °C and allowed to incubate on the column membrane for 5 minutes during elution. Ultraviolet microvolume spectrophotometry was used to assess RNA purity (260/280 > 2.0) and quantity (> 20 ng/µL) before inclusion in gene expression analyses. The RNA was stored at −80 °C for no longer than 2 weeks before NanoString gene expression analysis was performed.

Gene expression analysis

The NanoString nCounter MAX system (Bruker Spatial Biology) was used to perform a limited, targeted transcriptomic analysis of 31 genes involved in OA pathogenesis **(Supplementary Table S1)**, which utilized a custom codeset of equine probes designed for our laboratory.[23,27] These sequence-specific reporter and capture probes allow for counting of mRNA copies (ie, counts) using a digital analyzer. For each sample, 100 ng of RNA was hybridized with the custom codeset for 18 hours in a thermocycler at 65 °C with the lid heated to 70 °C; beyond 18 hours, samples were held at 4 °C for no more than 6 hours until analysis. The housekeeping genes beta-actin, glyceraldehyde-3-phosphate dehydrogenase, and hypoxanthine phosphoribosyl-transferase 1 were included and used for normalization of our 31 genes of interest included in the panel. Data processing, quality assessment, and normalization were performed in nSolver Analysis Software (Bruker Spatial Biology) and exported to Microsoft Excel for data management. Gene expression counts of interest were normalized to housekeeping genes in all instances before statistical analysis and are hereafter referred to as normalized RNA counts.

Statistical analysis

For experiment 1, gene expression data (normalized RNA counts) were $\log_2$ transformed before calculation of mean gene expression fold change from baseline for IL-6^{HIGH} and IL-6^{LOW} cells treated with Alpha2EQ for the generation of the heat map in Figure 2. Log_2-transformed data were used throughout due to high interhorse biologic variability and small sample size in experiment 1. Data were assessed for normality using the Shapiro-Wilk test and for equal variance using the Levene test. For each gene, a 1-tailed *t* test was used to assess differences between (1) IL-6^{HIGH} cells at baseline and IL-6^{HIGH} cells treated with Alpha2EQ (within-group comparison), and (2) IL-6^{HIGH} synovial fibroblasts treated with Alpha2EQ and IL-6^{LOW} synovial fibroblasts treated with Alpha2EQ (between-group comparison). One-tailed *t* tests were selected given our directional hypothesis that Alpha2EQ treatment would downregulate proinflammatory gene expression. These analyses were performed using JMP Student Edition 18.2.1 (JMP Statistical Discovery LLC).

For experiment 2, to first determine significant differences in gene expression resulting from various concentrations of IL-1β stimulation, log-transformed, normalized RNA counts were compared for baseline and following stimulation at 1, 2.5, 5, 7.5, and 10 ng/mL IL-1β using 2-tailed *t* tests, assuming unequal variance, for each gene. This analysis was performed in nSolver, software that uses a 2-tailed *t* test within each gene to determine significant differences in expression, regardless of direction of change (up- or downregulation), given our nondirectional hypothesis that some genes within the panel would be upregulated by stimulation and others downregulated. Based on these results (Table 2), 10 ng/mL IL-1β was carried forward for the remainder of experiment 2.

Next, the mean gene expression fold change from baseline (unstimulated, 0 ng/mL IL-1β) was calculated for 10 ng/mL IL-1β–stimulated cells and 10 ng/mL IL-1β–stimulated cells treated with Alpha2EQ for the generation of the heat map in Figure 3. Data were assessed for normality and equal variance as described above, and 1-tailed *t* tests were used to assess differences between these 2 groups. Differences in gene expression (normalized RNA counts) between unstimulated baseline (0 ng/mL IL-1β), 10 ng/mL IL-1β–stimulated, and 10 ng/mL IL-1β–stimulated cells treated with Alpha2EQ were also examined. Following Shapiro-Wilk and Levene tests, parametric data were analyzed using a 1-way ANOVA with Tukey test for multiple comparisons, and nonparametric data were analyzed using Wilcoxon signed-rank tests for pairwise comparisons within each gene. These analyses were performed in JMP Student Edition. Mean gene expression fold change between IL-1β–stimulated cells and IL-1β–stimulated cells treated with Alpha2EQ was calculated and reported using the results that were found to be significant in this analysis.

For all data reported for experiment 1 and experiment 2, the Benjamini and Hochberg correction[28] for false discovery rate (5%) was applied to correct for multiple comparisons. Adjusted *P* values are reported alongside original *P* values in all instances, and for all statistical testing, significance was defined as $P < .05$.

Results

Screening of cryopreserved synovial fibroblasts reveals distinct populations of cells based on IL-6 gene expression

Targeted transcriptomic analysis using NanoString nCounter MAX technology identified synovial fibroblasts from 4 horses (3 Thoroughbreds, 1 Saddlebred; all castrated males; mean age, 7.5; range, 2 to 11 years) with endogenous IL-6 expression 10-fold higher than background (≥ 200 normalized RNA counts; mean ± SD IL-6 expression of 16,043.50 ± 26,759.42 normalized RNA counts; range, 520.21 to 56,077.10 normalized counts). We determined this 10-fold increase to be biologically significant given that 2-fold gene expression changes are a standard threshold for functionally meaningful alterations in synovial transcriptomics.[29–31] These cells were classified as IL-6^{HIGH} synovial fibroblasts to be included in the first experiment of the study, examining the effect of Alpha2EQ on cells with endogenously high IL-6 expression. Synovial fibroblasts classified as IL-6^{HIGH} also exhibited marked upregulation (6- to 1,456-fold higher than background expression) of other proinflammatory genes associated with OA pathogenesis, including a disintegrin and metalloproteinase with thrombospondin motif 4 (ADAMTS4), C-C motif chemokine ligand 2/monocyte chemotactic protein 1 (CCL2/MCP1), C-X-C motif chemokine ligand 6/granulocyte chemotactic protein 2 (CXCL6/GCP-2), as well as MMP-1, -3, and -13. Four horses (2 Thoroughbreds, 1 warmblood, 1 Paint Horse; 2 castrated males, 2 intact females; mean age, 16; range, 12 to 22 years) were identified as having cells with IL-6 expression within background (≤ 20 counts; mean ± SD IL-6 expression of 16.49 ± 4.17 normalized RNA counts; range, 11.51 to 20.31 normalized counts) and were classified as IL-6^{LOW} synovial fibroblasts to be included in experiment 1 **(Table 1)**.

Four different horses were included in the second experiment in which cells were stimulated with IL-1β (4 Thoroughbreds; 1 castrated male, 1 intact male, 2 intact females; mean age, 13.5; range, 8 to 21 years). Synovial fibroblasts from these horses had IL-6 gene expression either within background (ie, same expression pattern as IL-6^{LOW} cells) or just slightly above background and were termed IL-6^{BASAL}. Therefore, horses were included in experiment 2 (IL-6^{BASAL} cells) if IL-6 expression was ≤ 30 normalized RNA counts (mean ± SD IL-6 expression of 19 ± 8.32 normalized RNA counts; range, 8.59 to 28.98 normalized counts). This IL-6 expression threshold allowed for stimulation of a consistent inflammatory model with IL-1β and differentiated these horses' cells from those used in experiment 1. All RNA samples passed quality control parameters in nSolver.

Table 1—Synovial fibroblasts were screened for IL-6 expression level.

	IL-6^{HIGH} synovial fibroblasts (RNA counts)					**IL-6^{LOW} synovial fibroblasts (RNA counts)**				
Gene	**Horse 1**	**Horse 2**	**Horse 3**	**Horse 4**	**Mean**	**Horse 5**	**Horse 6**	**Horse 7**	**Horse 8**	**Mean**
ADAMTS4	939.41	206.94	43.03	6,760.65	1,987.51	55.62	38.77	61.61	21.89	44.47
CCL2/MCP1	12,325.00	4,911.73	3,025.44	53,607.45	18,467.41	201.38	315.71	207.37	129.54	213.50
CCL5/ RANTES	155.74	7.48	21.51	335.17	129.98	38.36	55.39	43.58	25.54	40.72
CXCL6/GCP-2	29,194.62	6,081.07	2,012.4	79,166.8	29,113.72	257.00	295.40	359.15	229.89	285.36
IL-6	**5,230.54**	**2,346.16**	**520.21**	**56,077.10**	**16,043.50**	**11.51**	**20.31**	**19.54**	**14.60**	**16.49**
MMP-1	2,495.15	1,847.51	86.05	24,577.37	7,251.52	21.10	22.16	18.03	9.12	17.60
MMP-3	9,483.57	3,941.85	64.54	67,637.39	20,281.84	57.54	36.93	37.57	47.44	44.87
MMP-13	177.28	62.33	17.60	1,002.56	314.94	47.95	20.31	37.57	20.07	31.48
TNF-α	86.15	32.41	39.11	354.28	127.99	103.57	79.39	102.18	72.98	89.53

Cells with endogenous IL-6 expression 10-fold higher than background (≥ 200 normalized RNA counts) were classified as IL-6^{HIGH} synovial fibroblasts (horses 1 to 4; 3 Thoroughbreds, 1 Saddlebred; all castrated males; mean age, 7.5; range, 2 to 11 years), and cells with expression within background (≤ 20 counts) were classified as IL-6^{LOW} synovial fibroblasts (horses 5 to 8; 2 Thoroughbreds, 1 warmblood, 1 Paint Horse; 2 castrated males, 2 intact females; mean age, 16; range, 12 to 22 years). Also reported are select proinflammatory genes associated with osteoarthritis (OA), which demonstrated greater mean RNA counts for IL-6^{HIGH} compared to IL-6^{LOW} synovial fibroblasts. Results of IL-6 expression are bolded.

Alpha2EQ significantly downregulates expression of proinflammatory genes in IL-6^{HIGH} synovial fibroblasts

We first investigated changes in key proinflammatory genes implicated in the pathogenesis of OA in IL-6^{HIGH} synovial fibroblasts following 24-hour Alpha2EQ treatment in vitro. Compared to baseline, IL-6^{HIGH} synovial fibroblasts treated with Alpha2EQ showed 1.51- to 5.38-fold downregulation of genes encoding MMPs (ADAMTS4, MMP-1, MMP-3, and MMP-13), inflammatory cytokines (IL-6, IL-15, and TNF-α), and chemokines (CCL2/MCP1; C-C motif chemokine ligand 5 [CCL5]/regulated upon activation, normal T-cell expressed and secreted [RANTES]; and CXCL6/GCP-2; **Figure 2**). The IL-6^{HIGH} synovial fibroblast gene expression (mean ± SD log_2-normalized RNA counts) was significantly reduced from baseline for IL-6 (12.09 ± 2.82 vs 7.90 ± 1.83; P = .0268; adjusted P = .0320), IL-15 (6.58 ± 0.52 vs 4.70 ± 1.26; P = .0255; adjusted P = .0316), TNF-α (6.30 ± 1.57 vs 3.47 ± 0.96; P = .0139; adjusted P = .0187), CCL2/MCP1 (13.28 ± 1.82 vs 9.07 ± 1.09; P = .0056;

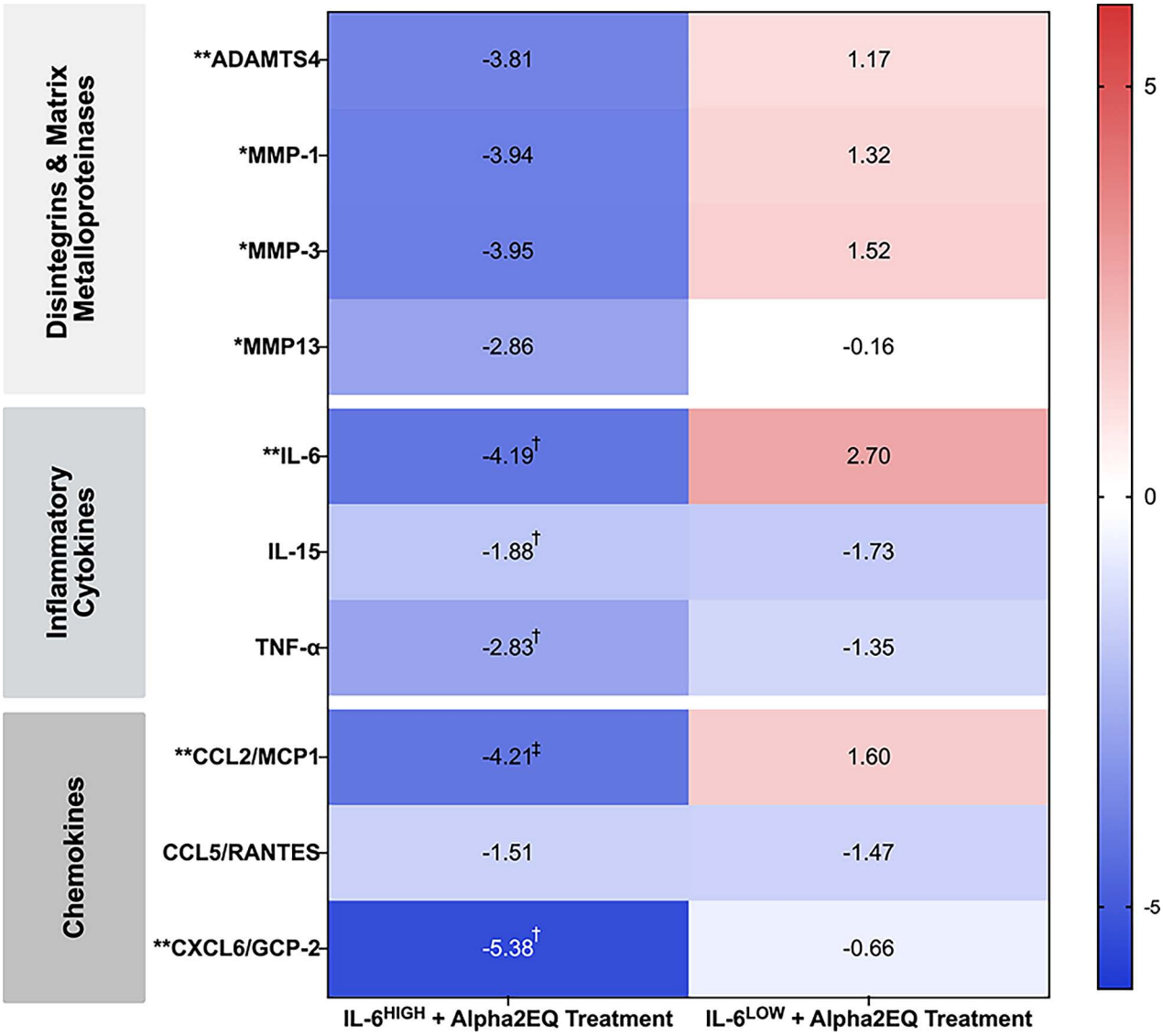

Figure 2—Alpha2EQ downregulates proinflammatory gene expression in IL-6^{HIGH} synovial fibroblasts in vitro. Heat map reports mean gene expression fold change from baseline for IL-6^{HIGH} (n = 4) and IL-6^{LOW} (4) synovial fibroblasts treated with Alpha2EQ, with red indicating gene upregulation, blue indicating downregulation, and white indicating expression equivalent to baseline. Gene expression (log_2-normalized RNA counts) of IL-6^{HIGH} synovial fibroblasts at baseline was compared to IL-6^{HIGH} synovial fibroblasts treated with Alpha2EQ using 1-tailed t tests for each gene. †P < .05 and by ‡P < .01, significant reduction in mean gene expression. *P < .05 and **P < .01, significant differences between IL-6^{HIGH} and IL-6^{LOW} synovial fibroblasts treated with Alpha2EQ, as determined by 1-tailed t test. Created with GraphPad Prism Version 10.1.1 and Biorender.com. ADAMTS4 = A disintegrin and metalloproteinase with thrombospondin motif 4. CCL2/MCP1 = C-C motif chemokine ligand 2/monocyte chemotactic protein 1. CCL5/RANTES = C-C motif chemokine ligand 5/regulated upon activation, normal T-cell expressed and presumably secreted. CXCL6/GCP-2 = C-X-C motif chemokine ligand 6/granulocyte chemotactic protein 2. MMP = Matrix metalloproteinase. TNF-α = Tumor necrosis factor-α.

adjusted P = .0079), and CXCL6/GCP-2 (13.66 ± 2.35 vs 8.28 ± 3.27; P = .0203; adjusted P = .0262) with Alpha2EQ treatment. The ADAMTS4 expression was downregulated (8.93 ± 3.11 vs 5.13 ± 0.85; P = .0442; adjusted P = .0507) but did not reach statistical significance. In addition, expression of several genes was decreased in IL-6^{HIGH} cells treated with Alpha2EQ when compared to IL-6^{LOW} cells treated with Alpha2EQ, including ADAMTS4 (−3.81 ± 2.80 vs 1.17 ± 0.80; P = .0003; adjusted P = .0004), MMP-1 (−3.94 ± 2.73 vs 1.32 ± 0.81; P = .0131; adjusted P = .0156), MMP-3 (−3.95 ± 3.27 vs 1.52 ± 0.61;

Table 2—Synovial fibroblasts (4 Thoroughbreds; 1 castrated male, 1 intact male, 2 intact females; mean age, 13.5 years; range, 8 to 21 years) were stimulated with 1, 2.5, 5, 7.5, or 10 ng/mL IL-1β for 24 hours to establish an in vitro model of inflammation and examine gene expression across this range of IL-1β stimulation concentrations.

	Mean ± SD log_2 fold change from baseline across IL-1β stimulation concentrations				
Gene	**1 ng/mL**	**2.5 ng/mL**	**5 ng/mL**	**7.5 ng/mL**	**10 ng/mL**
Disintegrins and MMPs					
ADAMTS4	**1.77 ± 0.50**	**2.80 ± 0.45**	**3.03 ± 0.29**	**3.45 ± 0.31**	**3.45 ± 0.28**
CCL2/MCP1	**2.03 ± 1.33**	**2.71 ± 1.36**	**3.16 ± 1.26**	**3.44 ± 1.15**	**3.47 ± 1.33**
MMP-1	**10.29 ± 2.30**	**11.69 ± 2.08**	**12.02 ± 1.83**	**12.30 ± 1.80**	**12.54 ± 1.71**
MMP-3	**7.58 ± 2.42**	**9.30 ± 1.59**	**9.82 ± 1.33**	**10.13 ± 1.36**	**10.30 ± 1.32**
MMP-13	**8.11 ± 2.64**	**9.44 ± 2.08**	**9.94 ± 1.94**	**10.24 ± 1.80**	**10.17 ± 2.04**
Inflammatory cytokines					
IL-10	0.00 ± 1.57	0.97 ± 1.74	0.96 ± 1.22	1.30 ± 1.52	**1.57 ± 1.13**
IL-17	0.35 ± 1.25	1.37 ± 1.54	1.47 ± 1.69	**1.77 ± 1.46**	**1.96 ± 1.48**
IL-1β	0.26 ± 1.02	**1.63 ± 0.80**	**1.51 ± 0.67**	**1.64 ± 0.64**	**1.76 ± 0.64**
IL-6	**6.73 ± 1.72**	**8.62 ± 1.82**	**9.18 ± 1.32**	**9.72 ± 1.08**	**9.85 ± 1.32**
TNF-α	**1.42 ± 0.25**	**1.31 ± 0.48**	**1.26 ± 0.21**	**1.51 ± 0.22**	**1.44 ± 0.22**
Chemokines					
CXCL6/GCP-2	**6.53 ± 1.76**	**7.57 ± 1.46**	**7.95 ± 1.27**	**8.19 ± 1.17**	**8.26 ± 1.22**
PPBP/CXCL7	**1.13 ± 0.44**	**1.17 ± 0.39**	**1.18 ± 0.45**	**1.28 ± 0.38**	**1.36 ± 0.29**
CCL5/RANTES	**2.14 ± 0.26**	**2.06 ± 0.45**	**2.31 ± 0.42**	**2.30 ± 0.76**	**2.57 ± 0.44**

Data are presented as mean ± SD log_2 fold change from baseline.
Significant upregulation from baseline, as determined by a 2-tailed t test with $P < .05$, is indicated by bold.
ADAMTS4 = A disintegrin and metalloproteinase with thrombospondin motif 4. CCL2/MCP1 = C-C motif chemokine ligand 2/monocyte chemotactic protein 1. CCL5/RANTES = C-C motif chemokine ligand 5/regulated upon activation, normal T-cell expressed and secreted. CXCL6/GCP-2 = C-X-C motif chemokine ligand 6/granulocyte chemotactic protein 2. MMP = Matrix metalloproteinase. PPBP/CXCL7 = Proplatelet basic protein/C-X-C motif chemokine ligand 7. TNF-α = Tumor necrosis factor-α.

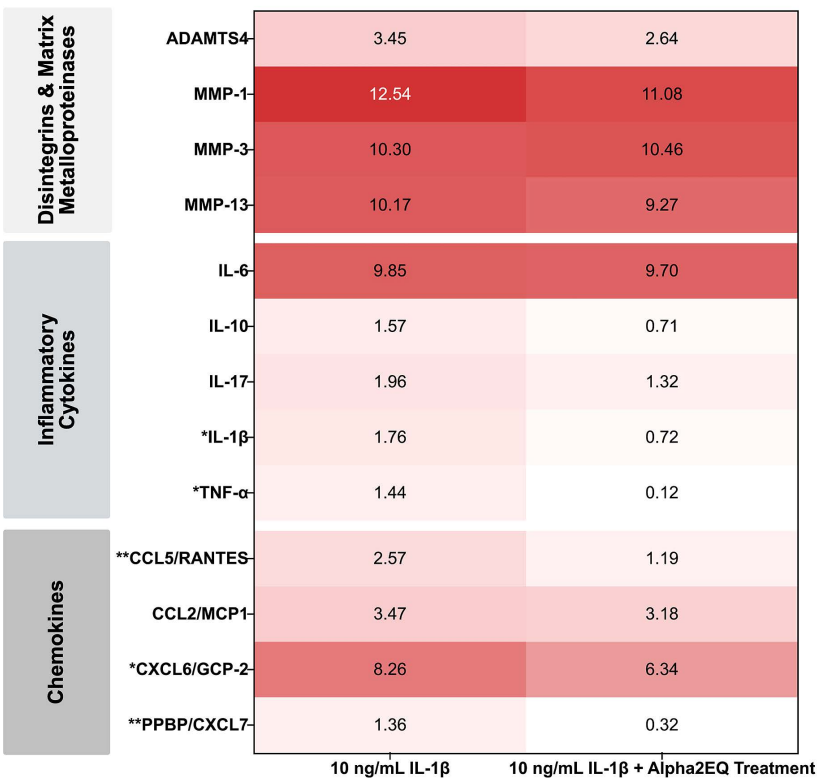

Figure 3—Alpha2EQ downregulates proinflammatory gene expression in an IL-1β model. Heat map reports mean gene expression fold change from unstimulated baseline (0 ng/mL IL-1β) for synovial fibroblasts stimulated with 10 ng/mL IL-1β and synovial fibroblasts stimulated with 10 ng/mL IL-1β with Alpha2EQ treatment (n = 4). Red indicates gene upregulation, blue indicates downregulation, and white indicates expression equivalent to unstimulated baseline cells. Mean gene expression fold change from unstimulated baseline was compared for synovial fibroblasts stimulated with IL-1β and IL-1β–stimulated cells treated with Alpha2EQ using 1-tailed t tests for each gene. *$P < .05$, **$P < .01$. Created with GraphPad Prism Version 10.1.1 and Biorender.com. PPBP/CXCL7 = Proplatelet basic protein/C-X-C motif chemokine ligand 7.

P = .0210; adjusted P = .0233), MMP-13 (−2.86 ± 1.46 vs −0.16 ± 0.40; P = .0150; adjusted P = .0172), IL-6 (−4.19 ± 1.23 vs 2.70 ± 0.57; P = .0002; adjusted P = .0003), CCL2/MCP1 (−4.21 ± 1.25 vs 1.60 ± 0.67; P = .0003; adjusted P = .0004), and CXCL6/GCP-2 (−5.38 ± 1.91 vs −0.66 ± 0.35; P = .0071; adjusted P = .0088).

IL-1β stimulation of synovial fibroblasts significantly upregulates proinflammatory genes in a concentration-dependent manner

To establish an in vitro inflammatory model and examine gene expression across a range of IL-1β concentrations, we stimulated IL-6^{BASAL} synovial fibroblasts with 1, 2.5, 5, 7.5, or 10 ng/mL IL-1β for 24 hours. Analysis of RNA counts across this range of concentrations revealed stimulation with 10 ng/mL IL-1β significantly upregulated expression of 13 proinflammatory genes from baseline (P < .05; **Table 2**). Of these 13 genes, only 12 were significantly different from baseline for 7.5 ng/mL, with IL-10 not significantly upregulated with stimulation (P > .05). For 2.5 and 5 ng/mL, only 11 genes were significantly upregulated (IL-10 and IL-17 did not reach significance; P > .05), and for 1 ng/mL, only 10 genes were significantly upregulated, as IL-1β also did not show a significant upregulation from baseline (P > .05). Given these results, 10 ng/mL IL-1β stimulation was carried forward for all in vitro testing of Alpha2EQ in this study.

Alpha2EQ significantly downregulates inflammatory cytokine and chemokine gene expression in a 10 ng/mL IL-1β model

We next examined changes in proinflammatory gene expression following 24-hour Alpha2EQ treatment of synovial fibroblasts in an IL-1β–induced inflammation model. Within the 13 genes significantly upregulated by 10 ng/mL IL-1β, the expression of 2 inflammatory cytokines and 3 chemokines was downregulated toward unstimulated (0 ng/mL

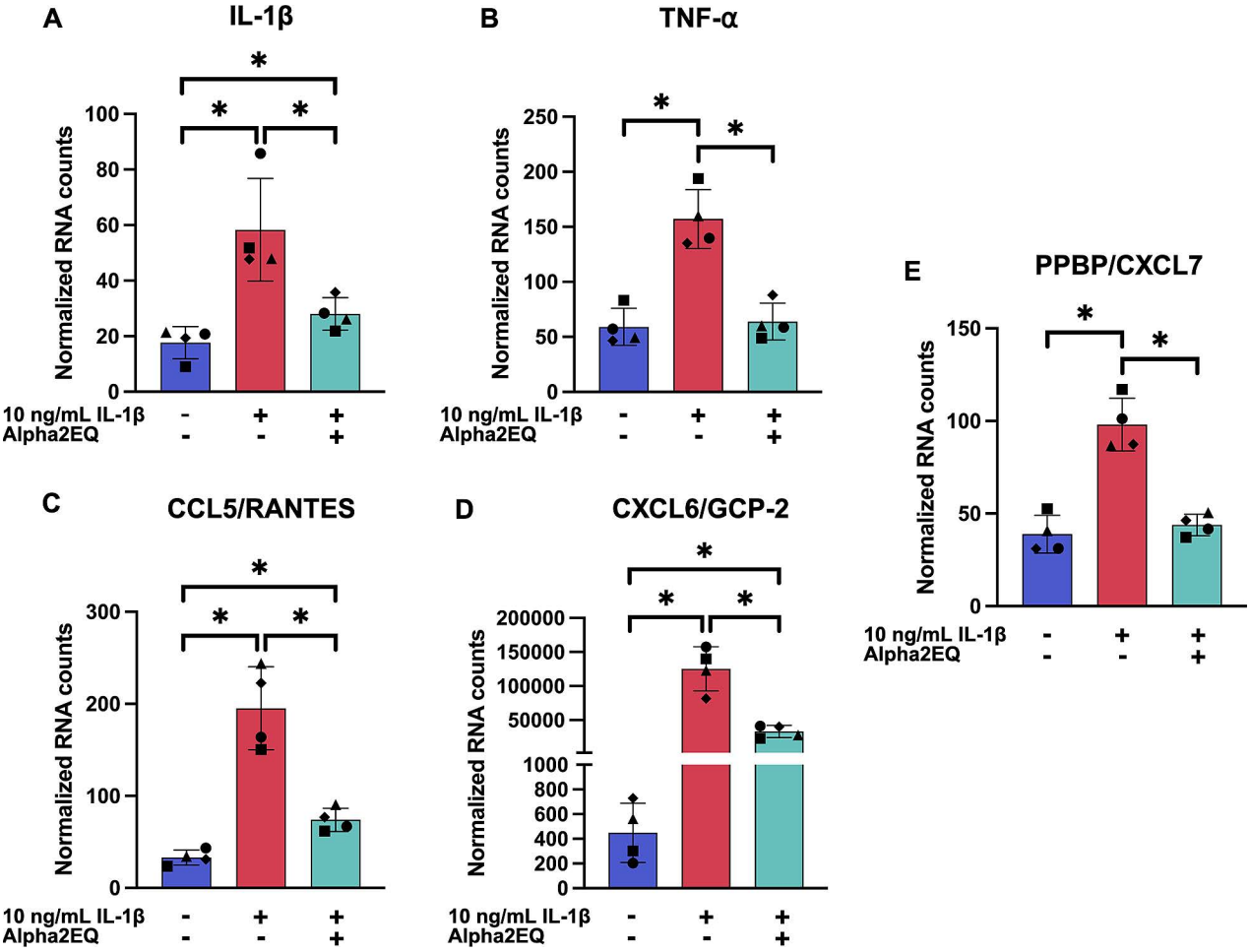

Figure 4—After stimulation with 10 ng/mL IL-1β, treatment with Alpha2EQ decreases expression of key inflammatory genes toward baseline levels. Normalized RNA counts for synovial fibroblasts without stimulation or treatment (blue), with 10 ng/mL IL-1β (red), and with 10 ng/mL IL-1β plus Alpha2EQ (green) are reported as mean ± SD for inflammatory cytokines IL-1β (A) and TNF-α (B) and chemokines CCL5/RANTES (C), CXCL6/GCP-2 (D), and PPBP/CXCL7 (E). Within each gene, parametric data were analyzed using 1-way ANOVA with Tukey test for multiple comparisons and nonparametric data with Wilcoxon signed-rank tests for pairwise comparisons among groups. *P < .05. Created with GraphPad Prism Version 10.1.1 and Biorender.com.

IL-1β) baseline levels **(Figure 3)**. Analysis of normalized RNA counts for unstimulated cells (0 ng/mL IL-1β), stimulated cells, and stimulated cells treated with Alpha2EQ revealed that 24-hour Alpha2EQ treatment of stimulated cells resulted in downregulation of TNF-α and proplatelet basic protein/C-X-C motif chemokine ligand 7 (PPBP/CXCL7) to levels that were not different from baseline ($P > .05$; **Figure 4**). Using these normalized RNA counts, gene expression fold change from stimulated cells was calculated. Compared to IL-1β–stimulated cells, stimulated cells treated with Alpha2EQ had a 2.14-fold downregulation of IL-1β ($P = .0212$; adjusted $P = .0299$) and a 2.64-fold downregulation TNF-α ($P = .0136$; adjusted $P = .0201$); CCL5/RANTES was downregulated 2.62-fold ($P = .0016$; adjusted $P = .0025$), CXCL6/GCP-2 by 4.07-fold ($P = .0245$; adjusted $P = .0330$), and PPBP/CXCL7 by 2.29-fold ($P = .0009$; adjusted $P = .0015$).

Discussion

We used a targeted transcriptomic approach to analyze gene expression changes resulting from Alpha2EQ treatment in vitro. By investigating synovial fibroblasts in 2 distinct models, our results reveal that 24-hour Alpha2EQ treatment downregulated expression of key proinflammatory genes involved in the pathogenesis of OA. Consistent with our hypothesis, Alpha2EQ treatment downregulated expression of several inflammatory cytokines and chemokines in IL-6^{HIGH} synovial fibroblasts, as well as in synovial fibroblasts stimulated with 10 ng/mL IL-1β. In contrast, none of the metalloproteinases examined in this study were significantly downregulated with Alpha2EQ treatment. Taken together, this evidence suggests Alpha2EQ reduces joint inflammation by modulating gene expression of synovial cells.

The pivotal role of IL-6 in OA pathogenesis and progression[4] has been demonstrated across species.[6,32–37] In our study, the identification of 2 populations of synovial fibroblasts with contrasting IL-6 gene expression from synovium collected exclusively from grossly normal joints suggests that IL-6 is upregulated before synovitis is macroscopically apparent. In cases of chronic OA, synovial fibroblasts have been shown to produce abundant IL-6 as part of their cytokine profile; the same has been shown in human patients with posttraumatic and postdysplastic chrondropathy.[38] Synovial fluid from human donors with symptomatic cartilage lesions was shown to have significantly more IL-6 than healthy donors, while this concentration was not significantly different from donors with OA.[39] Both studies provide evidence to suggest that IL-6 is one of the earliest genes upregulated in the pathogenesis of synovitis. Our finding that IL-6 expression was upregulated in our population before gross inflammatory changes to the joint were apparent is consistent with this prior evidence. Furthermore, IL-6^{HIGH} synovial fibroblasts demonstrated concurrent upregulation of numerous metalloproteinases, which are known to degrade the cartilage extracellular matrix in OA. Work by others[40] has shown that stimulation of chondrocytes with IL-6 induces proteoglycan loss via the dose-dependent increased expression of MMP-3, MMP-13, and ADAMTS4, all of which were upregulated in our IL-6^{HIGH} synovial fibroblast population.

In IL-6^{HIGH} synovial fibroblasts, treatment with Alpha2EQ, on average, downregulated IL-6 expression by 4.19-fold and IL-15 by 1.88-fold from baseline, which indicates that alteration of cytokine gene expression is responsible, at least in part, for Alpha2EQ's ability to modulate early inflammation. Interestingly, human patients with early knee OA were shown to have increased synovial fluid levels of IL-15 compared to end-stage patients.[41] In an in vitro explant system, exposure of articular chondrocytes to IL-15 induces the production of MMP-1 and MMP-3, suggesting a novel role for IL-15 in promoting protease release from cartilage.[42]

We found that CCL2/MCP1 was downregulated 4.21-fold from baseline in IL-6^{HIGH} synovial fibroblasts treated with Alpha2EQ. The CCL2 ligand has been shown in an experimental in vivo model to play a critical role in monocyte/macrophage recruitment and subsequent inflammation in OA. Blockade of the CCL2 pathway resulted in marked attenuation of macrophage accumulation, synovitis, and cartilage damage in this murine model.[43] In fact, work[44] by others has shown that interaction of the CCL2 ligand with its receptor on articular chondrocytes induces increased MMP-3 production, driving cartilage catabolism. In human knee OA patients, CCL2 chemokine concentration in synovial fluid has been positively associated with greater self-reported pain and disability.[45] Therefore, Alpha2EQ's ability to alter CCL2 expression may have implications both in altering the course of disease progression and in modulating pain in our equine patients.

Our investigation of the effects of IL-1β on equine synovial fibroblasts in vitro is the first, to the authors' knowledge, to report the repertoire of specific gene changes in this inflammatory model at a range of concentrations. Consistent with both gene expression changes seen in the equine in vivo metacarpophalangeal joint chip model recapitulating early posttraumatic OA and in human clinical cases,[8,31] we saw marked significant upregulation of MMP-1, MMP-13, and ADAMTS4 at all IL-1β concentrations examined. Although relatively little is known about OA-specific gene expression changes in the synovium,[31,46] emerging research in other in vitro models report substantial changes in synovial fibroblast gene expression including upregulation of MMP-1, MMP-3, MMP13, IL-1β, and IL-6.[23] Interestingly, these metalloproteinases and IL-6 were significantly upregulated compared to baseline across all concentrations of IL-1β stimulation, while IL-1β gene expression was not significantly upregulated by stimulation with 1 ng/mL in our study.

In accordance with the matrikine stimulation model of equine OA,[23] we also found CCL5/RANTES to be upregulated significantly from baseline with IL-1β stimulation at all concentrations. Alpha2EQ treatment significantly downregulated both IL-1β

and CCL5/RANTES expression following stimulation with 10 ng/mL IL-1β. In addition, CCL5/RANTES not only directly upregulates MMP-1 and MMP-13 expression in vitro but also acts as a mediator of IL-1β–induced MMP-1 and MMP-13 expression.[47] The absence of statistically significant downregulation of MMP-1, MMP-3, and MMP-13 with Alpha2EQ treatment in either model in our study was unexpected and not in agreement with our hypothesis. There was a clear trend toward downregulation of these MMPs in IL-6^{HIGH} cells (experiment 1); however, high intersubject biologic variability may have precluded detection of significant differences in this small population of horses. However, it is also important to note that we did not examine the expression of tissue inhibitors of MMPs (TIMPs), which is a limitation of our targeted transcriptomic approach. In an IL-1β–stimulated coculture of equine cartilage and synoviocytes, it was shown that synoviocyte gene expression of TIMP3 is upregulated 3-fold compared to control cultures without IL-1β.[10] The balance of inhibitors to MMPs may be of greater consequence to OA progression,[31,48] and it remains unknown if TIMP expression is possibly upregulated by Alpha2EQ.

Chemokines of the CXC family are also critical to the inflammatory response in OA, as they drive the migration of leukocytes to damaged synovium. Alpha2EQ treatment of synovial fibroblasts stimulated with 10 ng/mL IL-1β significantly downregulated PPBP/CXCL7 to a baseline level. Although less well characterized in OA, PPBP/CXCL7 expression in the synovium has been associated with early rheumatoid arthritis[49] and with bone erosion via osteoclastogenesis in this patient population.[50]

When we compared the response of IL-6^{HIGH} synovial fibroblasts to synovial fibroblasts stimulated with 10 ng/mL IL-1β, we saw that 2 genes were downregulated with Alpha2EQ treatment in both models: CXCL6/GCP-2 and TNF-α. Synovial membrane biopsies have demonstrated that CXCL6/GCP-2 is upregulated in the inflamed synovium of OA patients,[51] and due to its selective overexpression in osteoarthritic synovial fibroblasts in culture,[52,53] it has been implicated as a target for OA therapy. To the authors' knowledge, there are no reports of A2M's effects on CXCL6/GCP-2, but Alpha2EQ's ability to modulate its expression in both models in this study implies that downregulation of this gene is central to the orthobiologic's proposed mechanism of action. Similarly, TNF-α was significantly downregulated by Alpha2EQ treatment in both experiments in this study, with expression levels returning to baseline after treatment with Alpha2EQ in the IL-1β model. In vitro and in vivo, A2M has been shown to decrease TNF-α expression in both chondrocytes and in the synovium.[20,21] Across both an endogenous and stimulated inflammatory model, our results demonstrate that Alpha2EQ exerts a broad anti-inflammatory effect through downregulation of multiple pathways of cytokine and chemokine gene expression. Although our study investigated Alpha2EQ's effects on the synovial transcriptome, it has been suggested that A2M's cumulative anti-inflammatory effects are the sum of upstream regulation of gene expression and downstream binding of the translated effector proteins.[21] Exploration of Alpha2EQ's potential to modulate the function of translated proteins could further elucidate this commercial orthobiologic's mechanism of action.

The limitations of this study include the use of monolayer culture of synovial fibroblasts in isolation, rather than in coculture with chondrocytes, which would more closely recapitulate the native joint where synovial cross talk is critical both to normal joint homeostasis and to OA pathogenesis. Additionally, the study included a small number of biological replicates, with results showing high biological variability. Furthermore, it must be acknowledged that the constituents of the Alpha2EQ product were not evaluated in this study and that 2 discrete batches of Alpha2EQ were used in the endogenous IL-6^{HIGH} and IL-1β models. Importantly, at this time, due to the large and complex nature of A2M, there is no commercially available ELISA that is efficacious in quantifying this effector glycoprotein.[54,55] Moreover, Alpha2EQ in this study was collected from horses without musculoskeletal disease. In clinical practice, the orthobiologic would be used in an autologous manner and therefore be most often prepared from horses with OA, which may alter the composition of the biologic with regard to cytokine profile and subsequent IA effect. Alpha2EQ treatment was limited to 24 hours due to the feasibility of treatment beyond acute time points in cell culture; therefore, investigation of longitudinal cellular response remains unexplored. Finally, alterations in the expression of genes outside of the NanoString custom codeset for OA were not captured in this study. An unbiased approach such as RNA sequencing would be necessary to explore global changes in gene expression.

In conclusion, our findings demonstrate that Alpha2EQ treatment has potent anti-inflammatory effects on synovial fibroblast gene expression in vitro, supporting its current clinical use as equine orthobiologic treatment for OA. Downregulation of critical proinflammatory genes implicated in OA such as IL-6, CCL2/MCP1, IL-1β, CXCL6/GCP-2, and TNF-α was observed across synovial fibroblasts in an endogenous and in a stimulated inflammatory model following 24-hour Alpha2EQ treatment. Future studies will aim to further characterize the orthobiologic's composition and potential mechanisms of disease modification, as well as to elucidate Alpha2EQ's in vivo effects on osteoarthritic joints.

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Supplementary Materials

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