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In vitro inflammatory multi-cellular model of osteoarthritis

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ABSTRACT

Objective: Osteoarthritis (OA) is a chronic joint disease, with limited treatment options, characterized by inflammation and matrix degradation, and resulting in severe pain or disability. Progressive inflammatory interaction among key cell types, including chondrocytes and macrophages, leads to a cascade of intra- and inter-cellular events which culminate in OA induction. In order to investigate these interactions, we developed a multi-cellular *in vitro* OA model, to characterize OA progression, and identify and evaluate potential OA therapeutics in response to mediators representing graded levels of inflammatory severity.

Methods: We compared macrophages, chondrocytes and their co-culture responses to "low" Interleukin-1 (IL-1) or "high" IL-1/tumor necrosis factor (IL-1/TNF) levels of inflammation. We also investigated response changes following the administration of dexamethasone (DEX) or mesenchymal stromal cell (MSC) treatment via a combination of gene expression and secretory changes, reflecting not only inflammation, but also chondrocyte function.

Results: Inflamed chondrocytes presented an osteoarthritic-like phenotype characterized by high gene expression of pro-inflammatory cytokines and chemokines, up-regulation of ECM degrading proteases, and down-regulation of chondrogenic genes. Our results indicate that while MSC treatment attenuates macrophage inflammation directly, it does not reduce chondrocyte inflammatory responses, unless macrophages are present as well. DEX however, can directly attenuate chondrocyte inflammation.

Conclusions: Our results highlight the importance of considering multi-cellular interactions when studying complex systems such as the articular joint. In addition, our approach, using a panel of both inflammatory and chondrocyte functional genes, provides a more comprehensive approach to investigate disease biomarkers, and responses to treatment.

1. Introduction

Osteoarthritis (OA), characterized by joint pain and progressive cartilage degeneration, is due to an imbalance in matrix degradation and synthesis [1,2], ultimately resulting in diminished quality of life [3,4] and other comorbidities [5]. Common risk factors include aging and joint trauma, (posttraumatic OA (PTOA)) [6], wherein the initial injury leads to rapid chondrocyte death in the impacted area, followed by anabolic, catabolic and inflammatory changes that also occur during chronic OA [7]. Among the major OA pathogenetic mediators are Interleukin-1β (IΛ-1β) and tumor necrosis factor-α (TNF-α) [8,9], which regulate a plethora of downstream signaling pathways.

IL-1 stimulates chondrocytes and synovial cells to produce proteases, such as degradative matrix metalloproteinases (MMPs) [10]. TNF-α, which has been measured in synovial OA joints, has also been used to induce OA-like changes *in vitro* [10]. Nevertheless, despite a plethora of experimental systems, treatments have not been developed to prevent cartilage damage, reverse joint destruction or sufficiently improve OA symptoms [11].

For example, intra-articular (IA) corticosteroid injections are employed clinically, but only infrequently, due to the high doses needed to overcome its rapid clearance [12]. Dexamethasone (DEX), a glucocorticoid with anti-inflammatory and chondroprotective properties, inhibited inflammation and cartilage damage in PTOA models [7,13]. However, there are major safety concerns with the continuous use of steroid injections due to negative effects such as chondrocyte apoptosis [14,15]. Another approach to alter OA progression has been IA mesenchymal stromal cell (MSC) administration [11,16,17], as they secrete

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anti-inflammatory and regenerative factors. However, the target of MSC treatment is unknown and in either case, these cells are required in large numbers and are not long-lasting when freely administered [17]. We have previously demonstrated that alginate encapsulation of MSC (eMSC) promotes their secretory function and can be used to resolve tissue trauma [18], in traumatic central nervous system injury models [18–20].

Given the importance of minimizing animal experimentation, *in vitro* systems that recapitulate key aspects of the joint environment are needed [21]. However, most *in vitro* systems consist of only chondrocytes, in different culture configurations [21–24], while ignoring other cell components such as synoviocytes [24]. This is a critical omission, because synovial macrophages are important in perpetuating OA [25]. Furthermore, mono and co-culture systems, generally focus on only one aspect of OA, such as inflammation [26] and do not assess either degeneration or regeneration, both critical elements of OA [22].

While complex 3D models are more likely to represent all aspects of OA joints, these cultures require several weeks to be established [27], primary tissue sample variability is inherent, and overall are more expensive than monolayer cultures [28]. Recently, on-chip culture approaches have been explored, but these require unique technical skills and optimization [22]. Therefore, in generating animal-free predictive models for therapeutic testing there is a balance between complex 3D and simpler monolayer cultures.

We sought to establish a relatively rapid and inexpensive monolayer model to determine its efficacy in altering inflammatory, regenerative and degenerative gene expression, and compare the effect of different treatments on gene expression in mono- and co-culture configurations. Using this model, our studies were designed to treat chondrocytes stimulated with OA promoting factors, IL-1 and/or TNF, with eMSCs, free MSCs, or with DEX, and determine whether production of inflammatory proteins and/or inflammatory, chondrogenic, and extracellular matrix (ECM) remodeling gene expression, is affected by inflammatory disease severity. We also evaluated the critical role of macrophages in altering therapeutic responses to MSC.

2. Methods

2.1. Cell culture

All cells were maintained in a humidified 37 °C incubator with 5 % CO_2. Viability was assessed with a LIVE/DEAD cell assay (Fisher, Waltham, MA) as per manufacturer's instructions.

2.2. Bovine chondrocyte culture

Bovine chondrocyte-based *in vitro* models are commonly used to study OA due to the ease of cell procurement and cross-species reactivity with human factors [29]. In addition, studying cells from different species allowed us to test and differentiate the cell origins of secreted and intracellular products. We compared the inflammatory responses of our bovine cells with the our previous studies [30] using the human chondrocyte cell line, C28/I2 (obtained from Dr. Mary Goldring, Hospital for Special Surgery, NY, NY), to ensure a similar inflammatory pattern.

Articular cartilage was harvested from stifles of freshly slaughtered 2- to 4-week-old calves. Pooled cells harvested from 3 normal joints were expanded as monolayer cultures in humidified 37 °C, 5 % CO_2 incubators using high glucose Dulbecco's Modified Eagle's Medium (DMEM) (ThermoFisher, Boston, MA) supplemented with 10 % FBS (ThermoFisher, Boston, MA), 100 U/mL penicillin and 100 mg/mL streptomycin (1%v/v) (P/S), 2 mM L-glutamine, 50 μg/mL L-ascorbic acid (Sigma, St Louis, MO), 40 μg/mL L-proline (Sigma, St. Louis, MO), 100 μg/mL sodium pyruvate (ThermoFisher, Boston, MA), 1× ITS + premix (insulin, human transferrin, and selenous acid) (Corning, Corning, NY), 1 ng/mL transforming growth factor-β1 (Preprotech, Cranbury, NJ) and 5 ng/mL fibroblast growth factor (FGF)-2 (ThermoFisher, Boston, MA) [31,32]. Confluent chondrocytes were collected using trypsin-EDTA (TE 0.25 %) (ThermoFisher, Boston, MA) and plated at passage 1–2 for experiments.

2.3. MSC culture

Human bone marrow-derived MSC (Institute for Regenerative Medicine, Texas A&M University), were thawed at passage 2 and plated at 1714 cells/cm^2 in a humidified 37 °C, 5 % CO_2 incubator in Minimum Essential Medium-α (ThermoFisher, Boston, MA) containing no deoxy- or ribonucleosides, and supplemented with 10 % FBS (Premium Grade, Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine, 1 ng/mL FGF-2, 1 % P/S. Cells were grown to 70 % confluence, trypsinized and replated at 1714 cells/cm^2 in T-225 flasks until confluency and used for MSC experimental setups at passage 4–5 [33]. Alginate Poly-L-Lysine encapsulation of MSC was performed as previously described [18,34].

2.4. Monocyte isolation and differentiation

Human macrophages were obtained as described by Gray et al. [33]. Briefly, human peripheral blood (New York Blood Center, NY, NY) was fractionated utilizing density gradient centrifugation (Ficoll-Paque Premium, 1.077 g/mL, GE Healthcare, Chicago, IL). The buffy coat was collected and washed with 1× PBS and the mononuclear cells isolated by enriching the $CD14^+$ cell population, using magnetic bead cell sorting according to manufacturer's instructions (Miltenyi Biotech, Charleston, MA). Monocytes were seeded at 1 × 10^7 cells/T-175 cm^2 flasks in Advanced RPMI supplemented with 10 % FBS, 1 % P/S, 4 mM L-glutamine, and 5 ng/mL granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) to induce differentiation into proinflammatory M1 macrophages. After 7 days, differentiated cells were detached with trypsin-EDTA and cryopreserved at passage 1 in fully supplemented Advanced RPMI (ThermoFisher, Boston, MA) containing 10 % dimethyl sulfoxide. Passage 1 macrophages were used for our studies by quickly thawing and culturing them in fully supplemented Advanced RPMI overnight to allow cell attachment.

2.5. Monolayer inflammatory OA model

Bovine chondrocytes were plated in 12-well plates at a seeding density of 25,000 cells/cm^2 using chondrogenic media (hgDMEM supplemented with 1 % penicillin–streptomycin, 2 mM L-glutamine, 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, and 1 × ITS + premix [insulin, human transferrin, and selenous acid]) with 10 % FBS (to facilitate cell attachment) and attached overnight. Media was then changed to serum free chondrogenic media and chondrocytes were cultured in basal or pro-inflammatory conditions. IL-1 or IL-1 and TNF-α (IL-1/TNF) were used at a concentration of 10 ng/mL each to simulate "low" inflammation or "high" inflammation levels, respectively. These conditions were chosen based on prior studies describing IL-1 and TNF-α as key mediators of the inflammatory cascade in OA with both cytokines being upregulated independently and promoting cartilage degradation [35–38]. After 48 h in culture, media supernatants were collected while monolayer chondrocytes were trypsinized, and counted using Trypan Blue exclusion. Samples were stored in a −80 °C freezer.

2.6. DEX treatment

DEX, has been shown to have chondroprotective effects at low doses [12]. Therefore cells were treated with 50 μM of DEX to compare our results with previously reported studies [7]. After 48 h in culture, media supernatants were collected. Chondrocytes were detached with trypsin, counted using Trypan Blue exclusion, pelleted, and flash frozen. Samples were stored as described above.

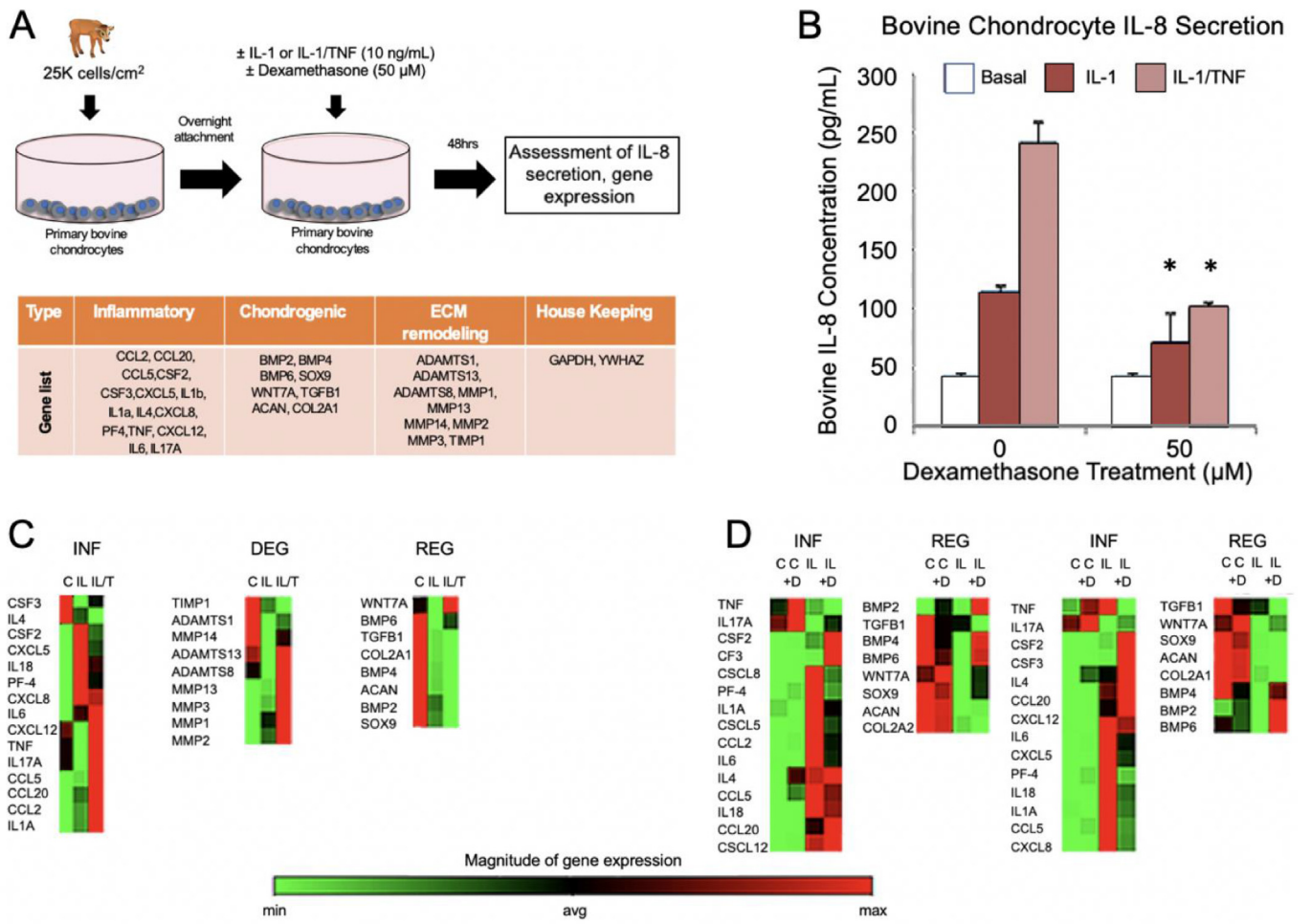

Fig. 1. Inflammatory model of OA: Chondrocyte responses to inflammatory stimuli and DEX treatment. A) Experimental model depicted including cell culture, treatment additions and culture evaluation metrics. B) IL-8 secretion levels by IL-1 and IL-1/TNF stimulated chondrocytes ± dexamethasone treatment. C) Differential gene expression in a panel of cytokines and chemokines (INF), matrix remodeling proteins (DEG), and chondrogenic genes (REG) after treatment with pro-inflammatory IL-1 (IL) or IL-1/TNF (IL/T) relative to basal control chondrocytes (C). D) Differential gene expression panel of cytokines and chemokines (INF) and chondrogenic genes (REG) after treatment with pro-inflammatory IL-1 ± dexamethasone (DEX) (IL + D) or after treatment with pro-inflammatory IL-1/TNF ± DEX (IL/T + D). Genes with higher expression levels, compared to the control group, are shown in red (max), whereas genes with lower expression levels are shown in green (min). Genes with average expression levels are shown in black (avg). Each group represents the mean fold change of 3 pooled samples ($n = 9$) from 3 independent experiments. Bar graph represents the mean ± SEM for $n = 9$ of 3 independent experiments. * = significance at $P < 0.05$ for DEX treated vs untreated cells. CSF3 = Colony Stimulating Factor 3, IL4 = Interleukin 4, CSF2 = Colony Stimulating Factor 2, CXCL5 = C-X-C Motif Chemokine Ligand 5, IL18 = Interleukin 18, PF-4 = Platelet Factor 4, CXCL8 = C-X-C Motif Chemokine Ligand 8, IL6 = Interleukin 6, CXCL12 = C-X-C Motif Chemokine Ligand 12, TNF = Tumor Necrosis Factor, IL17A = Interleukin 17A, CCL5 = C–C Motif Chemokine Ligand 5, CCL20 = C–C Motif Chemokine Ligand 20, CCL2 = C–C Motif Chemokine Ligand 2, IL1A = Interleukin 1A, TIMP1 = TIMP Metallopeptidase Inhibitor 1, ADAMTS1 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 1, MMP14 = Matrix Metallopeptidase 14, ADAMTS13 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 13, ADAMTS8 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 8, MMP13 = Matrix Metallopeptidase 13, MMP1 = Matrix Metallopeptidase 1, MMP2 = Matrix Metallopeptidase 2, WNT7A = Wnt Family Member 7A, BMP6 = Bone Morphogenetic Protein 6, TGFB1 = Transforming Growth Factor B1, COL2A1 = Collagen 2A1, BMP4 = Bone Morphogenetic Protein 4, ACAN = Aggrecan, BMP2 = Bone Morphogenetic Protein 2, COL2A2 = Collagen 2A2.

2.7. MSC co-culture studies

Bovine chondrocytes were plated in 12-well plates at 25,000 cells/cm^2 using chondrogenic media with 10 % FBS and left to attach overnight. Then media was changed to serum free media with or without 10 ng/mL of IL-1 or IL-1/TNF treatment and the chondrocytes were co-cultured with human free or eMSC using 0.4 μm pore size transwells fit for 12-well plates (PET, Falcon, ThermoFisher, Boston, MA). Co-cultures were maintained for 48 h. Culture supernatants and chondrocytes were collected and stored as described above.

2.8. Prostaglandin E_2 (PGE_2) dose response

Bovine chondrocytes and human macrophages were seeded at 25,000 cells/cm^2 in 24-well plates and allowed to attach overnight. The media was replaced with serum free chondrogenic media containing 0–20 ng/mL of PGE_2 (Cayman Chemicals, Ann Arbor, MI) for 48 h, cell culture supernatants were gently collected and stored at −80 °C as above.

2.9. Mixed culture studies

Mixed culture studies were performed by seeding all cell types at 25,000 cells/cm^2. Bovine chondrocytes were trypsinized, collected, seeded in 24-well plates, and allowed to attach overnight. In parallel, passage 1 human macrophages were thawed and seeded in 0.4 μm pore size transwells designed for 24-well plates using fully supplemented Advanced RPMI media and left to attach overnight. The following day, monolayer human MSCs (P3-4) were trypsinized, collected and resuspended in basal or stimulatory chondrogenic media containing 10 ng/mL

of IL-1 or IL-1/TNF. The macrophage and chondrocyte media were replaced with basal or stimulatory media and the MSCs were seeded in the transwells. Then transwells containing macrophages ± MSCs were placed on top of the chondrocyte cultures creating a mixed co-culture system. Alternatively, MSC and chondrocytes were co-cultured on the bottom. After 48 h, supernatants were collected and stored for further analysis.

2.10. qRT-PCR gene array

Gene expression data was obtained using a custom bovine RT^2 Profiler PCR Array (QIAGEN, Germantown, MD). Sample processing and qRT-PCR were performed by QIAGEN's Center for Genomic Services. Data analysis was performed using GeneGlobe platform. The gene panel used for qRT-PCR gene array analysis consisted of inflammatory genes, chondrogenic genes, ECM remodeling genes, and house-keeping genes. ECM remodeling genes were not analyzed in the DEX studies. Further information regarding statistical and data analyses utilized is provided in Supplementary Figure 1.

2.11. Cytokine measurement

Culture supernatants were removed from storage and thawed at room temperature. Bovine interleukin IL-8 (the only available bovine ELISA) levels were measured utilizing a bovine IL-8 Do-It-Yourself ELISA (Kingfisher Biotech, St. Paul,MN) following the manufacturer's instructions with some modifications. Briefly 1–2.5 μg/mL of capture antibody (PB0273B-100) was diluted in 1× ELISA Coating Buffer (Biolegend, San Diego, CA), loaded on an untreated 96 well ELISA plate (Nunc Maxi-Sorb) and incubated overnight at room temperature. Plates were blocked for non-specific binding with 4 % bovine serum albumin (BSA) (Sigma) in 1× PBS (w/v) for 1 h in a plate shaker. Samples were loaded and incubated for 2 h while mixing in a plate shaker. Biotinylated anti-bovine IL-8 polyclonal antibody (PBB1163B-050) was diluted in 4 % BSA solution at 0.05 μg/mL, added to the plate and incubated for 1hr while shaking. Streptavidin-Horseradish Peroxidase (Biolegend, San Diego, CA) was diluted 1:1000 in 4 % BSA solution.

Human Interleukin-8 (IL-8) and Interleukin-10 (IL-10) were measured using ELISA MAX Deluxe Sets (Biolegend, San Diego, CA) following manufacturer's instructions. PGE_2 levels were measured using the Prostaglandin E_2 Express ELISA kit (Cayman Chemicals, Ann Arbor, MI) as per manufacturer's instructions. Absorbances were recorded using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA).

2.12. Statistical analysis

qRT-PCR gene array data was analyzed using GeneGlobe RT^2 PCR Data Analysis software (QIAGEN). Data are reported as fold differences and only those with expression differences >2 were included, which is the accepted analysis standard [39]. Additional gene array analysis metrics are described in Supplementary Figure 1. Cytokine secretion data points represent the mean ± standard error of the mean (SEM) for the indicated number of independent observations (n). Statistical differences between the data were determined using analysis of variance followed by Fisher's least significant difference *post hoc* analysis with a significance level of $\alpha = 0.05$ in Kaleida-Graph software version 4.1 (Synergy Software, Reading, PA). A P-value of <0.05 was considered as significant.

3. Results and discussion

3.1. Monolayer OA inflammatory model

OA-like cell responses that more closely replicate the natural disease can be induced using cytokines [10]. As a first step in establishing an *in vitro* model of OA which presents a "low" and "high" level of inflammation, we stimulated monolayer chondrocytes with either IL-1 alone ("low") or IL-1 + TNF-α(IL-1/TNF) ("high"). After 48 h, we screened the efficacy of our model by initially evaluating the levels of IL-8 secretion into cell culture supernatants. IL-8 is a chemokine produced by OA chondrocytes involved in different aspects of the pathophysiology of the disease including the promotion of MMP production and leukocyte homing to the synovium [40]. IL-8 has been previously used in our OA model and by many others and is considered to be a reliable inflammatory metric [21,22,30,41,42]. Both inflammatory stimuli promoted the secretion of IL-8 with IL-1/TNF stimulated chondrocytes producing 11× more IL-8 than IL-1 stimulated cells (Fig. 1).

Table 1
IL-1 and IL-1/TNF stimulated chondrocyte gene expression changes in the presence of 50 μM DEX treatment.

Upregulated				
Gene	IL-1	IL-1/TNF	IL-1	IL-1/TNF
			+DEX	+DEX
CCL20	2401.34	7729.27	4099.27	11424.08
CXCL5	1875.74	3070.67	689.25	1179.34
CXCL8	560.74	2433.54	141.39	495.1
IL6	286.62	390.73	123.04	325.06
CCL2	166.02	434.05	76.18	199.19
IL1B	99.51	795.67	81.09	230.24
CSF2	54.63	48.12	317.6	828.65
IL1A	10.43	55.78	5.54	14.69
CXCL12	8.88	8.88	9.83	15.78
CCL5	4.72	22.04	3.86	4.92
CSF3	3.57	7.02	166.76	428.73
PF4	3.49	3.88	/	/
IL4	/	/	/	2.47
BMP2	/	/	/	2.28
Downregulated				
Gene	IL-1	IL-1/TNF	IL-1	IL-1/TNF
			+DEX	+DEX
ACAN	−17.1	−36.72	−13.02	−43.85
SOX9	−11.09	−7.67	−2.25	−8.3
COL2A1	−9.86	−14.63	−19.8	−39.27
BMP6	−6.58	−2.91	/	/
BMP4	−5.84	−4.41	/	/
TGFB1	/	/	−2.02	−2.19

Values represent the average gene fold regulation normalized to non-stimulated chondrocyte gene expression. Only values with a fold change higher that 2 are shown. Ordered in descending order with respect to IL-1 activated gene expression changes. IL-1/TNF, IL-1+Dex, IL-1/TNF + Dex columns are placed next to the IL-1 activated group for ease of comparison. "/" = expression differences <2. Bold = ECM remodeling/degenerative genes, Italic = chondrogenic/regenerative genes, Standard font = inflammatory genes. CCL20 = C–C Motif Chemokine Ligand 20, CXCL5 = C-X-C Motif Chemokine Ligand 5, CXCL8 = C-X-C Motif Chemokine Ligand 8, IL6 = Interleukin 6, CCL2 = C–C Motif Chemokine Ligand 2, IL1B=Interleukin1B, CSF2 = Colony Stimulating Factor 2, IL1A = Interleukin1A, CXCL12 = C-X-C Motif Chemokine Ligand 12, CCL5 = C–C Motif Chemokine Ligand 5, CSF3 = Colony Stimulating Factor 3, PF4 = Platelet Factor 4, IL4 = Interleukin 4, BMP2 = Bone Morphogenetic Protein 2, ACAN = Aggrecan, COL2A1 = Collagen 2A1, BMP6 = Bone Morphogenetic Protein 6, BMP4 = Bone Morphogenetic Protein 4, TGFB1 = Transforming Growth Factor B1.

Next, in order to gain a more comprehensive view of the cellular changes accompanying chondrocyte activation, gene expression changes using a panel of cytokine, chondrogenic, and ECM remodeling genes, were analyzed. As shown in Fig. 1, factors associated with the pathogenesis of OA (Table 1) were upregulated in our *in vitro* system. Both IL-1 and IL-1/TNF stimulated the up-regulation of pro-inflammatory cytokines (Fig. 1, Table 1). Both stimuli also increased the levels of matrix degrading proteins MMP1 (IL-1 36-fold, IL-1/TNF 82-fold), MMP 3(IL-1 226-fold, IL-1/TNF 2030 fold), and 13 (IL-1 13-fold, IL-1/TNF 295-fold). Several genes associated with chondrogenesis were down-regulated (Fig. 1, Table 1). Although IL-1 and IL-1/TNF stimulated chondrocytes had similar dysregulated genes, the fold regulation following IL-1/TNF stimulation was generally much greater.

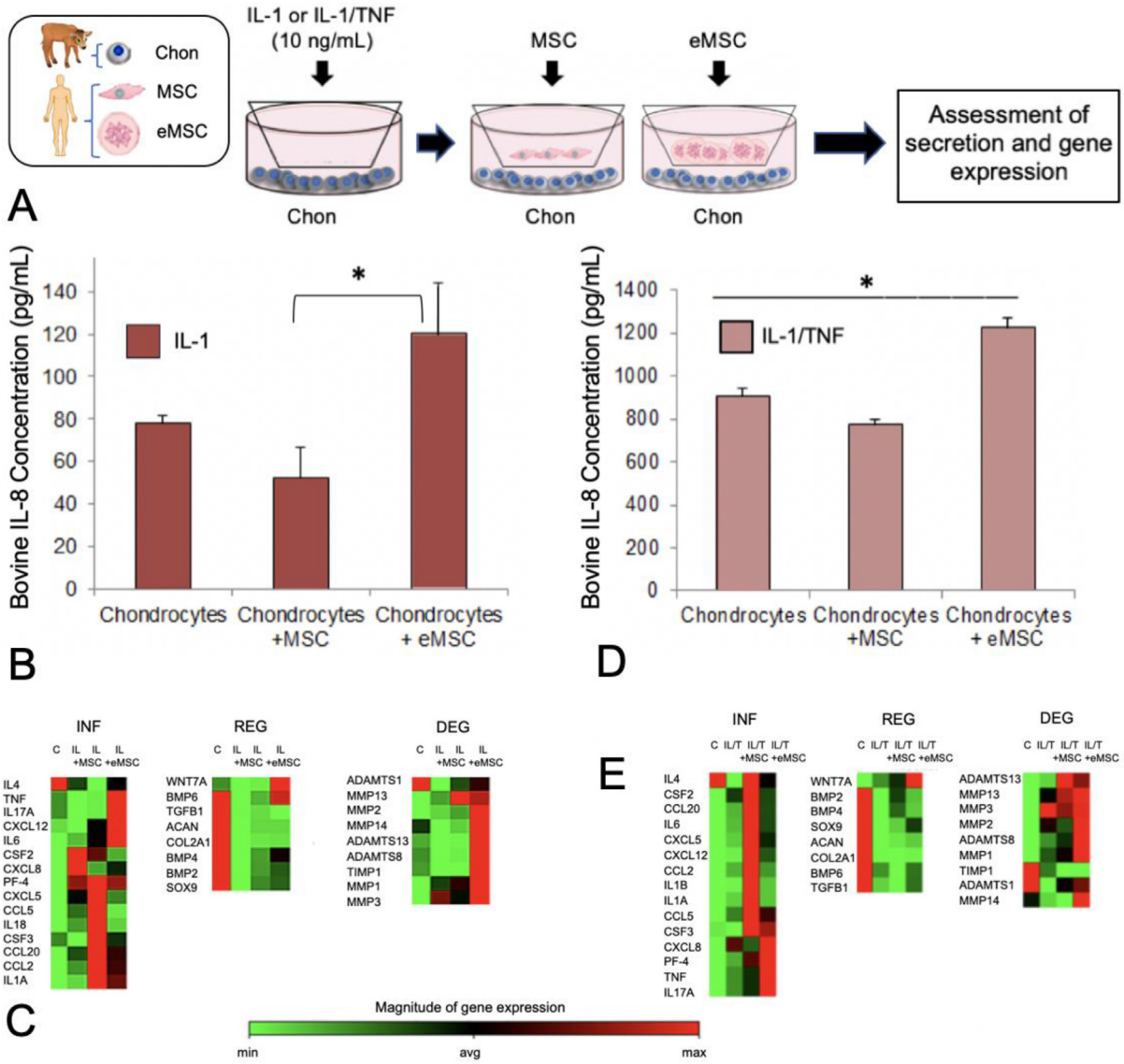

Fig. 2. Chondrocyte responses to MSC or eMSC: IL-1 or IL-1/TNF stimulated chondrocytes were treated with MSC or eMSC and both gene expression changes and IL-8 secretion were assessed. A) Experimental set-up depicted containing cell conditions and activation stimuli, B) IL-1 stimulated chondrocyte IL-8 secretion in response to MSC treatment; chondrocytes = 77.85 ± 3.93 pg/mL, chondrocytes + MSC = 51.98 ± 14.72 pg/mL, and chondrocytes + eMSC = 120.52 ± 23.33 pg/mL. Bar graph represents the mean ± SEM for n = 6–9 of 3 independent experiments. *$P < 0.05$ IL-1 + MSC chondrocytes vs IL-1 + eMSC chondrocytes. C) Differential gene expression in a panel of cytokines and chemokines (INF), matrix remodeling proteins (DEG), and chondrogenic genes (REG) after treatment with pro-inflammatory IL-1 (IL) treatment ± MSC or eMSC, relative to basal control chondrocytes (C), D) IL-1/TNF stimulated chondrocyte IL-8 secretion in response to MSC treatment, chondrocytes = 904.52 ± 37.65 pg/mL, chondrocytes + MSC = 774.71 ± 22.87 pg/mL, and chondrocytes + eMSC = 1230.66 ± 42.86 pg/mL. Bar graph represents the mean ± SEM for n = 6–9 of 3 independent experiments. *$P < 0.05$ among all groups, IL-1/TNF chondrocytes vs IL-1/TNF + MSC chondrocytes vs IL-1/TNF + eMSC chondrocytes. E) Differential gene expression in a panel of cytokines and chemokines (INF), matrix remodeling proteins (DEG), and chondrogenic genes (REG) after treatment with pro-inflammatory IL-1+TNF (IL/T) treatment ± MSC or eMSC, relative to basal control chondrocytes (C). Genes with higher expression levels are shown in red (max), whereas genes with lower expression levels are shown in green (min). Genes with average expression levels are shown in black (avg). Each group represents the mean fold change of 3 pooled samples (n = 9) from 3 independent experiments. Gene notations are defined in Fig. 1 legend.

This comprehensive gene array was then used to assess treatments effects. Here, we compared the gold standard treatment, DEX, with free MSC and eMSC, both known to have therapeutic affects for traumatic tissue injury resolution [18–20].

We first characterized the effect of DEX in the system as it is commonly used in the clinic to treat OA symptoms and its effects on chondrocytes have been previously studied by us and by others [43,44]. DEX can impact synovial cells as well as resident macrophages [44,45]. Differential gene expression changes were analyzed for stimulated and non-stimulated chondrocytes treated with 50 μM DEX for 48 h (Fig. 1, Table 1). DEX treatment of IL-1 stimulated chondrocytes down-regulated the gene expression of pro-inflammatory CCL2, CXCL5, CXCL8, PF4, IL-6 genes and up-regulated pro-chondrogenic BMP4, BMP6 and SOX9 genes. Similarly, IL-1/TNF stimulated chondrocytes treated with DEX down-regulated many pro-inflammatory genes. Some chondrogenic gene expression changes were also modified by DEX treatment, including BMP2, BMP4 and BMP6.

As previously observed, IL-8 secretion levels increased from IL-1 and IL-1/TNF stimulated chondrocytes. However, treatment with 50 μM DEX significantly decreased the secretion of IL-8 (Fig. 1) reflecting the downregulation of IL-8 gene expression observed at the mRNA level. Our results are consistent with previously published data where DEX has been

Table 2
IL-1 stimulated chondrocyte gene expression changes after 48 h in co-culture with free MSC or eMSC treatment.

Upregulated			Downregulated		
Gene	MSC	eMSC	Gene	MSC	eMSC
CSF3	6.73	/	CXCL8	−5.87	−2.34
MMP13	5.42	4.75	CSF2	/	−3.9
CCL2	4.93	3.14	CXCL5	/	−2.08
IL1B	4.19	/			
IL6	4.13	7.97			
CXCL12	3.63	6.31			
CCL20	2.91	/			
IL1A	2.88	2.32			
BMP6	2.49	11.57			
BMP4	2.42	5.33			
ADAMTS1	2.26	3.19			
CXCL5	2.08	/			
CCL5	2.02	/			
ADAMTS13	/	5.58			
CSF3	/	3.32			
MMP2	/	3.23			
TNF	/	2.91			
IL17A	/	2.91			
WNT7A	/	2.91			
ADAMTS8	/	2.91			
TIMP1	/	2.83			
MMP14	/	2.74			
MMP1	/	2.61			

Values represent the average fold regulation compared to IL-1 stimulated chondrocytes with no treatment. Ordered in descending order with respect to MSC treated group. Only values with a >2 fold change are shown. "/" = expression differences <2. Bold = ECM remodeling/degenerative genes, Italic = chondrogenic/regenerative genes, Standard font = inflammatory genes. CSF3 = MMP13 = Matrix Metallopeptidase 13, CCL2 = C–C Motif Chemokine Ligand 2, IL1B=Interleukin 1B, IL6 = Interleukin 6, CXCL12 = C-X-C Motif Chemokine Ligand 12, CCL20 = C–C Motif Chemokine Ligand 20, IL1A = Interleukin 1A, BMP6 = Bone Morphogenetic Protein 6, BMP4 = Bone Morphogenetic Protein 4, ADAMTS1 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 1, CXCL5 = C-X-C Motif Chemokine Ligand 5, CCL5 = C–C Motif Chemokine Ligand 5, ADAMTS13 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 13, CSF3 = Colony Stimulating Factor 3, MMP2 = Matrix Metallopeptidase 2, TNF = Tumor Necrosis Factor, IL17A = Interleukin 17A, WNT7A = Wnt family member 7A, ADAMTS8 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 8, TIMP1 = TIMP Metallopeptidase Inhibitor 1, MMP14 = Matrix Metallopeptidase 14, MMP1 = Matrix Metallopeptidase 1, CXCL8 = C-X-C Motif Chemokine Ligand 8, CSF2 = Colony Stimulating Factor 2.

reported to inhibit the induction of inflammatory cytokines [7,43] and promote chondrogenesis as observed by the upregulation of BMP 2, 4, and 6 [46,47]. The effects of DEX were more pronounced in the IL-1/TNF environment. These findings, which describe the effect of DEX on ECM gene expression, are also consistent with our previous 3D tissue engineering studies where more functional cartilage tissue is produced in the presence of DEX [44].

3.2. Co-culture studies

Following DEX treatment, we proceeded to challenge the system with a potential cellular treatment, MSC, which have been reported to decrease inflammation and promote tissue regeneration in several *in vitro* and *in vivo* studies including small clinical trials [48,49]. In addition, we previously demonstrated that eMSC is significantly more anti-inflammatory than MSC in a model of CNS injury [18,19]. To investigate the effects of MSCs and eMSCs in our OA model, IL-1 and IL-1/TNF stimulated chondrocytes were co-cultured with MSC or eMSCs and a qRT-PCR gene array was performed. The presence of MSC and eMSC induced significant gene expression changes in bovine chondrocytes (Fig. 2, Tables 2 and 3). However, unexpectedly, the chondrocyte response to the MSC and eMSC

Table 3
IL-1/TNF stimulated chondrocyte gene expression changes after 48 h in co-culture with free MSC or eMSC treatment.

Upregulated			Downregulated		
Gene	MSC	eMSC	Gene	MSC	eMSC
IL1B	58.07	6.2	CXCL8	−2.19	/
CSF3	16.15	13.67			
CXCL5	11.9	5.09			
IL6	8.24	2.95			
CCL20	7.36	2.58			
CCL2	7.14	/			
BMP4	6.74	2.71			
CXCL12	6.49	2.74			
BMP2	6.37	2.54			
IL1A	6.33	/			
CCL5	3.93	2.48			
PF4	2.78	3.87			
IL4	2.44	/			
ADAMTS13	2.12	2.07			
CSF2	2.09	/			
SOX9	/	3.19			
MMP1	/	3.03			
ACAN	/	2.42			
MMP13	/	2.06			

Values represent the average gene fold regulation compared to IL-1/TNF stimulated chondrocytes with no treatment. Ordered in descending order with respect to MSC treated group only values with a >2 fold change are shown. "/" = expression differences < 2-fold. Bold = ECM remodeling/degenerative genes, Italic = chondrogenic/regenerative genes, Standard font = inflammatory genes. IL1B = Interleukin 1B, CSF3 = Colony Stimulating Factor 3, CXCL5 = C-X-C Motif Chemokine Ligand 5, IL6 = Interleukin 6, CCL20 = C–C Motif Chemokine Ligand 20, CCL2 = C–C Motif Chemokine Ligand 2, BMP4= Bone Morphogenetic Protein 4, CXCL12 = C-X-C Motif Chemokine Ligand 12, BMP2 = Bone Morphogenetic Protein 2, IL1A = Interleukin 1A, CCL5 = C–C Motif Chemokine Ligand 5, PF4 = Platelet Factor 4, IL4 = Interleukin 4, ADAMTS13 = A Disintegrin and Metallopeptidase With Thrombospondin Type 1 Motif 13, CSF2 = Colony Stimulating Factor 2, MMP1 = Matrix Metallopeptidase 1, ACAN = Aggrecan, MMP13 = Matrix Metallopeptidase 13, CXCL8 = C-X-C Motif Chemokine Ligand 8.

treatment was characterized by exacerbated inflammation and catabolic activity.

When treated with MSC, IL-1 stimulated chondrocytes further up-regulated the expression of many pro-inflammatory genes and ECM remodeling genes (Table 2). Pro-chondrogenic BMP4 and BMP6 were both up-regulated. In contrast, eMSC-treated chondrocytes differentially expressed many inflammatory genes compared to MSC. More pronounced, however, was the effect of eMSC on ECM remodeling genes. TIMP1, ADAMTS1, ADAMTS8, ADAMTS13, MMP1, MMP2, MMP13, and MMP14 were all upregulated. Interestingly, pro-chondrogenic BMP4, BMP6 and WNT7A were also increased following eMSC treatment in IL-1 treated chondrocytes, (Table 2). The IL-8 levels in cell culture supernatants were also assessed. eMSC treated chondrocytes had significantly elevated levels of IL-8 when compared to MSC treated chondrocytes. IL-1/TNF stimulated chondrocytes treated with either MSC or eMSC had differential gene expression when compared to IL-1 stimulated chondrocytes (Table 3) and IL-8 secretion levels resulted in significantly lower levels for MSC treated chondrocytes, compared to eMSC treated chondrocytes, following IL-1/TNF treatment (Fig. 2).

3.3. PGE_2 secretion

Given the unexpected up-regulation of pro-inflammatory cytokines and ECM remodeling genes in IL-1 and IL-1/TNF stimulated chondrocytes treated with MSC or eMSCs, we questioned if MSC secretory function was being compromised in this *in vitro* inflammatory system. Previous studies performed by our group and others have highlighted the role of secreted PGE_2 on the anti-inflammatory and immunomodulatory

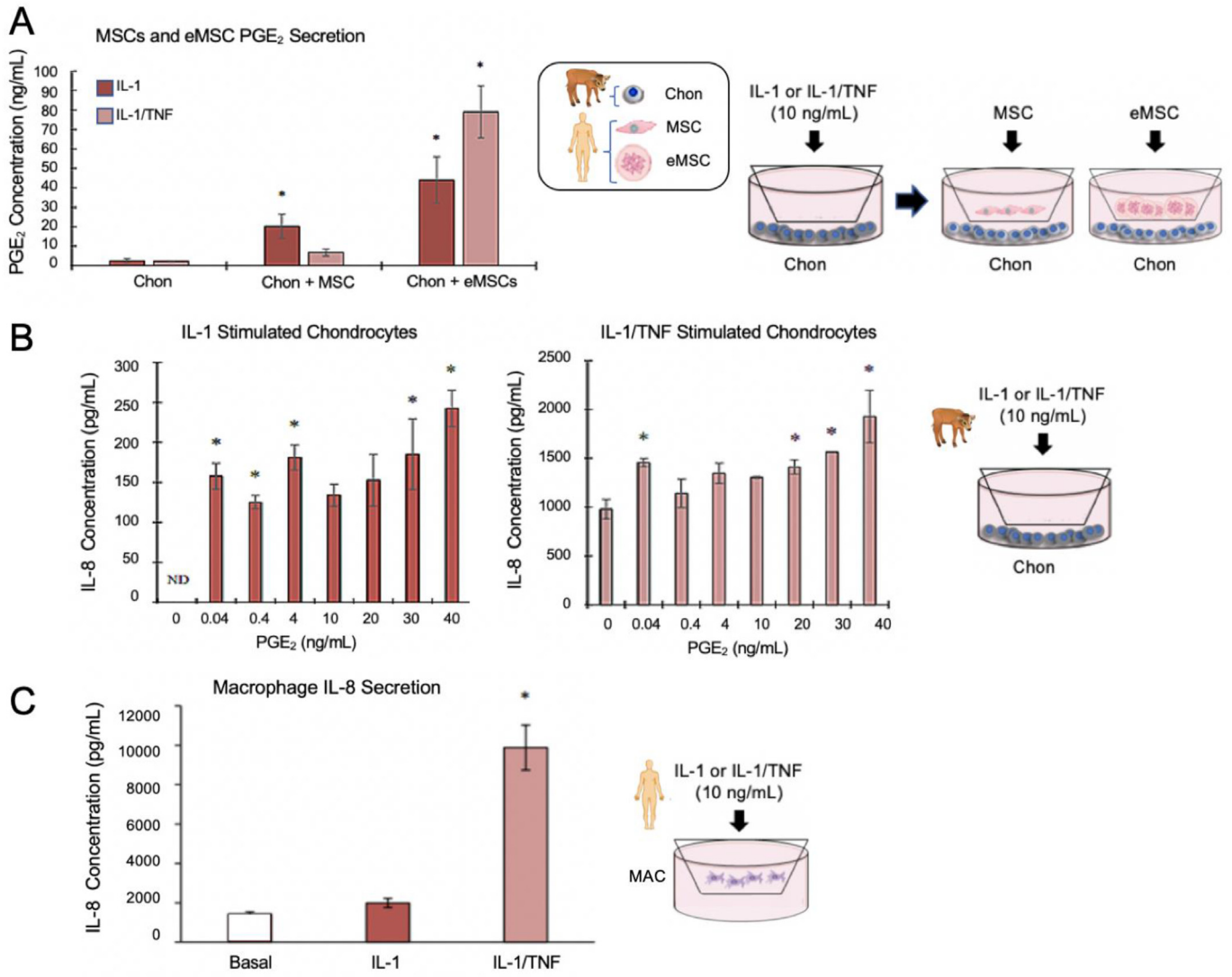

Fig. 3. Responses to stimulated chondrocytes or macrophages. A) PGE2 secretion by MSC or eMSC in co-culture with stimulated bovine chondrocytes. MSC and eMSC secrete significant levels of PGE2 when stimulated with IL-1 or IL-1/TNF compared to stimulated chondrocytes. PGE_2 secretion: IL-1 stimulated chondrocytes = 2.61 ± 1.16 ng/mL, IL-1/TNF stimulated chondrocytes = 2.32 ± 0.21 ng/mL, IL-1 chondrocytes + MSC = 20.20 ± 6.18 ng/mL, IL-1/TNF chondrocytes + MSC = 7.90 ± 1.38 ng/mL, IL-1 chondrocytes + eMSC = 44.04 ± 11.89 ng/mL, and IL-1/TNF chondrocytes + eMSC = 78.98 ± 13.33 ng/mL. Bar graphs represent the mean ± SEM for $n = 6$–9 replicates of 3 independent experiments. $^*P < 0.05$ MSC or eMSC treated chondrocytes vs their respective untreated controls. B) IL-8 secretion of IL-1 and IL-1/TNF stimulated chondrocytes doses with exogenous PGE_2. Bar graphs represent the mean ± SEM. $^*P < 0.05$ PGE2 treated chondrocytes vs pro-inflammatory chondrocytes. C) Peripheral blood-derived macrophage response to OA stimuli). LPS (data not shown) was utilized as a positive control for inflammatory responses. $^*P < 0.05$ basal macrophages (control) vs IL-1 or IL-1/TNF treated macrophages.

properties of MSC and eMSC in co-culture with macrophages [11,19,33,50–52]. Therefore, PGE_2 secretion was quantified in stimulated chondrocyte co-culture. MSC and eMSC both secrete high levels of PGE2 when stimulated with either IL-1 or IL-1/TNF in the presence of chondrocytes (Fig. 3A).

3.4. PGE_2 dose response

Recent studies have identified PGE_2 as a key mediator of the anti-inflammatory effect of MSC in osteoarthritic chondrocytes [11]. However, the role of PGE_2 in OA is not entirely understood as it has been shown to have both catabolic and anabolic effects [53]. Therefore, after demonstrating that MSC and eMSC produce PGE_2, we tested the effect of exogenous PGE_2 (Fig. 3B). In both, IL-1 and IL-1/TNF stimulated chondrocytes, PGE_2 did not attenuate IL-8 levels and in most conditions, it promoted IL-8 secretion. These results reflect the same observed trend with MSC or eMSC treatment of acutely stimulated chondrocytes (Fig. 3A).

3.5. *Characterization of stimulated macrophages*

Thus far, our results indicate that MSC treatment of IL-1 and IL-1/TNF stimulated chondrocytes does not attenuate inflammation. Yet, several *in vivo* studies have reported a decrease in pro-inflammatory cytokine secretion after IA injection of MSC into the synovium of inflamed joints [17]. Synovial macrophage inflammatory activity has been characterized as a key driver of chronic inflammation in OA [25]. We therefore thought that our observations could have resulted from the high PGE2 secretion levels by MSCs as only low PGE2 concentrations have decreased pro-inflammatory gene expression [54].

Our prior studies have shown that MSC effectively attenuate macrophage inflammation and promote macrophage phenotype transition from a pro-inflammatory (M1) to an anti-inflammatory (M2) macrophage and this was mediated by MSC secreted PGE2 binding to activated macrophage receptors [19]. We hypothesized that in our OA model, MSC immunomodulatory function may require the presence of macrophages

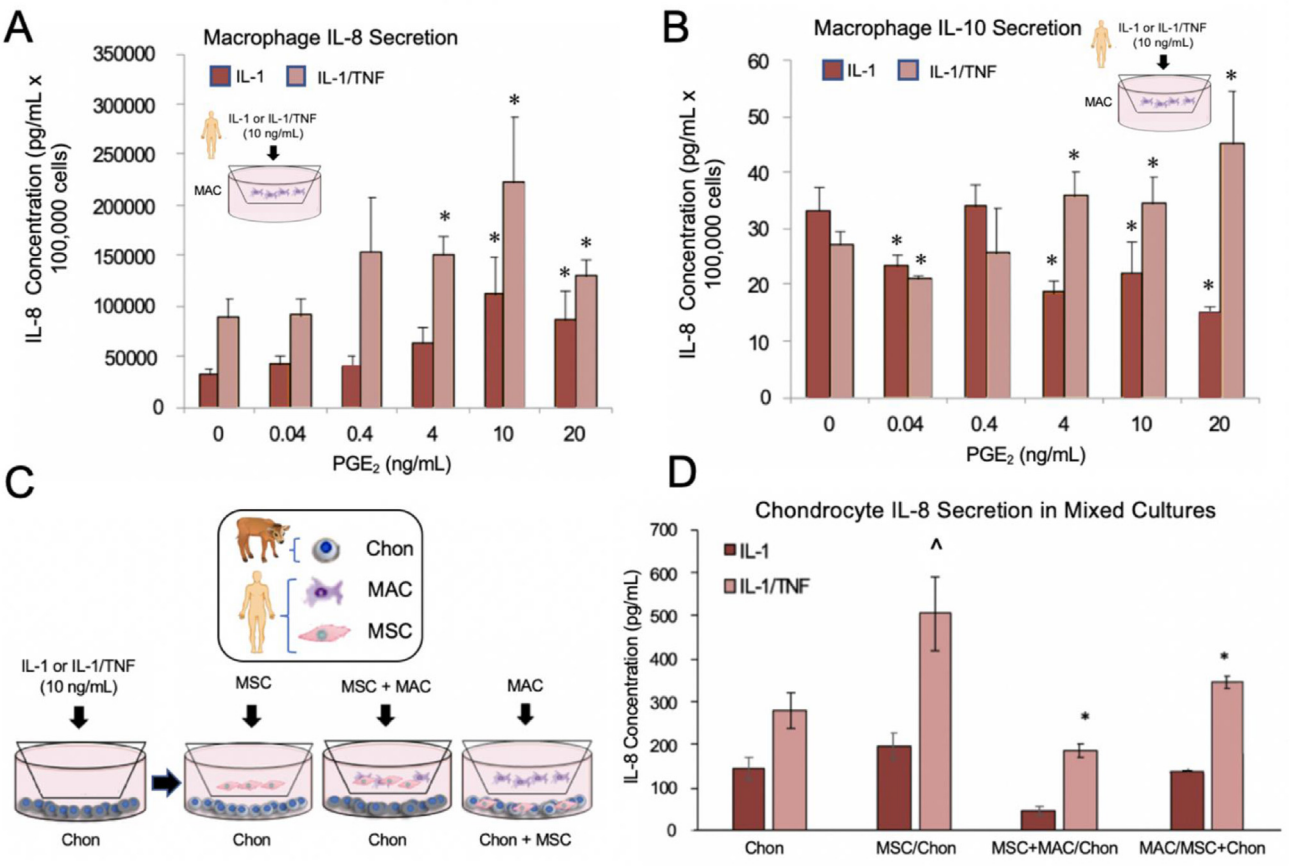

Fig. 4. Stimulated macrophages or mixed cultures. A,B) Stimulated macrophage response to PGE_2. IL-1 or IL-1/TNF stimulated macrophages were treated with different PGE_2 concentrations 0–20 ng/mL and their A) IL-8 and B) IL-10 secretion were quantified. *$P < 0.05$ pro-inflammatory macrophages (either IL-1 or IL-1/TNF) without exogenous PGE2 treatment vs PGE2 treated pro-inflammatory macrophages (either IL-1 or IL-1/TNF). C) Mixed culture model depicted including IL-1 or IL-1/TNF stimulated chondrocytes, macrophages and MSC. D) The abscissa corresponds to Fig. 4C where the slash (/) represents the porous filter. The notation of the graph represents the convention, cell(s) on upper well/cell(s) on lower well. Bar graphs represent the mean ± SEM of $n = 6$–9 of 3 independent experiments. ^$P < 0.05$ IL-1 or IL-1/TNF co-cultures relative to monoculture chondrocytes. *$P < 0.05$ IL-1 or IL-1/TNF activated mixed cultures containing macrophages relative to activated cultures without macrophages.

and not chondrocytes alone. In order to replicate the OA inflammatory environment, IL-1/TNF was used to induce pro-inflammatory M1 macrophages. We first characterized the effect of IL-1 and IL-1/TNF stimulation on M1 macrophages by measuring human IL-8 secretion after 48 h. Macrophages produced high levels of IL-8 when stimulated with IL-1/TNF, but not with IL-1 alone (Fig. 3C).

As mentioned above, PGE2 immunomodulation is characterized by a dose-dependent decrease in M1 markers and an increase in M2 markers including IL-10 secretion [19,33,52,55,56]. Therefore, the effects of PGE2 on activated macrophages were explored with exogenous PGE2. Macrophages responded to increasing concentrations of PGE_2 by secreting more IL-8 (Fig. 4A). IL-8 levels increased until 20 ng/mL PGE2 was added, at which point IL-8 levels approached baseline. In addition to measuring IL-8, levels of chondroprotective [57] IL-10 were measured (Fig. 4B). Interestingly, higher PGE2 concentrations decreased IL-10 secretion from IL-1 stimulated macrophages, but the opposite trend was observed with IL-1/TNF stimulated macrophages. These results highlight the complexity of macrophage phenotype regulation which could result in intermediate phenotypes with both pro- and anti-inflammatory characteristics both *in vitro* and *in vivo* [58,59]. It is important to note that our concentrations were selected based upon the fact that we have measured between 20 and 40 ng/mL PGE2 in the MSC secretome [18,19,33,50,52]. Nevertheless, our results indicate that, unlike chondrocytes, TNF/IL-1 activated macrophages were responsive to PGE2 levels known to be secreted by MSC.

3.6. Mixed cultures

After characterizing the individual macrophage and chondrocytes responses to OA stimuli and to PGE2, we proceeded to test cell interactions between chondrocytes, macrophages, and MSC after stimulation. Bovine IL-8 secretion was measured to tease out the chondrocyte inflammatory response. Remarkably, when macrophages were introduced into the culture, IL-8 secretion was significantly down-regulated for IL-1/TNF stimulated chondrocytes and the IL-1 stimulated chondrocytes followed a similar trend. Fig. 4C and D describes these results using two different transwell culture configurations, where MSC were in contact either with chondrocytes or with macrophages. The results indicated that in both configurations IL-8 was reduced in the presence of macrophages. These results suggest that the targets for MSC immunomodulatory action may require the presence of attenuated macrophages in order to also attenuate chondrocyte inflammation. These results are in agreement with studies showing macrophage reprograming from a pro-inflammatory to an anti-inflammatory state is highlighted as an effective treatment option for OA [60], and is consistent with our previous studies where local anesthetic treatment attenuated M1 macrophage TNF-α, which was sustained in co-culture with MSCs, but MSCs did not further enhance this anti-inflammatory effect [61]. However, we have not fully characterized changes in M1 (i.e. TNF) or M2 (i.e. IL-10) macrophage properties in our mixed culture experiments, which limits our mechanistic interpretation of these effects. Future studies may

compare human with bovine responses, which would also allow us to expand our inflammatory reagent panel for more comprehensive secretome studies.

Nevertheless, our results emphasize the importance of developing more comprehensive *in vitro* systems and suggest that unlike DEX, MSC may be used as an OA therapy, only when synovial cells are highly inflamed. In addition, we recognize that our model does not investigate mechanical injury components of OA, but these can also be incorporated into future studies as we have previously done for axonal injury [62,63]. Future studies can also incorporate chronically inflamed primary chondrocytes as well as synovial macrophages, to further investigate MSC or other therapies and our results can be compared directly with explant and/or other 3D culture systems.

4. Conclusions

We established a relatively rapid and inexpensive monolayer model and evaluated its efficacy in altering inflammatory, regenerative and degenerative gene expression. We compared DEX and MSC treatments on secreted mediators and gene expression in mono- and co-culture configurations and determined that macrophages, not chondrocytes, were likely the targets of anti-inflammatory MSC treatment. Using this relatively simple culture model, the current studies provide new insights to the cross-talk that may occur between chondrocytes, macrophages and therapeutic-delivery of MSCs *in situ*. It is important to note that while gene expression screening provides an important tool to compare an array of cellular changes, protein quantification is ultimately needed for a more mechanistic understanding of OA.

These data highlight the importance of considering multi-cellular interactions when studying complex systems. In addition, while our strategy included the inclusion of M1 macrophages to mimic the native inflammatory OA environment, the role of M2 macrophages, as well as the conversion of M1 to M2 following treatment can be explored in our system as well, using a combination of human ELISA and gene expression changes. In the future, our model can also be easily expanded to include additional synovial cells types and/or ECM proteins.

Author contributions

Conceptualization, IMB, RSS, MLY.
Formal Analysis, IMB.
Data Curation, IMB, SES, RH, CPR, AT.
Writing, IMB, RSS.
Review and editing, SES, CPR, CTH, RSS.
Supervision, RSS, MLY.
Project administration, MLY.
Funding acquisition, MLY, CTH.

Role of the funding source

The study sponsors were not involved in the study design, or in collection, analysis or interpretation of data. The sponsors were also not involved in the writing of the manuscript or in the decision to submit the manuscript for publication.

Declaration of competing interest

The authors declare no competing conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2023.100432.

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