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Differential modulation of inflammatory cytokines by recombinant IL-10 in IL-1β and TNF-α– stimulated equine chondrocytes and synoviocytes: impact of washing and timing on cytokine responses

Hoda A. Elkhenany[1,2], Renata L. Linardi[1] and Kyla F. Ortved[1*]

Abstract

Osteoarthritis (OA) remains a challenging joint disorder necessitating effective anti-inflammatory interventions. In this study, our primary objective was to establish an in vitro protocol that replicates the clinical investigation of anti-inflammatory drugs intended for OA management. Focusing on recombinant IL-10 (r.IL-10) as a potential anti-inflammatory treatment, we designed and implemented two distinct protocols to evaluate the efficacy of r.IL-10 in modulating chondrocyte and synoviocyte inflammation.

The experimental design involved sequential stimulation with IL-1β and TNF-α for 24 h, followed by washing (model 1) or not washing (model 2) the cells before r.IL-10 treatment. Samples were collected after 6–24 h of treatment. Cellular responses were evaluated by quantifying gene expression and synthesis of key inflammatory cytokines and proteases.

The expression and synthesis of inflammatory cytokines and proteases was significantly affected by washing and treatment time. The expression of IL-1β, TNF-α, IL-8, MMP-13, and ADAMTS5 were effectively reduced in r.IL-10-treated chondrocytes and synoviocytes in model 2 after 24 h, particularly at concentrations of 10 and 20 ng/mL. r.IL-10 treatment significantly increased IL-6 gene expression in chondrocytes at all time points. However, in synoviocytes, IL-6 expression was significantly lower in model 2 after 24 h of r.IL-10 treatment. r.IL-10 treatment significantly decreased IL-1β and TNF-α content in synoviocyte supernatants, particularly in model 2 at concentrations of 10 and 20 ng/mL after 6 and 24 h. r.IL-10 treatment in chondrocytes led to a significant decrease in IL-1β supernatant concentrations in model 2 after 24 h only.

This study demonstrated that r.IL-10 treatment effectively reduces key inflammatory markers and matrix metalloproteinase activity in both chondrocytes and synoviocytes, particularly in model 2 where cells were not washed prior to treatment. These findings highlight r.IL-10's potential as a robust anti-inflammatory agent for OA management and suggest its critical role in developing effective therapeutic strategies for OA.

*Correspondence:
Kyla F. Ortved
kortved@vet.upenn.edu

Full list of author information is available at the end of the article

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Introduction

Osteoarthritis (OA) is a prevalent degenerative joint disease characterized by the progressive breakdown of cartilage, synovial inflammation, and joint pain [1]. Despite its widespread impact, current treatment options primarily focus on symptom management rather than addressing the underlying inflammatory processes. Consequently, there is an urgent need for effective anti-inflammatory therapies that can mitigate the progression of OA.

Interleukin (IL)-10 is a potent anti-inflammatory cytokine known for its ability to inhibit the synthesis of pro-inflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α, which play crucial roles in the pathophysiology of OA [2]. Because of this, IL-10 has been investigated as a promising candidate for OA therapy due to its potential to modulate inflammatory responses and reduce cartilage degradation [3].

In vitro models are essential tools for preclinical drug evaluation, allowing for the controlled study of cellular responses to therapeutic agents [4]. However, the design of these models must accurately mimic the clinical environment to ensure translational relevance. There are several in vitro models used to study OA, including monolayer cultures of chondrocytes and synoviocytes [5, 6], co-culture systems [7], and three-dimensional tissue-engineered constructs [8]. Each model has its advantages and limitations. Monolayer cultures allow for easy manipulation and analysis of specific cellular responses but may not fully replicate the tissue architecture and cell-matrix interactions found in vivo. Co-culture systems, which involve multiple cell types such as chondrocytes and synoviocytes, provide a more physiologically relevant environment by mimicking cell-cell interactions [9]. Three-dimensional constructs offer even greater complexity, better simulating the extracellular matrix and mechanical properties of cartilage tissue [10].

One critical aspect of these in vitro models is the method of inducing inflammation to study the efficacy of anti-inflammatory treatments. Common approaches involve stimulating cells with pro-inflammatory cytokines such as IL-1β, TNF-α, or a combination of both. IL-1β is known to induce cartilage degradation and inflammation by promoting the expression of matrix metalloproteinases (MMPs) and other catabolic factors [11]. TNF-α also plays a significant role in OA pathogenesis by enhancing the production of inflammatory cytokines and MMPs [12]. Using these cytokines individually or in combination can provide insights into their specific and synergistic effects on cellular behavior and inflammation.

In this study, we aim to establish an optimized in vitro protocol for evaluating the anti-inflammatory effects of recombinant IL-10 (r.IL-10), synthetic version of the naturally occurring IL-10, in chondrocytes and synoviocytes. We implemented two distinct experimental protocols to evaluate the impact of washing and time on cytokine responses. Specifically, we investigated the effects of stimulation with IL-1β and TNF-α, followed by r.IL-10 treatment, on the expression of key inflammatory markers and MMPs.

Traditionally, in vitro experiments have included washing between two different treatments; however, we observed that the washing process itself could reduce pro-inflammatory markers, complicating the detection of the treatment effects under investigation. This finding highlights a gap in the literature regarding the impact of washing protocols on cytokine responses. We believe it is essential to address this aspect, as it can provide valuable insights for researchers designing similar experiments. While this may seem like a simple procedural step, it can significantly influence the outcomes of anti-inflammatory studies.

We hypothesized that r.IL-10 treatment of inflamed chondrocytes and synoviocytes would decrease expression and synthesis of key pro-inflammatory and degradative proteins and that there would be a temporal effect on these responses. This work, therefore, represents a significant step toward establishing a more predictive and reliable in vitro screening tool for anti-inflammatory drug development.

Materials and methods

Cell culture and stimulation protocol

Cartilage and synovium were aseptically collected from the femoropatellar and femorotibial joints of 4 young horses (2–8 years) that were euthanized for reasons unrelated to musculoskeletal disease following approval from the Institutional Animal Care and Use Committee (IACUC #806625). Horses were euthanized using an overdose of intravenous pentobarbital (75 mg/kg). Cartilage was digested overnight in 0.075% collagenase (Worthington Biochemical, Lakewood, NJ) as previously described [13]. Synovium was digested in 0.15% collagenase and 0.015% DNAse for 2 h at 37 °C as previously described [14]. Cells were stored in liquid nitrogen until future use.

Equine chondrocytes were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12, ThermoFisher Scientific), supplemented with 10% fetal bovine serum (FBS), HEPES (25mM, ThermoFisher Scientific), penicillin-streptomycin (100 µg/mL, Invitrogen),

L-ascorbic acid (50 mg/mL, A4544, Sigma-Aldrich), and alpha-ketoglutarate (30 mg/mL, Sigma-Aldrich) [15]. Synoviocytes were cultured in high-glucose DMEM, supplemented with 10% FBS, HEPES (25mM) and penicillin-streptomycin (100 µg/mL), following Nixon et al. with slight modifications [16].

Chondrocytes were pooled from 4 horses and synoviocytes from 3 horses. The cells, at passage 0, were retrieved from liquid nitrogen, and an equal number of cells were seeded together until confluence. Passage 1 cells were used for all experiments. The cells were seeded at a density of 5000 cells/cm² in 24-well plates. Prior to treatment, cells were cultured for 24 h or until they reached 80% confluence. Chondrocytes and synoviocytes were cultured separately throughout the experimental protocols.

To induce a pro-inflammatory state, cells were stimulated with IL-1β (10 ng/mL) and TNF-α (1 ng/mL), as described in our previous manuscript for effective inflammation induction in similar cell models [15]. Cells were then treated with recombinant equine IL-10 (r.IL-10, catalog number 1605-IL, R&D Systems, Minneapolis, MN, USA) at three concentrations: 10 ng/mL, 20 ng/mL, and 50 ng/mL.

Experimental groups

Experimental groups were established for analysis of the effects of r.IL-10 on equine chondrocytes and synoviocytes. The control groups consisted of unstimulated cells and stimulated cells without r.IL-10 treatment. The treatment groups consisted of stimulated cells treated with varying concentrations of r.IL-10. Following the defined incubation period, both cells and supernatant were harvested for subsequent analyses. All experiments were performed in triplicate.

The following culture models, along with the timelines for each treatment and stimulation, are shown in Fig. 1:

Model 1 Stimulation with IL-1β and TNF-α for 24 h, followed by a media change **with** cell washing using 1 mL of phosphate buffered saline (PBS) and treatment with r.IL-10 (10ng/mL, 20ng/mL, or 50ng/mL) for an additional 6–24 h.

Model 2 Stimulation with IL-1β and TNF-α for 24 h, followed by a media change **without** cell washing and treatment with r.IL-10 (10ng/mL, 20ng/mL, or 50ng/mL) for an additional 6–24 h.

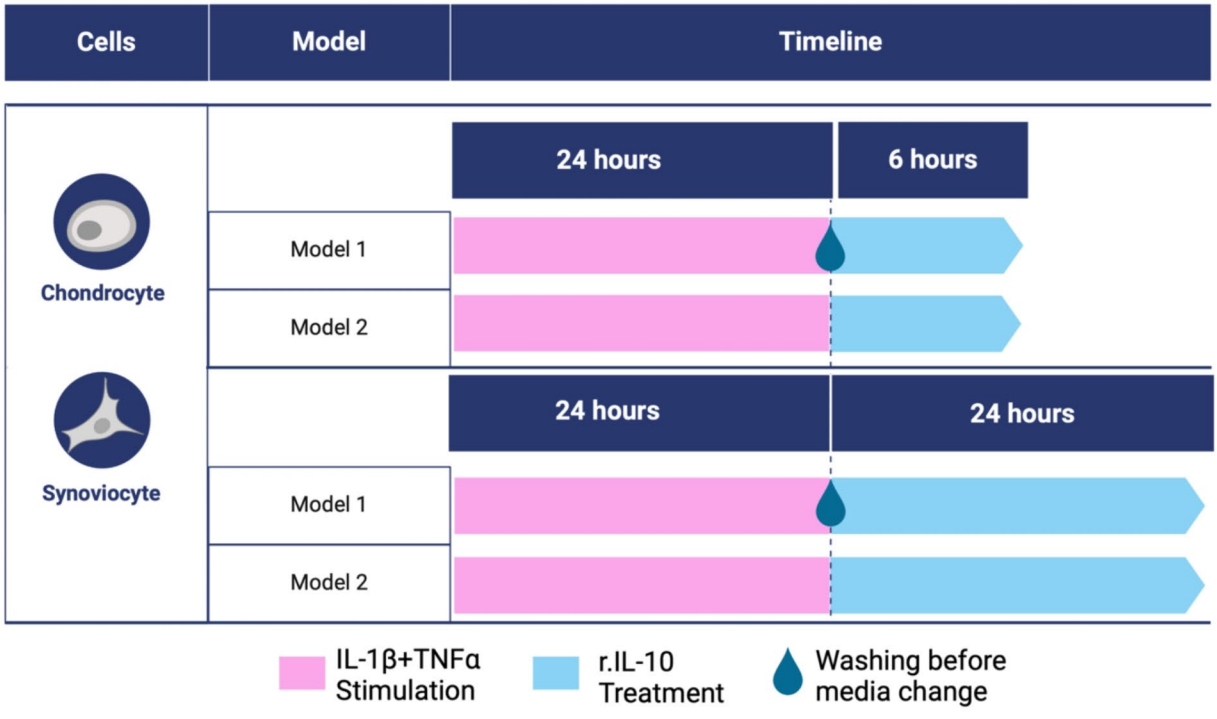

Fig. 1 Experimental design for evaluating IL-10 treatment in equine chondrocytes and synoviocytes. Two experimental models were used to examine the anti-inflammatory effects of equine recombinant IL-10 (r.IL-10) on chondrocytes or synoviocytes. Controls included unstimulated cells and stimulated cells without treatment. Supernatants were collected at the end of the culture period for quantification of cytokines with immunoassays. Cells were collected for gene expression analysis with qRT-PCR

Gene expression

Triplicate cell samples were pooled prior to RNA extraction to ensure a representative analysis of each experimental condition. RNA was extracted from chondrocytes and synoviocytes using RNeasy Mini kit (Qiagen, Germantown, MD) and reverse transcribed into cDNA with a High Capacity cDNA Reverse Transcription Kit (Appliedbiosystems, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to quantify expression of genes using TaqMan™ Universal Master Mix II (Applied Biosystems / ThermoFisher Scientific, Waltham, MA) and the QuantStudio 6 Flex Real-Time PCR Instrument (ThermoFisher Scientific, Waltham, MA). Each PCR reaction was run in triplicate. Genes of interest included immunomodulatory cytokines (*IL-1β*, *TNF-α*, *IL-6*, *IL-8*), degradative enzymes (*MMP-13* and *ADAMTS5*), and p16 as a marker of apoptosis. *18 S* served as the reference gene. The primer and probe sequence used for 18 S and p16 are detailed in Table 1. The primer and probe sequences for the remaining genes were obtained from ThermoFisher Scientific's proprietary equine-specific gene expression assay database. Relative mRNA expression was calculated by ΔΔCt method.

Supernatant analysis

Supernatant was collected at the end of the culture period, pooled from triplicate samples, and analyzed in duplicate. After collection, samples were centrifuged at 3,000 x g for 10 min and stored at -20 °C until further analysis. The concentrations of IL-1β, TNF-α, IL-6, and IL-8 were measured using a fluorescent bead-based multiplex immunoassay (Milliplex® Equine Cytokine/Chemokine Magnetic Bead Panel, catalog no. EQCYTMAG-93 K, Millipore, Burlington, MA) on the Luminex®200 instrument (Luminex, Austin, TX) following the manufacturer's instructions and as previously described [17]. Briefly, 25 µL of standard, control, or sample and 25 µL of antibody-immobilized beads were added to individual wells on a 96-well plate. Plates were incubated at 4°C overnight and then washed 3 times before adding 25 µL of detection antibody to each well. Plates were then incubated on a shaker at room temperature for 1 h followed by addition of 25 µL of streptavidin-phycoerythrin to each well. Plates were incubated for an additional 30 min on a shaker at room temperature before washing and addition of 150 µL of drive fluid. All plates were analyzed on the Luminex®200 instrument with xPONENT® software with 50 events per bead and a sample size of 100 µL.

Prostaglandin E2 (PGE2) concentration in supernatants was quantified using a competitive enzyme-linked immunosorbent assay (ELISA) (SKGE004B, R&D Systems, MN, USA) according to the protocol provided by the manufacturer. Briefly, 150 µL of calibrator diluent was added to wells followed by 150 µL of standard, control, or sample. Next, 50 µL of primary antibody solution was added to each well and plates were incubated on a shaker for 1 h at room temperature. Following incubation, 50 µL of PGE2 conjugate was added to each well and plates were incubated for 2 h at room temperature on a shaker. Plates were then washed 4 times and 200 µL of substrate solution was added to each well. Plates were incubated for 30 min at room temperature, 100 µL of stop solution was added to each well, and the optical density was determined using a microplate reader at 450 nm.

Statistical analysis

Data was tested for normal distribution using the Shapiro-Wilk test accompanied by Q-Q plots for further validation of normality. Statistical comparisons were executed through two-way Analysis of Variance (ANOVA), which allowed for the evaluation of both individual effects of the treatment conditions (e.g., presence or absence of r.IL-10) and the experimental models (washed vs. unwashed), as well as any interaction effects between them on inflammatory marker expression.

Table 1 Equine primer sequences/id used to analyze gene expression

Gene	Role		Sequence/Assay ID
18 S ribosomal	Reference gene	Forward	GCCGCTAGAGGTGAAATTCT
		Reverse	TCGGAACTACGACGGTATCT
		Probe	AAGACGGACCAGAGCGAAAGCAT
p16 (CDKN2A)	Cell-cycle inhibitor	Forward	CTCTTGGCCGACATGCT
		Reverse	CCCATCATCACGACCTGAAT
		Probe	ACCTCACCCAACGCGCTGAA
IL-1β	Pro-inflammatory cytokine	Assay ID	Ec04260298_s1
TNF-α	Inflammatory cytokine	Assay ID	Ec03467871_m1
IL-6	Pro-inflammatory cytokine and Anti-inflammatory myokine	Assay ID	Ec03468678_m1
IL-8	Chemoattractant cytokine	Assay ID	Ec03468860_m1
MMP-13	Degradation of extracellular matrix	Assay ID	Ec03467796_m1
ADAMTS-5	degrading articular cartilage matrix	Assay ID	Ec03470669_m1

Dunnett's multiple comparisons test was used as a post-hoc analysis to compare each experimental group to the stimulated non-treated control group. The significance threshold was set at $p<0.05$. All statistical analyses were performed using GraphPad Prism version 10.3.1. Results are presented as mean ± standard deviation (SD), except for supernatant cytokine concentrations, which are presented as mean ± coefficient of variation (CV%). Statistical significance denoted by asterisks (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Results

Gene expression in chondrocytes in response to stimulation and r.IL-10 treatment

The effect of culture model and r.IL-10 treatment on gene expression in chondrocytes is shown in Fig. 2.

Stimulation led to a significant increase in *IL-1β* expression at 6 h in model 1 and model 2. However, at 24 h, only cells that were not washed continued to show upregulation of *IL-1β*.

IL-1β gene expression was significantly decreased in chondrocytes treated with r.IL-10 in model 2, where cells were not washed. Specifically, chondrocytes treated with 20 ng/mL and 50ng/mL of r.IL-10 had significantly decreased expression of *IL-1β* at 6 h ($p<0.0001$), while only chondrocytes treated with 20ng/mL had significantly decreased expression of *IL-1β* at 24 h ($p<0.001$, Fig. 2A).

Interestingly, stimulation of chondrocytes did not elicit a significant increase in *TNF-α* gene expression. Expression was only detectable in stimulated, untreated chondrocytes in both models after 24 h but not at 6 h. *TNF-α* expression was significantly decreased in both model 1 and model 2 at 24 h when treated with r.IL-10 at a concentration of 20 ng/mL ($p<0.001$ and $p<0.05$, respectively). However, *TNF-α* expression was significantly higher at 6 h in chondrocytes treated with r.IL-10 at a

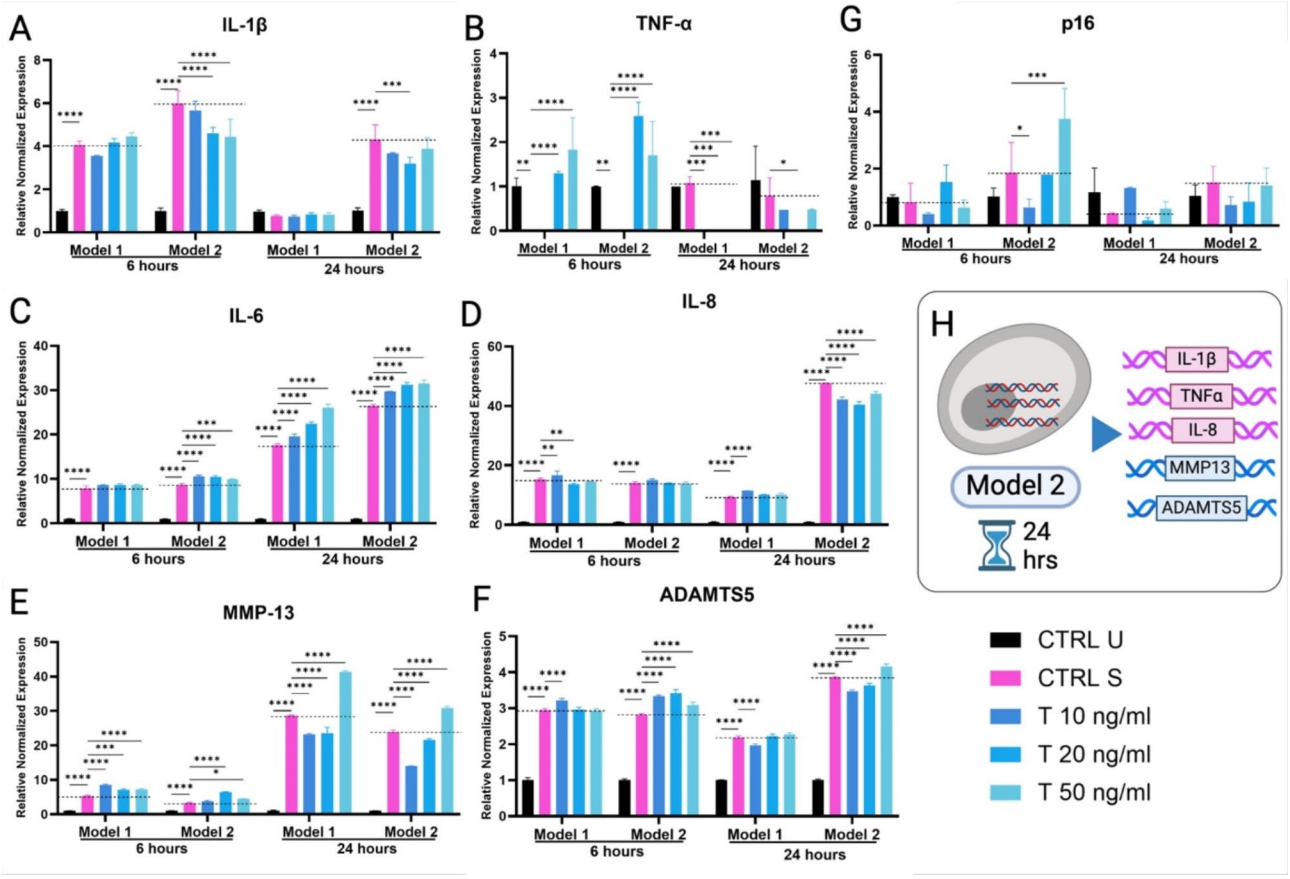

Fig. 2 Relative mRNA expression of (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6, (**D**) IL-8, (**E**) MMP-13, (**F**) ADAMTS5 and (**G**) p16 in chondrocytes. PCR runs were performed in triplicate for each treatment group. Treatment groups included unstimulated control (CTRL U), stimulated control (CTRL S), and stimulated followed by IL-10 treatment (T) at three different concentrations (10, 20, and 50 ng/mL). Cells were collected 6–24 h after initiation of r.IL-10 treatment. The data are expressed as mean ± SD. Significant differences between CTRL S and other treatment groups are denoted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. The dotted line demarcates the level of the stimulated group without IL-10 treatment. (H) This schematic diagram illustrates the inflammatory genes that were suppressed as a result of specific treatment protocols at defined time point, highlighting the optimal model for downregulating the majority of these inflammatory genes

concentration of 20 ng/mL and 50 ng/mL ($p<0.0001$, Fig. 2B).

IL-6 gene expression was increased in both models at all time points following stimulation with expression increasing between 6 and 24 h. r.IL-10 treatment at all concentrations led to a further increase in *IL-6* gene expression compared to stimulated, untreated chondrocytes cultured using model 1 at 24 h and using model 2 at both 6 and 24 h ($p<0.0001$, Fig. 2C).

The expression of *IL-8* was also increased in both models at all time points following stimulation. There was a temporal effect noted in chondrocytes cultured in model 2 with expression increasing from 6 to 24 h. The expression of *IL-8* was significantly decreased with r.IL-10 treatment at all concentrations in chondrocytes cultured in model 2 at 24 h ($p<0.0001$, Fig. 2D).

The expression of *MMP-13* increased significantly with stimulation over time with small increases noted at 6 h in both models and larger increases noted at 24 h. At 24 h, chondrocytes cultured in both models and treated with 10 ng/mL or 20 ng/mL r.IL-10 had significantly decreased expression of *MMP-13* compared to stimulated controls ($p<0.0001$, Fig. 2E). Interestingly, treatment with 50 ng/mL r.IL-10 led to a significant increase in *MMP-13* expression compared to stimulated controls.

ADAMTS5 expression was significantly increased following stimulation at 6 and 24 h ($p<0.0001$). Treatment with r.IL-10 at 10 ng/mL in model 1 at 24 h led to significant decreases in *ADAMTS5*, while treatment with r.IL-10 at both 10 ng/mL and 20 ng/mL in model 2 at 24 h led to significant decreases in *ADAMTS5* gene expression ($p<0.0001$, Fig. 2F).

The expression of *p16* did not vary significantly between different groups, except for significantly decreased expression at 6 h in chondrocytes in model 2 treated with r.IL-10 at a concentration of 20 ng/mL ($p<0.05$) and significantly increased expression at 6 h in chondrocytes in model 2 treated with r.IL-10 at a concentration of 50 ng/mL ($p<0.001$, Fig. 2G).

Gene expression in Synoviocytes in response to stimulation and r.IL-10 treatment

The effect of culture model and r.IL-10 treatment on gene expression in synoviocytes is shown in Fig. 3.

Stimulation led to a significant increase in *IL-1β* only at 24 h in model 2, where the cells were not washed. Treatment with r.IL-10 at the three tested concentrations significantly reduced *IL-1β* expression in synoviocytes, observed exclusively in model 2 after 24 h ($p<0.0001$, Fig. 3A).

Similar to chondrocytes, *TNF-α* gene expression in synoviocytes appeared to be minimally affected by culture model or stimulation. Interestingly, at 24 h, expression of *TNF-α* was significantly decreased in synoviocytes treated with all concentrations of r.IL-10 at 24 h in model 2 compared to both stimulated and unstimulated controls (Fig. 3B).

Unlike chondrocytes, expression of *IL-6* was not affected by stimulation at 6 h; however, *IL-6* was significantly upregulated by stimulation at 24 h in both models. Treatment with r.IL-10 at all concentrations significantly decreased expression of IL-6 in model 2 ($p<0.0001$), while in model 1, *IL-6* expression was significantly increased with r.IL-10 treatment compared to stimulated controls ($p<0.0001$, Fig. 3C).

Stimulation led to significant increases in *IL-8* expression in both models with expression increasing over time. Treatment with r.IL-10 led to downregulation of *IL-8* in model 1 at 6 h at a concentration of 10 ng/mL only, while *IL-8* was significantly downregulated by r.IL-10 at all concentrations in model 2 at 6 h ($p<0.001$). At 24 h, *IL-8* expression was significantly increased in model 1 cultures treated with r.IL-10 at 10 and 20 ng/mL. In model 2, *IL-8* continued to be significantly downregulated at 24 h at all concentrations of r.IL-10 ($p<0.0001$, Fig. 3D).

A similar effect was observed in *MMP-13* gene expression. Expression of *MMP-13* was upregulated with stimulation and this effect increased over time. *MMP-13* expression was decreased in model 1 at 6 h treated with r.IL-10 at a concentration of 10 ng/mL but was increased in model 1 at 6 h treated with r.IL-10 at a concentration of 50 ng/mL ($p<0.0001$). No downregulation in *MMP-13* was noted at 24 h following r.IL-10 treatment in model 1. In model 2, *MMP-13* was significantly decreased at 6 and 24 h at all concentrations of r.IL-10 ($p<0.0001$, Fig. 3E).

Expression of *ADAMTS5* was also upregulated with stimulation but only cells cultured in model 2 showed an increase in expression over time ($p<0.0001$). In model 1, treatment with r.IL-10 at a concentration of 10 ng/mL led to significant downregulation of *ADAMTS5* at 6 h, while treatment with higher concentrations of r.IL-10 led to increased expression of *ADAMTS5*. At 24 h, *ADAMTS5* expression was decreased in cells treated with 10 and 50 ng/mL of r.IL-10. In model 2, expression of *ADAMTS5* was significantly downregulated at 24 h at all concentrations of r.IL-10 ($p<0.0001$, Fig. 3F).

p16 gene expression was only slightly affected by model, stimulation, and treatment. Interestingly, treatment with r.IL-10 at 20 and 50 ng/mL led to significantly decreased p16 expression in model 2 at 24 h compared to both stimulated and unstimulated controls ($p<0.01$, $p<0.001$ respectively, Fig. 3G).

Cytokine quantification in chondrocyte cultures in response to stimulation and r.IL-10 treatment

The concentration of the immunomodulatory cytokines IL-1β, TNF-α, IL-6, and IL-8 in chondrocyte culture supernatants is shown in Fig. 4.

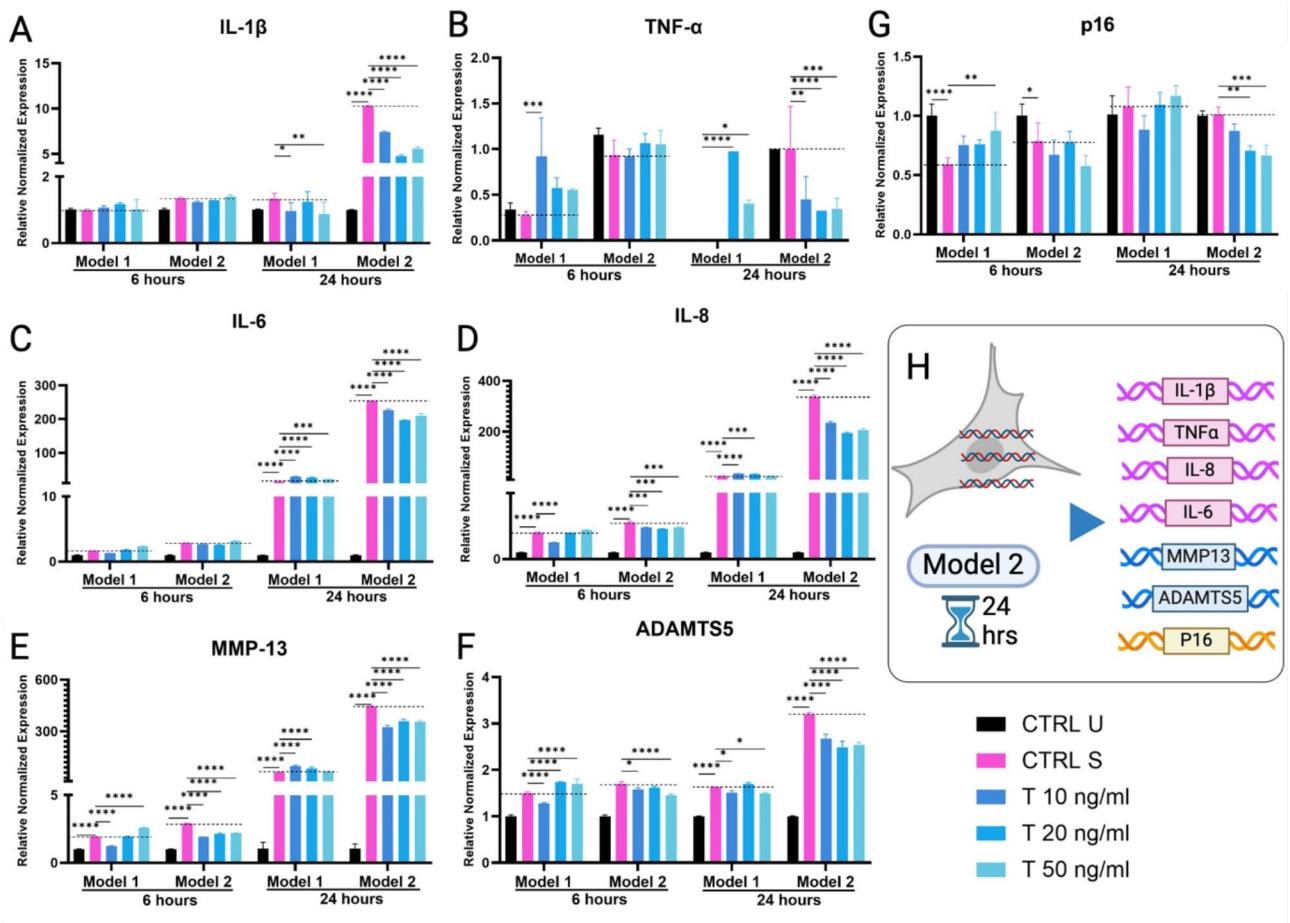

Fig. 3 Relative mRNA expression of (**A**) IL-1β, (**B**) TNF-α, ((**C**) IL-6, (**D**) IL-8, (**E**) MMP-13, (**F**) ADAMTS5 and (**G**) p16 in synoviocytes. PCR runs were performed in triplicate for each treatment group. Treatment groups included unstimulated control (CTRL U), stimulated control (CTRL S), and stimulated followed by IL-10 treatment (T) at three different concentrations (10, 20, and 50 ng/mL). Cells were collected 6–24 h after initiation of r.IL-10 treatment. The data are expressed as mean ± SD. Significant differences between CTRL S and other treatment groups are denoted as $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$. The dotted line demarcates the level of the stimulated group without IL-10 treatment. (H) This schematic diagram illustrates the inflammatory genes that were suppressed as a result of specific treatment protocols at defined time points, highlighting the optimal model for downregulating the majority of these inflammatory genes

IL-1β concentration was only significantly increased by stimulation in chondrocytes cultured using model 2 at 6 and 24 h. No effect of r.IL-10 treatment was noted at 6 h, however, at 24 h, r.IL-10 treatment at 10, 20, and 50 ng/mL ($p<0.0001$, 0.001, 0.01, Fig. 4A) led to significant decreases in IL-1β concentration compared to stimulated controls.

TNF-α concentration was also minimally increased by stimulation in chondrocytes cultured using model 2. Treatment with r.IL-10 did not lead to decreased TNF-α supernatant concentrations. In fact, r.IL-10 treatment at 10 and 50ng/mL was associated with significantly higher concentrations of TNF-α at both 6 and 24 h (Fig. 4B).

IL-6 concentration was significantly increased by stimulation in both models at 6 and 24 h. This was especially notable in model 2 at 24 h. Treatment with r.IL-10 was associated with further increased IL-6 concentration in model 2 at all concentrations at 6 h and at 20 and 50ng/mL at 24 h (Fig. 4C).

Finally, IL-8 concentration was significantly increased by stimulation in both models and at both time points. Treatment with r.IL-10 was not associated with decreased IL-8 concentration in either model or at any time point (Fig. 4D).

Cytokine quantification in synoviocyte cultures in response to stimulation and r.IL-10 treatment

The concentration of the immunomodulatory cytokines IL-1β, TNF-α, IL-6, and IL-8 in synoviocyte culture supernatants is shown in Fig. 5.

IL-1β concentrations were significantly higher in both models at 6 h and further increased at 24 h following stimulation with higher concentrations of IL-1β noted in model 2. Treatment with r.IL-10 at concentrations of 10 and 20 ng/mL showed a significant reduction of IL-1β

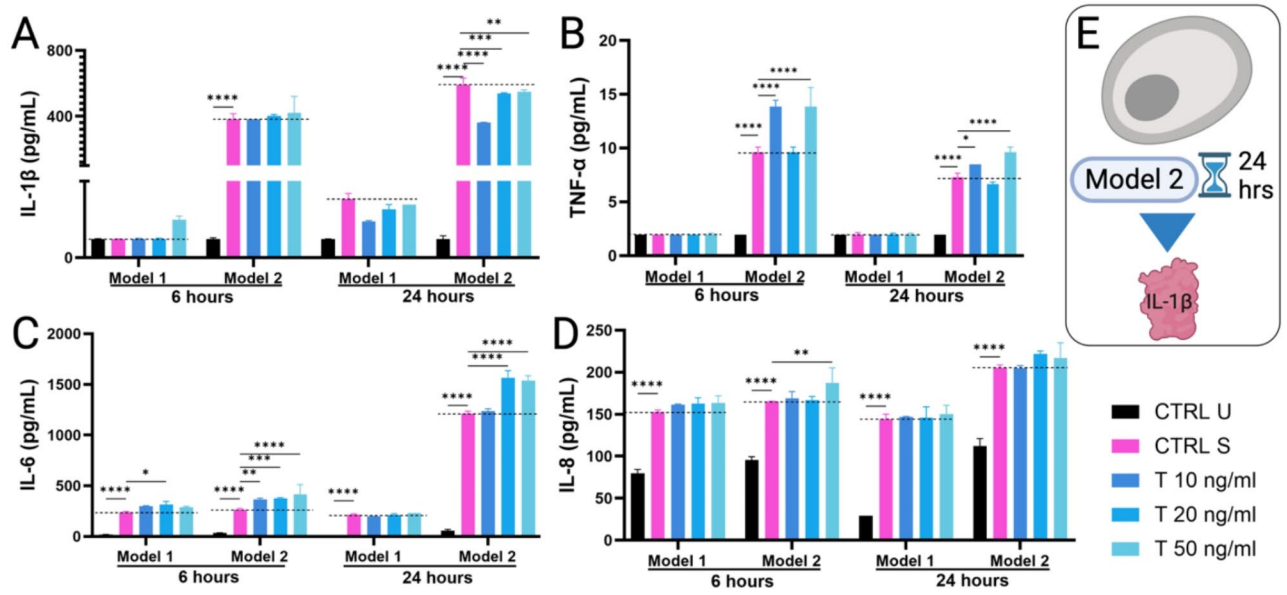

Fig. 4 Concentration of immunomodulatory cytokines (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6, and (**D**) IL-8 in the chondrocyte culture supernatants from different treatment groups: unstimulated (CTRL U), stimulated (CTRL S), and stimulated followed by IL-10 treatment (T) at three different concentrations (10, 20, and 50 ng/mL). Supernatants were collected 6–24 h after initiation of r.IL-10 treatment. Experimental runs were performed in duplicate for each treatment group. The data are expressed as mean ± coefficient of variation (CV). Significant differences between CTRL S and other treatment groups are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. The dotted line demarcates the level of the stimulated group without IL-10 treatment. (H) This schematic diagram illustrates the inflammatory cytokines that were suppressed as a result of specific treatment protocols at defined time points, highlighting the optimal model for downregulating the majority of inflammatory cytokines

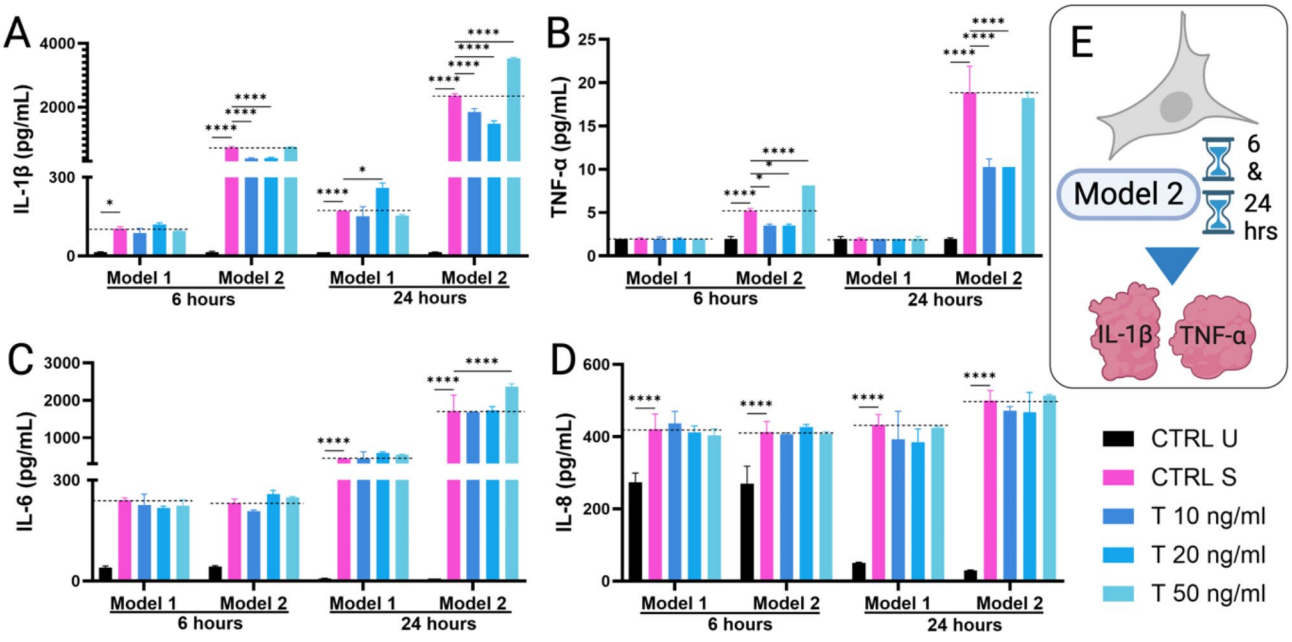

Fig. 5 Concentration of immunomodulatory cytokines (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6, and (**D**) IL-8 in synoviocyte culture supernatants from different treatment groups: unstimulated (CTRL U), stimulated (CTRL S), and stimulated followed by IL-10 treatment (T) at three different concentrations (10, 20, and 50 ng/mL). Supernatants were collected 6–24 h after initiation of r.IL-10 treatment. Experimental runs were performed in duplicate for each treatment group. The data are expressed as mean ± coefficient of variation (CV). Significant differences between CTRL S and other groups are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. The dotted line demarcates the level of the stimulated group without IL-10 treatment. (H) This schematic diagram illustrates the inflammatory cytokines that were suppressed as a result of specific treatment protocols at defined time points, highlighting the optimal model for downregulating the majority of inflammatory cytokines

in model 2 at both time points (6 and 24 h) ($p<0.0001$, Fig. 5A).

Similar to chondrocyte cultures, TNF-α concentrations were minimally increased with stimulation in model 2. Treatment with r.IL-10 at 10 and 20 ng/mL led to significantly decreased TNF-α concentrations at 6 and 24 h (Fig. 5B).

IL-6 concentration was significantly increased by stimulation in both models at 24 h but not at 6 h. There was no notable difference between stimulation and r.IL-10 treatment in model 1 at both 6 and 24 h. Similarly, in model 2, IL-6 levels were not significantly different at 6 h, but r.IL-10 at a concentration of 50 ng/mL exhibited significantly higher IL-6 levels after 24 h (Fig. 5C).

Finally, IL-8 concentration was significantly increased by stimulation in both models and at both time points. Treatment with r.IL-10 was not associated with significant decrease in IL-8 concentration in either model or at any time point (Fig. 5D).

PGE2 concentration in chondrocyte and synoviocyte cultures in response to stimulation and r.IL-10 treatment

The concentration of PGE2 in chondrocyte and synoviocyte culture supernatants is shown in Fig. 6. Stimulation led to significant increases in PGE2 concentrations in chondrocyte cultures in both models and both time points. r.IL-10 treatment at 50 ng/mL led to a significant reduction in PGE2 concentration in chondrocyte supernatants compared to the stimulated controls, but only in model 1 after 6 h ($p<0.001$). Notably, PGE2 levels were significantly higher after r.IL-10 treatment at all concentrations in model 2 after both 6 and 24 h (Fig. 6A).

In synoviocyte cultures, stimulation also led to significant increases in PGE2 concentrations from both models and both time points. PGE2 levels were further increased above stimulated controls in synoviocytes treated at all concentrations of r.IL-10 in model 1 at 24 h ($p<0.0001$, Fig. 6B).

Discussion

Our study aimed to elucidate the differential modulation of inflammation by r.IL-10 in IL-1β and TNF-α-stimulated chondrocytes and synoviocytes, with a focus on the impact of cell washing and time on cytokine responses. The findings demonstrated that r.IL-10 effectively reduces several key inflammatory markers and degradative enzymes, particularly in model 2 where cells were not washed prior to treatment. This response was also more profound after 24 h of r.IL-10 treatment versus 6 h of treatment.

In our study, the effect of washing the cells prior to treatment significantly influenced the outcomes observed. Washing the cells likely removed residual pro-inflammatory cytokines and other mediators from the culture medium, which may have contributed to the observed differences in cytokine expression. In model 2, where cells were not washed before treatment, there appeared to be a prolonged inflammatory response in the cells. This in turn provided a more suitable environment to examine the immunomodulatory effects of r.IL-10 treatment with reduction of several key inflammatory markers and degradative enzymes noted. This finding aligns with previous studies indicating that washing, such as joint lavage, can influence inflammatory responses. For instance, histological evaluations in rabbit models have shown that joint lavage reduced the breakdown of articular cartilage and inflammation of the synovium, with a significant decrease in *IL-1β* and *TNF-α* levels in synovial fluid compared to control [18]. In our study, the stimulation groups that underwent washing may have lost these critical factors more rapidly, which would explain why cells in model 1 had diminished gene expression and protein synthesis, and a less robust response to r.IL-10 treatment when compared to the unwashed model 2 cultures.

In contrast, while Wu et al. [19] demonstrated significant downregulation of pro-inflammatory markers, including *IL-1β*, *TNF-α*, *MMP-3*, *MMP-9*, and

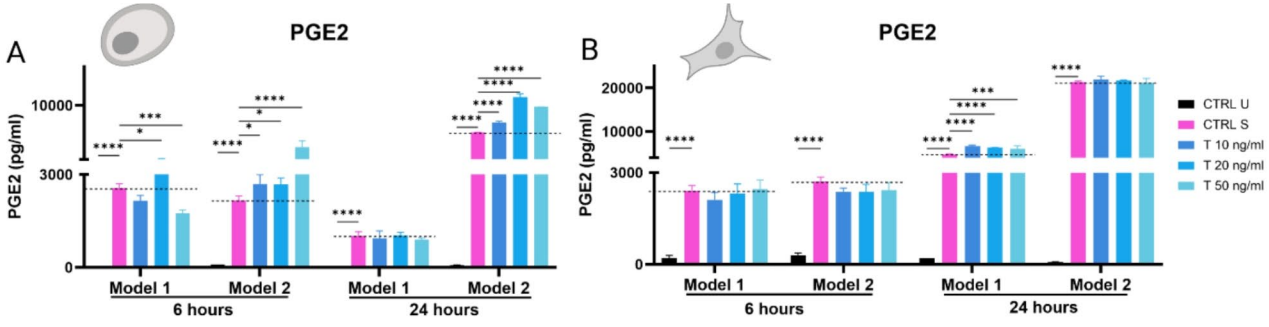

Fig. 6 PGE2 concentration in supernatants from (**A**) chondrocyte and (**B**) synoviocyte cultures. Treatment groups included: unstimulated (CTRL U), stimulated (CTRL S), and stimulated followed by IL-10 treatment (T) at three different concentrations (10, 20, and 50 ng/mL) 6 and 24 h after initiation of r.IL-10 treatment. Experimental runs were performed in duplicate for each treatment group. The data are expressed as mean ± SD. Significant differences between CTRL S and other groups are denoted as *$p<0.05$, ***$p<0.001$, and ****$p<0.0001$. The dotted line demarcates the level of the stimulated group without IL-10 treatment

Ki-67 following baicalein treatment, they specifically noted washing of the treated cells between the stimulation and treatment phases. However, they did not detail whether the stimulated non-treated cells were washed in a similar manner. This raises questions about the potential impact of residual cytokines in their non-treated controls, as the absence of this detail leaves the washing protocol unclear. The differences in methodology highlight the complexities involved in designing in vitro studies for evaluating anti-inflammatory treatments. While our no-wash approach aimed to reflect in vivo conditions, the lack of washing in Wu et al.'s study for the non-treated cells may have influenced their results, emphasizing the need for clarity in experimental protocols to better understand the effects of therapeutic agents.

A clear temporal response in cytokine expression and synthesis was also observed. Interestingly, at the 6-hour time point, an immediate early response to stimulation was evident, showing initial upregulation of pro-inflammatory cytokines and degradative enzymes, however, this early response was more robust in chondrocytes than synoviocytes. By 24 h, the sustained effects became more apparent with further increases in expression of pro-inflammatory mediators, especially in model 2 cultures where cells were not washed. Interestingly, at 24 h, the increased expression of pro-inflammatory mediators was more profound in synoviocytes, not chondrocytes, as was noted at 6 h. These temporal changes in gene expression were reflected in the quantification of *IL-1β*, *TNF-α*, *IL-6* and *IL-8* in the supernatants of chondrocyte and synoviocyte cultures. Taken together, our observations demonstrate a significant temporal effect on gene expression and protein synthesis in response to stimulation and also demonstrate how different cell types respond to stimulation. These observations are consistent with previous research reported that the effect of IL-10 has been observed to be more pronounced after prolonged exposure, with significant anti-inflammatory effects detected even after 48 h in vitro [20].

Researchers have investigated the potential use of IL-10 to treat OA due to its potent anti-inflammatory properties and studies have demonstrated that IL-10 can mitigate the inflammatory cascade in osteoarthritic conditions. For instance, AAV-mediated overexpression of IL-10 has been shown to reduce inflammation in stimulated equine chondrocyte pellets [21] and sustained IL-10 transgene expression following intra-articular AAV5-IL-10 administration in horses has provided evidence of its long-term therapeutic potential [22]. Additionally, *IL-10* is upregulated in response to pro-inflammatory cytokines such as IL-1β and TNF-α, potentially counteracting their detrimental effects in osteoarthritic cartilage [23]. Our results align with these findings, as we observed that r.IL-10 treatment significantly reduces *IL-1β*, *TNF-α*, *IL-8*, *MMP-13*, and *ADAMTS5* gene expression in both chondrocytes and synoviocytes, particularly in model 2. This model, which did not involve washing the cells prior to r.IL-10 treatment, more closely mimics the in vivo environment where continuous cytokine signaling occurs. The concentration of cytokines in culture supernatants further reinforced the efficacy of r.IL-10, showing a significant decrease in IL-1β and TNF-α content in synoviocyte supernatants, particularly in model 2, at concentrations of 10 and 20 ng/mL after 6 and 24 h. In chondrocytes, r.IL-10 treatment led to a significant decrease in IL-1β in model 2 after 24 h only. These results suggest that the timing and context of cytokine exposure are critical determinants of r.IL-10's anti-inflammatory effects.

The pro-inflammatory cytokines IL-1β and TNF-α have been reported to induce tissue degradation by decreasing anabolism and inducing the expression and activation of proteases, including *MMP-13* and *ADAMTS5* [24, 25]. In our study, stimulation in both models successfully induced similar effects, significantly increasing expression of *MMP-13* and *ADAMTS5* in both chondrocytes and synoviocytes at all time points. IL-10 treatment reduced the expression of *ADAMTS5*, an enzyme responsible for aggrecan degradation [21] and the expression of *MMP-13*, an enzyme that contributes to degradation of collagen type II [26]. The *NF-κB* signaling pathway has been shown to play a critical role in the progression of OA by upregulating the expression of pro-inflammatory cytokines such as *IL-1β* and catabolic enzymes like *MMP-13*, thereby accelerating the deterioration of articular cartilage [27]. IL-10 has demonstrated the ability to inhibit NF-κB activity, which in turn mitigates these deleterious effects. Similar results have been reported by Behrendt et al. in which IL-10 significantly reduced the expression of *MMP-3*, *MMP-13*, and *ADAMTS4* [26]. Limiting expression of these degradative enzymes has the potential to effectively protect the extracellular matrix from degradation.

Interestingly, r.IL-10 treatment of stimulated cultures increased *IL-6* gene expression in chondrocytes in both models at 24 h, while significantly lowering *IL-6* gene expression in synoviocytes in model 2. This contrasts with a previous study where IL-10 had no effect on IL-6 in TNF-α stimulated human synovial fibroblasts [28], suggesting that the experimental model influence IL-10's anti-inflammatory effects. In a tri-culture model of OA, the addition of regulatory T cells (Tregs), known producers of IL-10, was associated with increased concentrations of the anti-inflammatory cytokines IL-10 and IL-4 in the medium. While Tregs were associated with increased expression of tissue inhibitor of metalloproteinase (TIMP)-1, an important protective cytokine, in chondrocytes and synoviocytes, Tregs also led to

increased expression of *IL-6* and decreased expression of *collagen type 2* and *aggrecan* in IL-1β-stimulated chondrocytes [29]. This indicates that while IL-10 promotes chondroprotective conditions, it may also support the synthesis of IL-6 in response to inflammatory stimuli. Additionally, IL-10-treated chondrocytes showed significantly higher *IL-6* levels compared to non-treated, stimulated chondrocytes, though *IL-6* levels remained lower in chondrocytes than in synoviocytes under the same condition. Notably, during OA, *IL-6* expression differs significantly between cell types, with a recent study using single cell RNA sequencing showing that less than 1% of chondrocytes expressed *IL-6* compared to 5–36% of synoviocytes [30]. Another study by Cameron et al. (2021) demonstrated that AAV-mediated *IL-10* overexpression in equine BM-MSCs reduced *IL-1β* and *IL-6* expression in stimulated cartilage explants, though BM-MSCs transduced with AAV null produced similar effects, suggesting IL-10 alone may not drive the anti-inflammatory response [31]. *IL-6* likely mediates both pro-inflammatory and anti-inflammatory responses depending on the cytokine environment and cellular context [32]. Its increase after IL-10 treatment may reflect a complex regulatory interaction balancing inflammation and cartilage repair by promoting the survival and proliferation of chondrocytes under certain conditions [17].

p16, also known as CDKN2A, is a key regulator of cellular senescence, a state of stable cell cycle arrest that cells enter in response to various stressors, including DNA damage, oxidative stress, and inflammatory cytokines [33]. Senescence serves as a protective mechanism to prevent the proliferation of damaged cells, but it can also contribute to tissue aging and dysfunction if senescent cells accumulate [34]. *p16* inhibits cyclin-dependent kinases 4 and 6 (*CDK4/6*), preventing the phosphorylation of the retinoblastoma (RB) protein and blocking cell cycle progression from the G1 to the S phase, thereby inducing senescence [35]. Our findings suggest that chondrocytes are less responsive to IL-1β and TNF-α stimulation in terms of *p16* expression, which might indicate a more robust mechanism to avoid senescence under inflammatory conditions. In contrast, synoviocytes showed a significant increase in *p16* expression upon stimulation, suggesting a higher sensitivity to inflammatory signals and a potential predisposition to senescence. However, treatment with r.IL-10 effectively downregulated p16 expression in synoviocytes, demonstrating its protective effect against inflammation-induced senescence in these cells. A previous study found that *IL-10* overexpression in chondrocytes rescued proliferation and suppressed apoptosis, as well as reduced the expression of degeneration markers like *collagen type X*, *IL-6*, and *TNF-α* in IL-1β-pretreated chondrocytes [36].

Our study assessed PGE2 synthesis in response to stimulation and r.IL-10 treatment. We found that a significant reduction in PGE2 was observed only at the highest concentration of 50 ng/mL in model 1, where chondrocytes were washed and supernatants collected after 6 h of treatment. In synoviocytes, PGE2 levels were either similar to stimulated controls or significantly higher following r.IL-10 treatment, particularly in model 1 after 24 h. These findings contrast with a previous report in which 5 ng/mL IL-10 inhibited PGE2 release and downregulated the *TNF* receptor and *cyclo-oxygenase-2* in TNFα-induced synovial fibroblasts [37]. On the other hand, conditioned medium from adipose-derived stem cells (ASCs) was shown to increase PGE2 produced by ASCs and shown to be directly involved in their anti-inflammatory effects, primarily through the EP4 receptor [38]. Increased PGE2 levels may act through EP2 and EP4 receptors to elevate cAMP, inhibiting pro-inflammatory cytokines and promoting anti-inflammatory pathways [39]. IL-10 may upregulate PGE2 as part of a strategy to enhance anti-inflammatory mediators [40], or as a feedback mechanism to control excessive inflammation and maintain tissue homeostasis [41]. Additionally, PGE2 signaling has been demonstrated to suppress the release of inflammatory cytokines by macrophages, including TNF, IL-1β, and interferon (IFN)-β [42, 43].

One notable limitation of our study is the pooling of cells from individual horses for culture, which limits our ability to assess variability between horses and may lead to falsely decreased standard deviations and coefficients of variance. Additionally, we were unable to assess the protein synthesis of MMP-13 and ADAMTS5 as we did with the other cytokines in this study. This limitation arises from the lack of reliable antibodies that specifically bind to equine MMP-13 and ADAMTS5. Consequently, our findings regarding these enzymes are limited to gene expression analysis, which may not fully capture the post-transcriptional regulation and functional activity of these proteins. Furthermore, this study relies on monolayer, single-cell culture systems, which have inherent limitations when evaluating osteoarthritis as this system does not fully capture the complexity of the in vivo environment, where multiple cell types, tissue interactions, and systemic factors play crucial roles. It remains uncertain whether residual IL-1β and TNF-α are present in the unwashed cell culture supernatants at the time of analysis. This potential residual presence could impact the measured cytokine concentrations and, consequently, the interpretation of our results.

In summary, this study highlights the potential of r.IL-10 as a potential anti-inflammatory agent for OA management. By optimizing the in vitro conditions to better reflect the clinical environment, we demonstrated the significant impact of washing and timing on the modulation

of inflammatory responses. These findings pave the way for developing more effective therapeutic strategies for OA, emphasizing the need for carefully designed preclinical models that consider the dynamic nature of cytokine signaling in vivo.

Given the findings of this study, future research should explore the application of this optimized in vitro model for testing new generations of anti-inflammatory drugs and plasmids. Additionally, investigating different time points, including long-term effects, as well as assessing cytotoxicity and the stimulation protocol, will be important for a comprehensive understanding of the treatment effects. Utilizing a broader range of equine samples could help assess inter-animal variability, providing a more robust dataset. Furthermore, in vivo studies could be designed to validate our in vitro findings, examining the therapeutic potential of r.IL-10 in induced or naturally occurring osteoarthritis. Further investigating the downstream signaling pathways affected by r.IL-10, particularly in relation to other pro-inflammatory cytokines, could also yield deeper insights into its mechanism of action, ultimately informing better strategies for the management of osteoarthritis and other inflammatory conditions.

Conclusion

This study demonstrates that r.IL-10 significantly reduces inflammatory markers and degradative enzyme expression in IL-1β and TNF-α-stimulated chondrocytes and synoviocytes. Notably, the efficacy of r.IL-10 was significantly enhanced in a model where cells were not washed prior to treatment, highlighting the importance of experimental conditions in preclinical evaluations. These findings highlight the potential of r.IL-10 as an effective anti-inflammatory agent for OA management and underscore the importance of experimental conditions in preclinical evaluations. Overall, this study advances our understanding of r.IL-10's role in modulating joint inflammation and provides a foundation for developing effective therapeutic strategies for OA.

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Author contributions
H.E. performed most of the cell culture experiments, interpreted the results, and prepared the manuscript. R.L. assisted with logistical support and provided technical assistance during experiments. K.O. supervised the project, edited the manuscript, and was responsible for funding acquisition.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study involving equine cartilage and synovium tissue collection was approved by the Institutional Animal Care and Use Committee (IACUC), the University of Pennsylvania (Animal Use Protocol No. #806625).

Consent for publication
All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Clinical Studies-New Bolton Center, University of Pennsylvania, 382 West Street Road, Kennett Square, PA 19348, USA
[2]Department of Surgery, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt

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