DEVELOPMENT OF IN VITRO OSTEOARTHRITIS MODELS TO STUDY THE EFFECTS OF MESENCHYMAL STROMAL CELL TREATMENTS

by

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ABSTRACT OF THE DISSERTATION

Development of *In Vitro* Osteoarthritis Models to Study the Effects of Mesenchymal Stromal Cell Treatments

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Osteoarthritis (OA), the principal source of physical disability and impaired quality of life in the US, is a chronic age-related disease characterized by the progressive destruction of articular cartilage, leading to total joint deterioration. OA severely burdens the US healthcare system with overall cost of ~185 billion dollars a year. Recent evidence suggests that inflammatory cytokine and chemokine release signals and cellular infiltration ultimately lead to matrix degradation and cartilage destruction. There is currently no cure for OA. Existing treatments alleviate symptoms initially; however, they are not able to alter disease progression and disease development eventually proceeds. Therefore, there is a need to develop effective therapies that could alter OA progression and promote healing in osteoarthritic joints.

One approach to alter the progression of OA has been intra-articular administration of mesenchymal stromal cells (MSC) which secrete anti-inflammatory and regenerative factors that could alter the underlying pathophysiology of OA. However, these cells are not long-lasting when freely administered. We have previously demonstrated that alginate encapsulation of MSC

lengthens their survival and promotes their secretory function, a characteristic that could serve as long term treatment for OA. In this dissertation, we investigated whether treatment with MSC or alginate-encapsulated MSC can provide sustained reduction of OA mediated joint inflammation and destruction, and promote healing in an *in vitro* model of OA.

In addition, we aimed to improve on current OA *in vitro* models which often rely on chemically or mechanically stimulated chondrocytes, the sole cell component of articular cartilage, without taking into consideration other cell types and their interactions in the articular joint. We developed a multi-culture stackable insert system that allows for the 3D co-culture and investigation of multiple cell types, cell-cell interactions, and cell responses to their environment. Such experiments could provide powerful new tools and therapies in an otherwise irreversible progressive disease.

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CHAPTER 1: INTRODUCTION

1.1 Osteoarthritis

Osteoarthritis (OA), sometimes referred as degenerative joint disease or degenerative arthritis, is characterized by the slow progressive degeneration of articular cartilage, associated with hypertrophy of the bone and thickening of the capsule [1] often as a result of an imbalance in matrix degradation and synthesis [2, 3]. Disease progression involves the remodeling of all joint tissues (bone, synovium, ligaments) with subsequent joint space narrowing [4]. With an increasing prevalence and incidence due to the growth in aging population and the obesity epidemic, OA is the principal cause of mobility-related disability in the US [5]. By the year 2030 over 67 million Americas will suffer from some form of OA [6, 7]. OA has been implicated in diminishing the quality of life of patients, increasing mortality [8, 9], and comorbidities such as depression and anxiety[10], making it a considerable burden to both the patient and society. OA is the most common chronic condition of joints resulting in impaired quality of life and leads to increased healthcare and aggregate costs amounting to approximately $185 billion per year [2, 11]. Common risk factors include aging, obesity, previous joint injury, joint malalignment, and genetic predisposition; however, the pathogenesis of OA is still largely unknown [2, 12]**.**
[2, 3].

1.2 Pathophysiology of Osteoarthritis

1.2.1 The role of chondrocytes

Chondrocytes, the single cellular component of articular hyaline cartilage, are considered to be terminally differentiated cells that maintain a dynamic equilibrium between synthesis and degradation of extracellular matrix (ECM) components [2, 3]. The cartilage ECM is comprised mostly by type II collagen and the proteoglycan aggrecan which interact with other cartilage-

specific collagens (types IX and XI), small proteoglycans and other proteins [3]. In early OA, chondrocytes become aberrant and start proliferating (clonal expansion) and secreting catabolic cytokines and matrix-degrading enzymes (Table 1.1) [12]. These processes, in conjunction with a downregulation of anabolic signaling, lead to a disruption of matrix equilibrium which results in the progressive loss of cartilage tissue [1, 13]. A population of aberrant chondrocytes might undergo apoptosis, resulting in diminished local cell number and cartilage defects. As a result of cartilage loss, pathological remodeling of subchondral bone gives rise to sclerosis and osteophyte formation [2, 3]. These processes eventually lead to reduction of joint space due to cartilage degradation which causes bone to bone contact resulting in pain and disability for the patient (Figure 1.1).

Table 1.1. Important mediators of OA [2]

Inflammatory mediators	Proteases
TNF	MMP-1, 3, 9, 13
IL- 1β, 6, 8 15, 17, 21	ADAMTS- 4, 5
PGE_2 (conflicting data)	TACE
Substance P	
NGF	
EGF	
VEGF	
FGF-2	

1.2.2 The role of synoviocytes

Synoviocytes, the resident cells of the synovial membrane (synovium) consist of two distinct cell types: synovial fibroblasts and macrophages which are present in the synovium at percentage of 93% and 7%, respectively [2, 14]. The synoviocytes are a major source of synovial fluid components which contribute to the functional properties of articular surfaces and modulate chondrocyte activity, such as hyaluronic acid (HA) and lubricin [15]. It is widely accepted that synovial inflammation and the production of pro-inflammatory and catabolic mediators from the

OA synovium are important for the symptomatic progression of OA [14]. During OA progression, the synovial membrane becomes a source of pro-inflammatory and catabolic cytokines and proteinases, which contribute to ECM degradation (Figure 1.1) [15]. Among the synovial cells, macrophages are mostly responsible for maintaining synovial inflammation by inducing the production of pro-inflammatory cytokines and proteases such as IL-6, IL-8, MCP-1, ADAMTS4 and MMPs by synovial fibroblasts and generally perpetuating inflammation in OA joints by secreting high levels of IL-1 and TNF-α [14, 16]. Pathophysiological changes in synoviocytes facilitate angiogenesis and innervation in the synovium, which may result in pain responses.

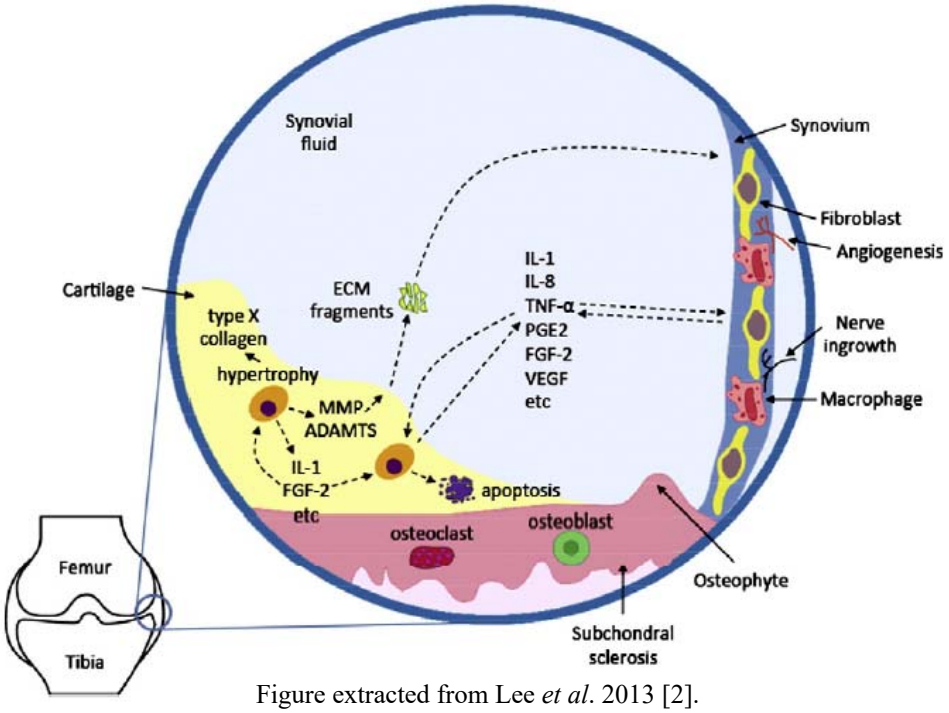

Figure extracted from Lee *et al*. 2013 [2].

Figure 1.1. Molecular aspects of knee OA [2]. OA is a disease that involves the "whole joint". Aberrantly activated chondrocytes will start proliferating and secreting pro-inflammatory cytokines and proteinases that cleave the main cartilage building blocks (collagen and proteoglycans). Cytokines and ECM fragments will travel through the synovial fluid and reach the synovial membrane where the synoviocytes will become activated. Synoviocytes, especially synovial macrophages, will start secreting more inflammatory and catabolic signals and perpetuate the inflammatory state in the joint. After undergoing clonal expansion, the chondrocytes will go through apoptosis leaving defects in the cartilage. These processes induce remodeling in the subchondral bone and lead to osteophyte formation.

1.3 Standard of care

Currently, while there is no cure for OA, symptoms can be managed with lifestyle changes, physical therapy, medications and surgery. Common non-surgical treatments consist of physical and occupational therapy, nonsteroidal anti-inflammatory drugs, intra-articular injections of corticosteroids, and hyaluronic acid. However, these interventions reduce symptoms initially but are not disease-modulating and OA progression continues. Eventually, the patient might need surgical intervention which may include arthroscopy, joint resurfacing, osteotomy, synovectomy, arthrodesis (joint fusion), and total joint arthroplasty. Nonsurgical and surgical treatments are outlined in Table 1.2.

Table 1.2. OA standard of care *

Nonsurgical treatment	**Consists of**
Physical and occupational therapy	Balanced fitness program, modification in work, or sport activities. Strengthening muscles around OA-affected joints helps ease the burden on those joints and reduce pain.
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Commonly used drugs to ease inflammation and pain. NSAIDs include aspirin, ibuprofen, and naproxen.
Intra-articular steroid injection	Corticosteroids are powerful anti-inflammatory medicines. Limited to 3-4 procedures per year due to adverse side effects (i.e. chondrolysis)
Hyaluronic acid (HA) injections	HA a major component of synovial fluid acts as a shock absorber and lubricant. HA injections alleviate symptoms of synovial fluid thinning (i.e. Synvisc, Euflexxa, Orthovisc)
Surgical treatment	**Consists of**
Arthroscopy	Fix tears in soft tissue around the knee, repair damaged cartilage, remove bone spurs, cyst, and loose fragments in the joint
Osteotomy	Cutting and removing bone or adding a bone wedge near a damaged joint. Realignment of bones to shift weight from a damaged area to an undamaged area by OA.
Joint fusion	Eliminates joint by fastening together the ends of bone. Procedure performed on ankles, wrists, thumbs, fingers or spine.
Knee arthroplasty (total knee replacement)	Removes part of the bone and creates artificial joint with metal or plastic components

* Information obtained from the Arthritis Foundation webpage (http://www.arthritis.org/about-arthritis/types/osteoarthritis/treatment.php).

Existing treatments for OA provide, at best, symptomatic relief for pain and fail to prevent cartilage damage and subsequent destruction of other joint tissues [17]. None of the current treatment options replace damaged cartilage or delay the progression of OA.

1.4 Limitations in assessing drug efficacy

The development of disease modifying therapies that can stop the progression of OA or even cure it remains elusive, in part due to the fact that the field is still limited by the lack appropriate preclinical models that mimic clinical phenotypes of human disease such as *in vitro* models that recapitulate the physiology and native tri-dimensional (3D) architecture of articular joint tissues [18]. Drug failures in clinical trials are generally due to the poor predictive power of existing preclinical models [19] where major differences in drug efficacy exist between 2D and 3D substrates [20]. For example, many drugs exhibiting cytotoxic behavior in cancer cells *in vitro* fail to show efficacy *in vivo,* and in many cases, this is due to the poorly understood chemo-resistant effects conferred by cell-cell and cell-ECM interactions in the cancer microenvironment [20]. These same challenges apply to OA research as several disease modifying drugs have failed during the clinical translation period (see section 1.5 Disease Modifying Drugs).

Several *in vivo* models have been developed to study the complex mechanisms involved in joint homeostasis and disease [21]. The main benefit of animal models is that they allow for the study of the disease in the context of the whole joint. However, many successful therapeutic treatments in animal studies have failed in human clinical trials due to species-specific differences [22]. In addition, the push to reduce animal experimentation due to ethical concerns has increased the need for the development of advanced *in vitro* models that can more accurately represent stages of human disease [22].

Given the complexity of OA progression and the interactive multi-cellular responses, an optimal OA model should combine cartilage, synovium, and other tissues to form an interactive physiological system which fully captures the biological complexity and mechanical functions of the articular joint [23]. OA *in vitro* models that evaluate the responses of synoviocytes, chondrocytes and their interactions in co-culture have been established in monolayer, with tissue explant cultures, and engineered cartilage constructs [14, 17, 24-33].

1.5 Disease modifying therapies (DMOADs)

The development of joint structure-modifying treatments for OA has been extremely challenging as evidenced by several late-stage clinical programs resulting in early termination due to adverse effects or not meeting the minimum significant changes to continue. In addition, recognition that OA is a heterogeneous disease that manifests with different pathophysiology in patients has brought the understanding that a "one-size-fits-all' approach will not be appropriate to treat OA. Here we describe several DMOADs that have been tested for the treatment of OA (extracted from Karsdal etl al. 2016 [34]):

1. *Matrix metalloproteinase (MMPs) inhibitors* – selective inhibition of MMPs could stop the degradation of articular cartilage. However tested inhibitors with published result have shown systemic adverse reactions or no efficacy at all. Other clinical prospects to selectively inhibit MMP-13 are under clinical review.
2. *Bisphosphonates* - are anti-resorptive drugs that could slow down the progression of OA and protect the bone and cartilage. Risedronate, an oral bisphosphonate, showed promising results in a phase II clinical trial; however, it failed during phase III. It is still unclear if these drugs can slow the progression of OA.
3. *Calcitonin* – a commonly used treatment for osteoporosis due to its anti-resorptive action. As an oral formulation, it has been shown to inhibit bone and cartilage degradation.

Nevertheless, calcitonin treatment did not decrease joint narrowing or provide symptomatic relief in 2 phase III clinical trials.

4. *Aggrecanase (ADAMTS-) inhibitors* – as their name implies, aggrecanases degrade aggrecan, a major component of the ECM in articular cartilage. Two main aggrecanases' inhibitors for ADAMTS-4 and ADMTS-5 have been tested. The ADAMTS-4 inhibitor is a small molecule named AGG-523 which was tested in phase I clinical trials; however, no results have been shared. ADAMTS-5 antibodies have been developed and are currently being tested.
5. *Nitric oxide synthase (iNOS) inhibitors*- cindunistat hydrochloride maleate an iNOS inhibitor was tested in clinical trials in patients with symptomatic knee OA; however, no positive effects where observed.
6. *Stronium* – anti-resorptive drug that has been used to treat osteoporosis. It has shown cartilage protection from degradation and beneficial effects decreasing joint narrowing. However, the level of evidence is still low and further studies should be conducted.
7. *Cathepsin K inhibitors* – blocks the osteolytic effects of this protease. Several inhibitors are being studied due to slowing the progression of OA in models of chronic and posttraumatic OA. Currently being investigated in a phase I trial.
8. *Estrogen* – replacement therapy in post-menopausal women could decrease cartilage turnover. Selective estrogen-receptor modulators (SERM), levormeloxifene and raloxifene both inhibited bone and cartilage degradation.
9. *Parathyroid hormone (PTH)* – teriparatide is the only FDA/EMA approved bone anabolic treatment, which has shown effects in cartilage homeostasis. It has been reported that it can inhibit chondrocyte hypertrophy and cartilage degradation. There are no current OA clinical studies for this drug.

10. *Sprifermin (rhFGF18)* –fibroblast growth factor 18 protein has been engineered to target chondrocytes intraarticularly. FGF18 promotes chondrocyte proliferation and cartilage ECM production. This drug is currently in phase II clinical trials.
11. *Anti-inflammatory agents (IL-1, -6, TNF)* – receptor antibodies and antagonist have been tested to decrease the cartilage destruction promoted by pro-inflammatory cytokines. For example, adalimumab a TNF antagonist that is commonly used to treat rheumatoid arthritis did not provide any benefit to OA patients. However, several drugs targeting other ytokines are in clinical development.
12. *TPX-100* – peptide under clinical investigation that promotes cartilage and bone regeneration.

1.6 Mesenchymal stromal cells treatment

Mesenchymal stromal cells (MSCs) are a heterogeneous population of adult stem-like cells commonly isolated from various tissues including bone marrow, adipose tissue, placenta, umbilical cord blood and even the synovial membrane [35]. MSCs are clonal, plastic adherent cells which possess tri-lineage differentiation capacity into osteoblastic, adipogenic and chondrogenic cell lines [36]. Initially, the interest in MSCs as a cellular therapeutic focused on their multipotent tissue replacement potential [35]. However, a shift in the paradigm resulted following *in vivo* studies demonstrating therapeutic effects of MSCs with little to no engraftment in host tissues. Therefore, MSCs therapeutic effects are now attributed to other functions [17]. Studies have shown that they also have tissue-regenerative properties, mainly via secretion of bioactive factors that exert potent immunomodulatory, proangiogenic, anti-apoptotic, anti-fibrotic, neuroprotective, and anti-inflammatory effects [17, 35, 37-40]. In osteoarticular diseases, preclinical studies in animal models have also suggested that MSCs may be used in the development of innovative applications for the treatment OA [17]. In particular, it has been shown that intra-articular injections of bone marrow -derived MSCs might be effective in

preventing the progress of OA in animal models (Table 1.3). However, the mode and target of MSC disease modulation is unclear.

Table 1.3. Pre-clinical *in vivo* studies utilizing intra-articular injection of MSCs for OA treatment [21].

Study	Species	Type of Lesion	Treatment	Study Period	Results
Murphy et al., 2003	Goat	OA induced via cranial cruciate ligament and medial meniscus	1X10^7 allogeneic BM-MSC* and HA	5 months	Regeneration of the meniscus which helped retard the progression of the OA. **No engraftment in articular cartilage.**
Sato et al., 2007	Guinea pig	Spontaneous OA of stifle	7X10^6 human BM-MSC with HA	5 weeks	Partial cartilage repair in the experimental group, but not in the other groups. **By week 5 MSCs could not be detected.**
Mcllwraith et al., 2011	Horse	Osteochondral defect by medial femorotibial joint followed by microfrature	2X10^7 autologous BM-MSC	12 months	**No significant clinical improvement in the joints.** BM-MSC-treated joints showed an increase in the firmness of the repair tissue.
Van Buul et al., 2012	Wistar Rat	Monoiodoacetate (MIA) injection	1x10^6 rat or human MSCs	4 weeks	Significant increase in weight bearing in affected paw compared to controls. **No significant histological changes of cartilage**.
ter Huurne et al., 2012	Mouse C57BL/6	Collagenase-induced OA of stifle	2X10^6 allogeneic AD-MSC+	6 weeks	Cartilage destruction was retarded. At 5 days after injection, **no AD-MSC could be detected.**
Pei et al., 2013	Miniature pig	Partial thickness cartilage defects on medial femoral condyle	Allogeneic S-MSC#	3 months	Tissue positive for GAG and collagen II. However, **no engraftment of injected S-MSC detected in repair tissue.**
Desando et al., 2013	Rabbit	Mild OA via transection of cranial cruciate ligament	2x10^6 or 6X10^6 autologous AD-MSC	6 months	AD-MSC retarded progression of OA. **No engraftment of AD-MSC into endogenous cartilage.**
Javanmard et al., 2015	Sprague-Dawley rats	Monoiodoacetate (MIA) injection	2.5x10^5 rat MSCs or MSCs conditioned media (CM)	2 weeks	**Better histological outcome** in knees treated with MSCs and MSCs CM.

*BM-MSCS= bone marrow-derived MSCs, +AD-MSC= adipose tissue-derived MSC, #S-MSC= synovium- derived MSC

Key observations from these studies include: 1) MSCs do not engraft into cartilage defects; however they can be detected in the synovial membrane, medial meniscus, fat pad, and lateral meniscus, 2) MSC treated joints are mechanically stronger with greater levels of aggrecan, 3) MSCs may retard the progression of OA lesions in the short term, 4) MSC injection showed chondrogenic benefits by retarding the progression of cartilage destruction and reducing inflammation, 5) MSC reduce pain related behaviors and, 6) nevertheless, despite treatment potential, millions of cells are required for short term efficacy whereas long term efficacy is generally absent due to lack of MSC engraftment. Therefore, if they are to be effective, new strategies to deliver and localize cell therapies are needed.

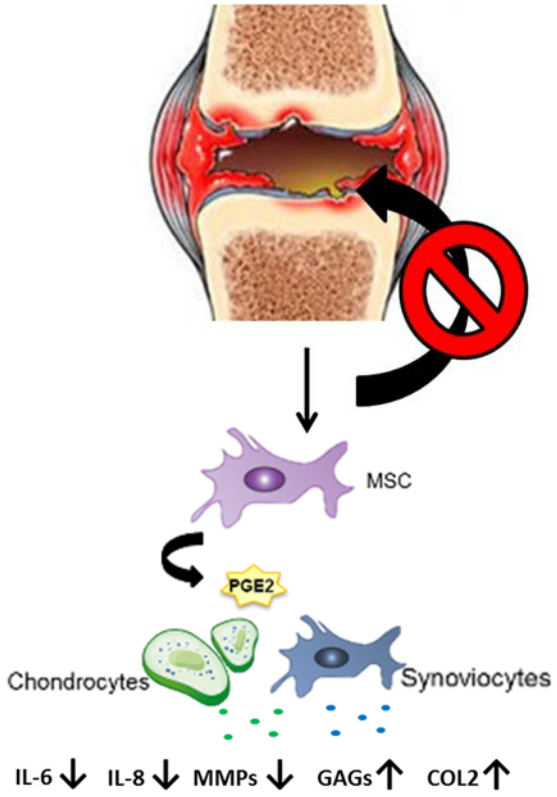

Figure 1.2. Intra-articular injection of MSCs. According to pre-clinical studies intra-articular injection of MSC does not result in cell engraftment. MSC injected into the synovial capsule will not engraft into cartilage defects and differentiate into chondrocytes. However, transient beneficial effects have been observed in multiple studies where MSC secreted factors promote ECM production and attenuate inflammatory markers. Several weeks post-injection MSC cannot be detected in the joint space.

Currently, there are 120 registered clinical trials (clinicaltrials.gov) to evaluate the safety and efficacy of MSC from different sources as a therapy for OA. From these studies, 41 have been completed (only 3 have reported results), 31 are actively recruiting, 16 are not yet recruiting, 14 have an unknown status, 13 are active but not recruiting, and 5 have been withdrawn/terminated. These numbers evidence the popularity and high demand for treatments that could potentially modify the pathophysiology of OA. Generally, studies have shown positive outcomes with improved knee pain and self-reported function. In addition, adverse effects due to intra-articular injection of cell therapies have been rare making it an apparent safe procedure [41]. Nevertheless, poor study design, high risk of bias, large heterogeneity, and low confidence in the reported methods have raised questions about the actual efficacy of the therapy [42]. A recent meta-analysis using the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) approach to evaluate the effectiveness of MSC treatment for knee OA determined that the quality of evidence in these studies was very low to low [43].

As observed in several pre-clinical studies, MSC are short-lived after injection in the body[44]. Due to the transient effects of freely migrating MSC intraarticular injection, an optimal strategy should include delivery and localization of MSC without compromising their chondrogenic and anti-inflammatory function. We have previously demonstrated enhanced MSC anti-inflammatory and regenerative function after alginate encapsulation [45]. Compared with freely migrating MSC, the eMSC secretome is characterized generally, by higher cytokine concentrations, including anti-inflammatory IL-10 and prostaglandin E2 (PGE_2) [40, 45]. We have also shown that eMSC pre-conditioned with inflammatory IL-1β and LPS significantly promote macrophage secretion of anti-inflammatory IL-10 and reduced secretion of inflammatory TNF-α *in vitro* (FIG 1.3) [35, 38] and that eMSCs promote tissue protection and the attenuation of inflammation following injury in a rat organotypic hippocampal slice culture [39, 40].

Furthermore, alginate encapsulation offers a delivery vehicle for MSCs in a rat spinal cord contusion model leading to attenuation of macrophage activation with drastically smaller cell numbers than previously used [45-47]. An important point to note is that compared with soluble anti-inflammatory drugs such as dexamethasone which rapidly dissipate over time, MSC represent an "active" therapy in that they respond to changes in the microenvironment and adjust their secretome accordingly.

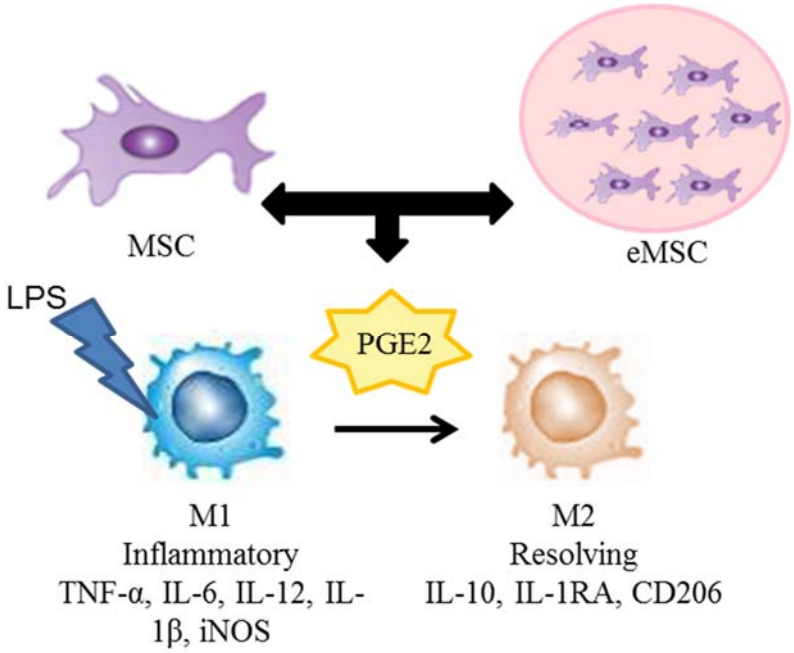

Figure 1.3. MSC are immunomodulatory. MSC and eMSC secrete a plethora of cytokines and growth factors with anti-inflammatory and regenerative functions. Prostaglandin E2 (PGE_2) has been identified as a major factor in reprograming pro-inflammatory (M1) macrophages to anti-inflammatory (M2) macrophages in several disease models.

To extend and promote the effect of MSC in the joint, this dissertation will explore the use of alginate- encapsulated human MSC (eMSCs) to reduce joint inflammation and destruction characterized by OA and promote re-growth and healing. We will explore MSC and eMSC OA resolving function using co-cultures of inflamed target cells, including chondrocytes and macrophages and compare the MSC therapeutic effects with dexamethasone, a widely used glucocorticoid as part of the standard of care for OA. In addition, to study the interactions

between multiple MSC target cells more precisely, we set to develop a 3D stackable *in vitro* system that would allow for the study of cellular physiology and degenerative joint diseases such as OA. This model will serve as a more clinically relevant *in vitro* model as we move forward in developing a translational therapy.

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CHAPTER 2: IN VITRO INFLAMMATORY MODEL OF POSTTRAUMATIC OSTEOARTHRITIS: PLATFORM TO TEST DISEASE MODIFYING TREATMENTS

Note: This chapter is reproduced from the following manuscript written by **Ileana Marrero - Berrios**:

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2.1 Introduction

An estimated 1 in 3 adults between the ages of 18-64 years old exhibit symptoms of arthritis. Osteoarthritis (OA) or degenerative joint disease is the most common form of arthritis with an estimated 30.8 million adults suffering from this disease in the US [1]. OA is characterized by joint pain and progressive degeneration of articular cartilage due to an imbalance in matrix degradation and synthesis [2, 3]. OA has been implicated in diminishing the quality of life of patients, increasing mortality [4, 5], and comorbidities such as depression and anxiety[6], making it a considerable burden to both the patient and society. Common risk factors include aging, obesity, joint malalignment, and genetic predisposition; however, the pathogenesis of OA is still largely unknown [3, 7]**.**

Another risk factor that considerably increases the rate of OA is joint trauma. For example, patients who suffer a knee injury are 4.2 times more likely to develop OA compared with uninjured persons. Osteoarthritis that develops after joint injury is deemed posttraumatic OA (PTOA) [8]. The initial injury leads to disruption of the ECM in cartilage and fast

chondrocyte death in the area of impact. This disruption triggers further chondrocyte death, decrease in ECM production, promotion of catabolism resulting in matrix degradation, changes in the subchondral bone, and exacerbated inflammation [9]. Pro-inflammatory cytokines such as interleukin (IL-) -1, -8, -6, tumor necrosis factor – α (TNF-α), interferon-γ and others are elevated soon after joint injury [10, 11]. Diffusion of these cytokines and wear particles from the cartilage into the synovial fluid contribute to the development of synovitis and further cartilage degeneration [9]. Eventually the persistent imbalance in the joint leads to OA development.

Existing treatments for OA provide, at best, symptomatic relief for pain and fail to prevent cartilage damage and subsequent destruction of other joint tissues [12]. Since PTOA develops after an evident joint injury, it provides the opportunity to perform early therapeutic interventions. Intra-articular injection of dexamethasone, a synthetic glucocorticoid with pleiotropic anti-inflammatory and chondroprotective properties, has been shown to inhibit inflammation and cartilage damage in PTOA models [9, 13]. However, there are still major safety concerns with the continuous use of steroid injections due to negative effects such as chondrocyte apoptosis [14, 15]. One approach to alter the progression of OA has been intra-articular administration of mesenchymal stromal cells (MSC) [12, 16, 17] which secrete anti-inflammatory and regenerative factors that could alter the underlying pathophysiology of OA. However, 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) lengthens their survival and promotes their secretory function [18], a characteristic that could serve as long term treatment for OA.

To develop effective therapies, comprehensive *in vitro* systems that recapitulate the joint environment are needed. However, most OA-based *in vitr*o systems consist of chondrocytes, the sole cell component of cartilage, in different culture configurations while ignoring other cell components, such as synoviocytes, and the effects of cell-cell interactions. This is a critical

omission, as we demonstrate in our studies, because synovial macrophages are important in perpetuating OA progression [19]. Furthermore, current studies are generally limited to the anti-inflammatory MSC effects and fail to integrate potential chondrogenic MSC function within an inflammatory environment. Using a monolayer cell culture model, our studies were designed to treat chondrocytes and macrophages stimulated with OA promoting factors *in vitro* with eMSCs and free MSCs, or with the soluble anti-inflammatory drug, dexamethasone, and determine whether the production of inflammatory markers, chondrogenic potential, and extracellular matrix remodeling is affected.

2.2 Materials and Methods

2.2.1 Cell Culture and Maintenance

All cells were maintained in a humidified 37°C incubator with 5% CO_2 and cultured in their respective media until used for experiments.

2.2.2 Bovine chondrocyte culture

Primary bovine chondrocytes at passage 0-1, kindly donated by Dr. Clark T. Hung (Columbia University), were expanded as a monolayer culture in a humidified 37°C, 5% CO_2 incubator using high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin and 100mg/mL streptomycin (1%v/v) (P/S), 2mM L-glutamine, 50μg/mL L-ascorbic acid (Sigma), 40μg/mL L-proline (Sigma), 100 μg/mL sodium pyruvate (Gibco), 1× ITS + premix (insulin, human transferrin, and selenous acid) (Corning), 1 ng/mL transforming growth factor (TGF)-β1 (Preprotech) and 5 ng/mL fibroblast growth factor (FGF)- 2 (Gibco)[20, 21]. Confluent chondrocytes were collected using trypsin-EDTA (TE 0.25%) (Gibco) and plated at passage 1-2 for experiments.

2.2.3 Mesenchymal stromal cell culture

Human bone marrow-derived mesenchymal stromal cells purchased from the Institute of Regenerative Medicine (Texas A&M) were thawed at passage 2 and plated as a monolayer culture at 1,714 cells/cm^2 in a humidified 37°C, 5% CO_2 incubator. Cells were cultured in Minimum Essential Medium - α (MEM-α) (Gibco) containing no deoxy- or ribonucleosides, and supplemented with 10% FBS (Premium Grade, Atlanta Biologicals), 2mM L-glutamine, 1ng/mL FGF-2, 1% P/S. The cells were grown to 70% confluence, trypsinized and re-plated at 1,714 cells/cm^2 in T-225 flasks until confluency and used for MSC experimental setups at passage 4-5 [22].

2.2.4 Monocyte isolation and differentiation into macrophages

Human macrophages were obtained following protocols previously described by Gray et al [22]. Briefly, human peripheral blood (New York Blood Center) was fractionated utilizing density gradient centrifugation (Ficoll-Paque Premium, 1.077 g/mL, GE Healthcare). The buffy coat was gently collected and washed twice with 1X PBS and the mononuclear cells were isolated by enriching the $CD14^+$ cell population, using magnetic bead cell sorting according to manufacturer's instructions (Miltenyi Biotech). Monocytes were seeded at $1X10^7$ cells/T-175 cm^2 flasks in Advanced RPMI supplemented with 10% FBS, 1% P/S, 4mM L-glutamine, and 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems) to induce differentiation into proinflammatory macrophages (M1 macrophages). After 7 days of culture, the differentiated cells were detached with trypsin-EDTA and cryopreserved at passage 1 in fully supplemented Advanced RPMI (Gibco) containing 10% DMSO. Passage 1 macrophages were used for our studies by quickly thawing and culturing them in fully supplemented Advanced RPMI the night prior an experiment to allow cell attachment.

2.2.5 MSC alginate encapsulation and eMSCs viability

Alginate Poly-L-Lysine encapsulation of MSC was performed as previously described [18, 23]. Briefly, MSCs were mixed with 2.2% alginate solution (w/v, in Ca^{2+} free DMEM) at a seeding density of $4x10^6$ cells/mL and extruded into a crosslinking solution (100mM $CaCl_2$, 5g/L glucose, 145mM of NaCl, and 10mM MOPS, pH 7.2) using an electrostatically assisted cell encapsulation unit (Nisco). Encapsulated MSCs (eMSCs) were allowed to further crosslink for 10 min. Then, the crosslinking solution was removed and the eMSC were transferred into a 50mL conical tube, washed with 1X PBS and strained using a cell strainer (100 μm). The PBS was removed and the eMSC were coated with poly-l-lysine (PLL) to provide further structural support by resuspending them in 5mL of PLL (Sigma-Aldrich, MW: 68,600 g/mol) (0.05% w/v) for 2 min while gently agitating the conical tube. After PLL coating, the PLL was removed by straining and replaced with 1X PBS for a last wash. The eMSC were ultimately resuspended into 5mL of MEM-α media in an upright T-25 flask until needed for experiments. LIVE/DEAD cell assay was performed to assess the viability of the eMSCs as per manufacturer's instructions. LIVE/DEAD-stained cells were visualized using a spinning disk confocal microscope (Olympus IX81) to acquire 400-500 μm Z stacks at 20 μm intervals for 15 capsules per condition. Images were analyzed using SlideBook 5 software (3i, CO) [27]. This assay allowed us to quantify the initial number of viable cells per capsule to estimate the number of capsules needed for future experiments.

2.2.6 Monolayer inflammatory model of osteoarthritis

In order to establish an *in vitro* inflammatory OA model, bovine chondrocytes (passage 1-2) 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, 2mM 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 left to attach overnight. After initial cell attachment, the media was changed to serum free chondrogenic

media and chondrocytes were cultured in basal conditions or stimulated with IL-1 or IL-1/ TNF-α (10ng/mL) to induce a pro-inflammatory OA state. After 48 hours in culture, media supernatants were gently collected while monolayer chondrocytes were trypsinized, counted using Trypan Blue exclusion method, pelleted, and flash frozen. All samples were stored in a -80°C freezer until further use.

2.2.7 Dexamethasone treatment of monolayer inflammatory model of osteoarthritis

Dexamethasone (DEX), a glucocorticoid commonly used in the clinic, has been shown to have chondroprotective effects at low doses[24] . Therefore, stimulated and non-stimulated monolayer cultures were treated with 50μM of DEX to validate our observations with previously reported studies [9]. After 48 hours in culture, media supernatants were gently collected while monolayer chondrocytes were detached with trypsin, counted using Trypan Blue exclusion method, pelleted, and flash frozen. Samples were stored as previously described.

2.2.8 Co-culture studies with free or eMSCs

Bovine chondrocytes (passage 1-2) were plated in 12-well plates at a seeding density of 25,000 cells/cm^2 using chondrogenic media with 10% FBS and left to attach overnight. On the following day, the media was changed to serum free media with or without 10ng/mL of IL-1 or IL-1/TNF treatment and the chondrocytes were co-cultured with free or eMSC by using 0.4 μm pore size transwells fit for 12-well plates (PET, Falcon). Co-cultures were maintained for 48hrs and then cell culture supernatants and chondrocyte monolayers were collected and stored as previously described.

2.2.9 Prostaglandin E_2 dose response

Chondrocytes and macrophages were seeded at 25,000 cells/cm^2 in 24-well plates and allowed to attach overnight. Then, the media was replaced with serum free chondrogenic media containing

0- 40 ng/mL of prostaglandin E_2 (PGE_2) (Cayman Chemicals) for 48hrs. Then, cell culture supernatants were gently collected, stored at -80C and replaced with 4% (w/v) paraformaldehyde (PF) for 20 min at room temperature to fix the cells. After fixing, enough 1X PBS was added to dilute the PF to 1% (w/v) and the samples were stored at 4°C for further analysis.

2.2.10 Mixed culture studies

Mixed culture studies were performed by seeding all cell types at a seeding density of 25,000 cells/cm^2. Chondrocytes were trypsinized, collected and seeded in 24-well plates, and allowed to attach overnight. In parallel, passage 1 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 MSCs (P3-4) were trypsinized, collected and resuspended in basal or stimulatory chondrogenic media containing 10ng/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 providing cell-cell contact with macrophages. Then transwells containing macrophages and MSCs were placed on top of the chondrocyte cultures creating a mixed co-culture system. After 48hrs in culture, supernatants were collected and stored for further analysis.

2.2.12 qRT-PCR gene array

Gene expression data was obtained using a custom bovine RT^2 Profiler PCR Array (QIAGEN). Sample processing and qRT-PCR was performed by QIAGEN's Center for Genomic Services. Data analysis was performed using GeneGlobe platform.

2.2.13 Cytokine measurement

Cell culture supernatants were removed from storage and allowed to thaw at room temperature. Bovine interleukin (IL) -8 levels were measured from the supernatants utilizing a bovine IL-8 Do-

It-Yourself ELISA (Kingfisher Biotech) following the manufacturer's instructions with some modifications. Briefly 1-2.5µg/mL of capture antibody (PB0273B-100) was diluted in 1X ELISA Coating Buffer (Biolegend), 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 1X PBS (w/v) for 1 hour in a plate shaker. Then samples were loaded and incubated for 2 hours while mixing in a plate shaker. Biotinylated anti-bovine IL-8 polyclonal antibody (PBB1163B-050) was diluted in 4% BSA solution at 0.05µg/mL, added to the plate and incubated for 1hr while shaking. Streptavidin-Horseradish Peroxidase (Biolegend) was diluted 1:1000 in 4% BSA solution ------ Human IL-8, IL-10, and IL-6 were measured using ELISA MAX Deluxe Sets (Biolegend) following manufacturer's instructions. PGE_2 levels were measured using the Prostaglandin E2 Express ELISA kit (Cayman Chemicals) as per manufacturer's instructions. Absorbances were recorded using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA).

2.2.14 Macrophage cell counting

Fixed cells stored at 4°C were transitioned to room temperature, then the PF was removed and replaced with 300 µL of Hoechst stain (Molecular Probes) diluted 1:5000 in PBS and incubated for 30 min in the dark. Cells were then washed twice with PBS and maintained in 200 µL of PBS for imaging. Stained nuclei were imaged at 4X using an inverted fluorescent microscope (IX81, Olympus) with SlideBook software version 5.0 (Intelligent Imaging Innovations) software. Nuclei were counted using Fiji Image J (NIH).

2.2.15 Statistical analysis

qRT-PCR gene array data was analyzed using GeneGlobe RT^2 PCR Data Analysis software (QIAGEN). 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 (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis with a significance level of $\alpha = 0.05$ in Kaleida-Graph software version 4.1 (Synergy Software, Reading, PA, USA).

2.3 Results and Discussion

2.3.1 Monolayer inflammatory model of OA

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 inflammation) or with IL-1 + TNF (high inflammation), key mediators of the inflammatory OA cascade. After 48 hrs of stimulation in the presence of IL-1 alone or IL-1 in combination with TNF-α (IL-1/TNF), chondrocytes were analyzed for gene expression changes using a panel of cytokine, chondrogenic, and ECM remodeling genes (Table 2.1). As expected, inflammatory induction with both IL-1 and IL-1/TNF promoted differential gene expression in monolayer chondrocytes. As shown in Figure 1, various factors associated with the pathogenesis of OA (Table 2.2) were upregulated in our *in vitro* system. Both, IL-1 or IL-1/TNF stimulated the up-regulation of multiple pro-inflammatory cytokine and matrix degrading proteins genes, particularly, CCL2, CCL20, CSF2, CXCL5, IL1B, CXCL8, IL6, CCL5, MMP1, MMP13, and MMP3 (FIG 2.1A-B, Table 2.2A). While genes associated with chondrogenesis such as BMP2 were down-regulated (FIG 2.1C, Table 2.2B). Although IL-1 and IL-1/TNF stimulated chondrocytes had similar dysregulated genes, the fold regulation on IL-1/TNF was significantly higher especially for ECM remodeling proteins (Table 2.2). This comprehensive gene array that evaluates the inflammatory, chondrogenic and metabolic state of chondrocytes was then used as a baseline screening method for potential OA biomarkers to assess the therapeutic efficacy of different treatments, particularly disease modifying therapies.

Table 2.1: Gene Panel used for qRT-PCR gene array analysis

Type	Inflammatory	Chondrogenic	ECM remodeling*	House Keeping
Gene list	CCL2, CCL20, CCL5, CSF2, CSF3, CXCL5, IL1b, IL1a, IL4, CXCL8, PF4, TNF, CXCL12, IL6, IL17A	BMP2, BMP4 BMP6, SOX9 WNT7A, TGFB1 ACAN, COL2A1	ADAMTS1, ADAMTS13, ADAMTS8, MMP1, MMP13 MMP14, MMP2 MMP3, TIMP1	GAPDH, YWHAZ

*ECM remodeling genes were not analyzed in the dexamethasone studies.

In addition to gene expression changes, we evaluated the secretion levels of IL-8 in cell culture supernatant. IL-8 is a chemokine produced by OA chondrocytes involved in different aspects of the pathophysiology of the disease including the promotion of matrix metalloproteinase production, neutrophil accumulation and activation and leukocyte homing to the synovium [25]. Both inflammatory stimuli promoted the secretion of IL-8 with IL-1/TNF stimulated chondrocytes producing 11X more IL-8 than IL-1 stimulated cells (FIG 2.1D). However, the magnitude of both gene expression and IL-8 secretion changes was more pronounced in the IL-1/TNF group.

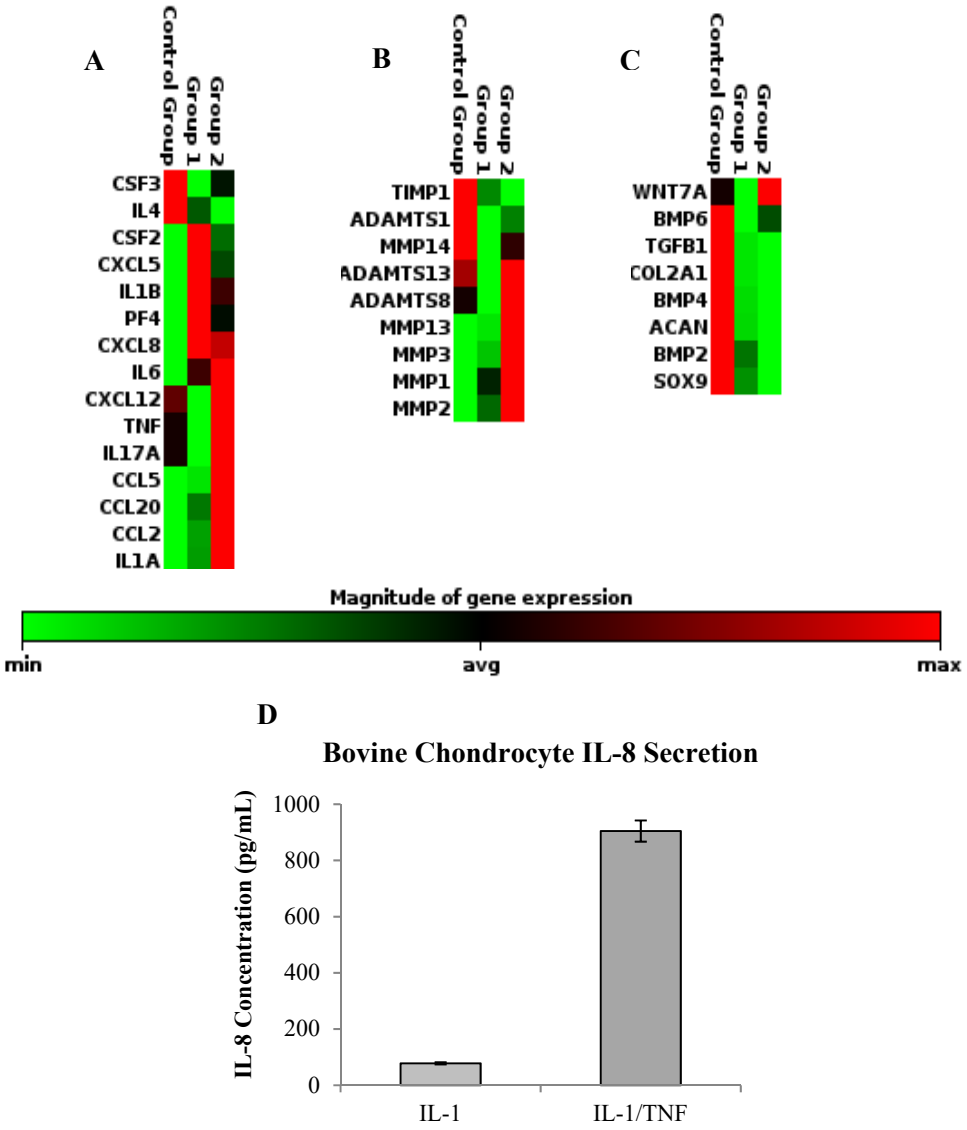

Figure 2.1: Monolayer inflammatory model of OA. Differential gene expression in a panel of A) cytokines and chemokines, B) matrix remodeling proteins, and C) chondrogenic genes after treatment with pro-inflammatory IL-1 or IL-1/TNF. Control group= basal chondrocytes, group 1= IL-1 stimulated chondrocytes, group 2= IL-1/TNF stimulated chondrocytes. D) IL-8 secretion levels by IL-1 and IL-1/TNF stimulated chondrocytes. Genes with higher expression levels are shown in red, whereas genes with lower expression levels are shown in green. Genes with average expression levels are shown in black. 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= 6-9 of 3 independent experiments.

Table 2.2: Chondrocyte gene expression changes in the presence of IL-1 or IL-1 and TNF stimuli. 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.

Upregulated		
Gene	**IL-1**	**IL-1/TNF**
CCL2	24.72	131.94
CCL20	312.32	1183.54
CSF2	8.11	3.04
CXCL5	133.99	48.93
IL1B	3.53	2.56
CXCL8	273.19	241.72
PF4	3.08	/
IL6	22.15	35.19
CCL5	/	14.96
MMP1	36.57	82.91
MMP13	13.72	295.32
MMP3	226.42	2030.89

Downregulated		
Gene	**IL-1**	**IL-1/TNF**
CSF3	-2.3	/
BMP2	-3.11	-15.34
BMP4	-9.03	-20.18
BMP6	12.41	-2.48
SOX9	-3.02	-6.69
TGFB1	-2.94	-3.24
ACAN	-7.9	-17.27
COL2A1	-10.8	-20.16
ADAMTS1	-4.61	-2.44
IL4	/	-2.43

Since stimulation of chondrocyte monolayers with IL-1 + TNF resulted in the development of inflammatory OA characteristics *in vitro*. We proceeded to characterize the effect of Dexamethasone (DEX) in the system as this glucocorticoid is commonly used in the clinic to treat OA symptoms and its effects on chondrocytes have been previously studied [26]. Differential gene expression changes were analyzed for stimulated and non-stimulated chondrocytes treated with 50μM DEX for 48hrs (FIG 2.2, Table 2.3). DEX treatment of IL-1 stimulated chondrocytes down-regulated the gene expression of pro-inflammatory CCL2, CXCL5, CXCL8, PF4, IL-6 and up-regulated pro-chondrogenic BMP4, BMP6 and SOX9 (FIG 2.2 A-B). Similarly, IL-1/TNF stimulated chondrocytes treated with DEX showed downregulation of pro-inflammatory CCL2, CCL5, CXCL5, IL1B, IL1A, CXCL8, PF4 and upregulation of pro-chondrogenic BMP2, BMP4 and BMP6 (FIG 2.2C-D). However, CSF2 and CSF3 were up-regulated while COL2A1 expression was still downregulated in both conditions.

As previously observed, IL-8 secretion levels increased in the IL-1 and IL-1/TNF stimulated chondrocytes; however, treatment with 50μM DEX significantly decreased the secretion of IL-8 (FIG 2.2E) reflecting the downregulation of IL-8 gene expression observed at

the mRNA level. Generally, our results are consistent with previously published data where DEX has been reported to inhibit the induction of inflammatory cytokines [9, 26] and promote chondrogenesis as observed by the upregulation of BMP 2, 4, and 6 [27, 28]. The effects of DEX were more pronounced in the more inflammatory IL-1/TNF environment.

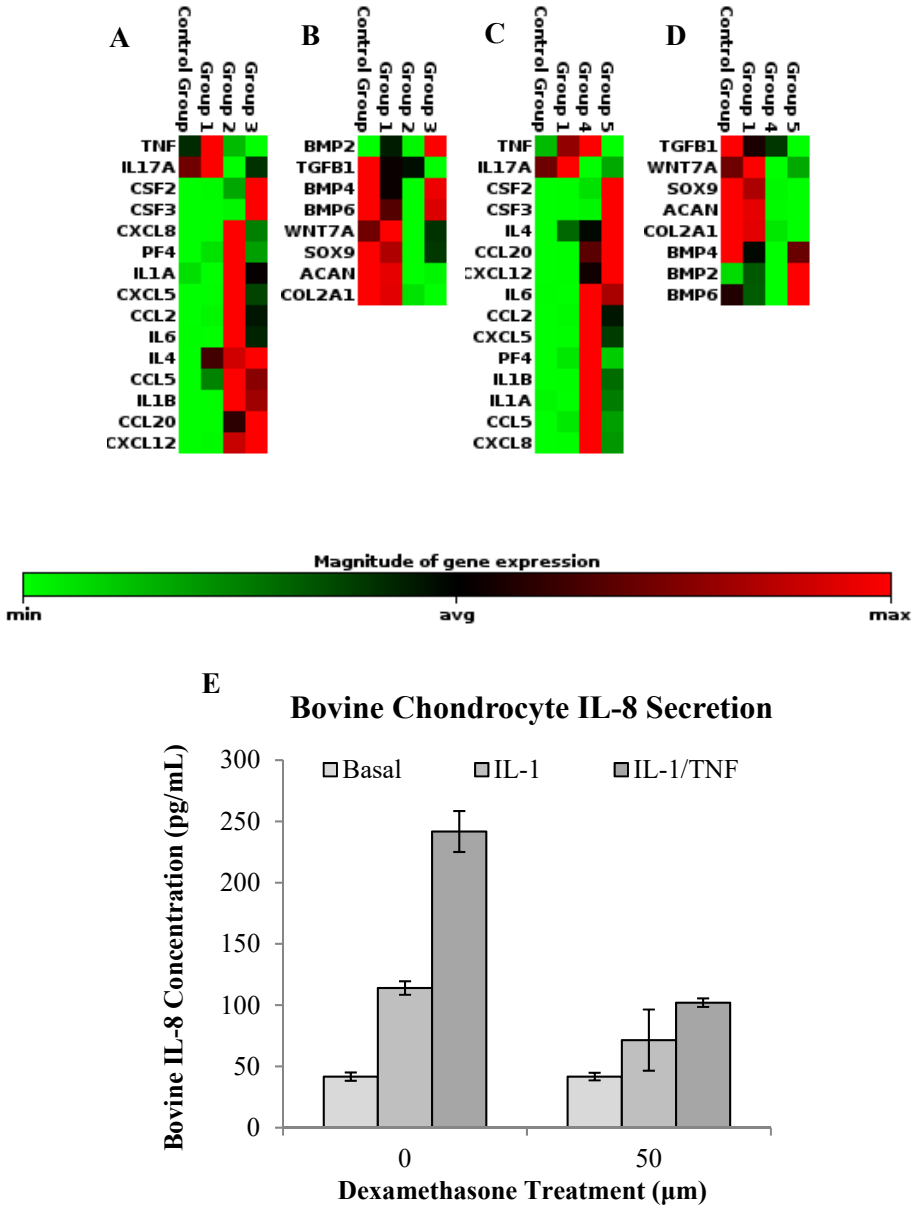

Figure 2.2: Stimulated and non-stimulated chondrocytes treated with 50µM DEX for 48hrs. Differential gene expression panel of A) cytokines and chemokines, B) chondrogenic genes after treatment with pro-inflammatory IL-1+/- dexamethasone (DEX) or C) cytokines and chemokines,

D) chondrogenic genes after treatment with pro-inflammatory IL-1/TNF+/- DEX. Control group = basal chondrocytes, Group 1= basal chondrocytes + DEX, Group 2= IL-1 stimulated chondrocytes, Group 3=IL-1 stimulated chondrocytes + DEX, Group 4= IL-1/TNF stimulated chondrocytes, Group 5= IL-1/TNF stimulated chondrocytes + DEX. Genes with higher expression levels are shown in red, whereas genes with lower expression levels are shown in green. Genes with average expression levels are shown in black. 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.

Table 2.3: IL-1 and IL-1/TNF stimulated chondrocyte gene expression changes in the presence of 50 μM DEX treatment. 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.

Upregulated				
Gene	**IL-1**	**IL-1/TNF**	**IL-1+ DEX**	**IL-1/TNF+ DEX**
CCL2	166.02	434.05	76.18	199.19
CCL20	2401.34	7729.27	4099.27	11424.08
CCL5	4.72	22.04	3.86	4.92
CSF2	54.63	48.12	317.6	828.65
CSF3	3.57	7.02	166.76	428.73
CXCL5	1875.74	3070.67	689.25	1179.34
IL1B	99.51	795.67	81.09	230.24
IL1A	10.43	55.78	5.54	14.69
CXCL8	560.74	2433.54	141.39	495.1
PF4	3.49	3.88	/	/
CXCL12	8.88	8.88	9.83	15.78
IL6	286.62	390.73	123.04	325.06
IL4	/	/	/	2.47
BMP2	/	/	/	2.28

Downregulated				
Gene	**IL-1**	**IL-1/TNF**	**IL-1+ DEX**	**IL-1/TNF +DEX**
BMP4	-5.84	-4.41	/	/
BMP6	-6.58	-2.91	/	/
SOX9	-11.09	-7.67	-2.25	-8.3
ACAN	-17.1	-36.72	-13.02	-43.85
COL2A1	-9.86	-14.63	-19.8	-39.27
TGFB1	/	/	-2.02	-2.19

2.3.2 Co-culture studies with free MSC or alginate-encapsulated MSCs (eMSC)

Following the characterization of stimulated chondrocyte responses to DEX treatment, we proceeded to challenge the *in vitro* system with a potential cellular disease modifying treatment, MSC. MSC treatments have been reported to decrease inflammation and promote tissue regeneration in several *in vitro* and *in vivo* studies including small clinical trials. However, the level of evidence is still inadequate to fully demonstrate MSC therapeutic efficacy in the treatment of OA [29]. To investigate the effects of MSCs and eMSCs, IL-1 and IL-1/TNF

(10ng/mL) stimulated chondrocytes were co-cultured with MSC or eMSCs at a 1:1 ratio for 48hrs and a qRT-PCR gene array was performed as previously described. The presence of MSC and eMSC induced significant gene expression changes in bovine chondrocytes (FIG 2.3). Unexpectedly, the chondrocyte response to the MSC and eMSC treatment was characterized by exacerbated inflammation and catabolic activity (FIG 2.3). In addition, the MSC delivery method promoted differential gene regulation as shown in FIG 3A-3D. When treated with MSC, IL-1 stimulated chondrocytes further up-regulated the expression of pro-inflammatory and ECM remodeling genes including CCL2, CCL20, CCL5, CSF3, CXCL5, IL1B, IL1A, CXCL12, IL6, ADAMTS1, and MMP13 (Table 2.4). Although pro-chondrogenic BMP4 and BMP6 were up-regulated, CXCL8 was the only down-regulated gene from the inflammatory panel. In contrast, eMSC treated chondrocytes further upregulated pro-inflammatory CCL2, CSF3, IL1A, TNF, CXCL12, IL6, and IL17A; ECM remodeling ADAMTS1, ADAMTS8, ADAMTS13, MMP1, MMP2, MMP13, and MMP14; and pro-chondrogenic BMP4, BMP6, WNT7A, and TIMP1. In this case, CSF2, CXCL5, and CXCL8 were down-regulated (Table 2.4). In addition to evaluating the gene expression changes, the IL-8 levels in cell culture supernatants were assessed resulting in significantly elevated levels of IL-8 in eMSC treated chondrocytes when compared to MSC treated chondrocytes (FIG 2.3D).

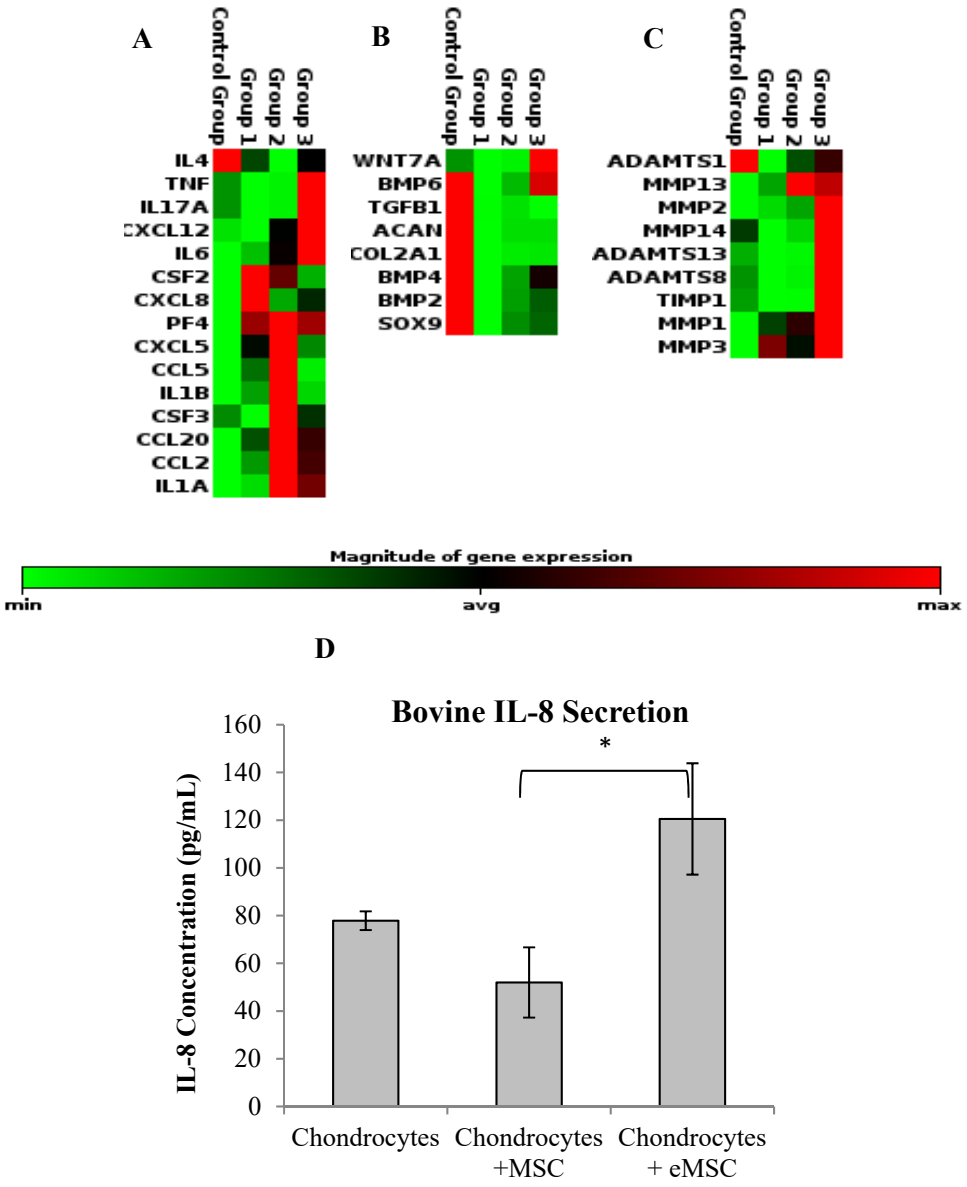

Figure 2.3: Differential gene expression panel of IL-1 stimulated chondrocytes treated with MSC or eMSC. A) cytokines and chemokines, B) chondrogenic genes C) matrix remodeling proteins. Control group = basal chondrocytes, Group 1= IL-1 stimulated chondrocytes, Group 2= IL-1 + MSC, Group 3=IL-1 stimulated chondrocytes + eMSC. D) IL-8 secretion of IL-1 stimulated chondrocytes treated with MSC or eMSC. IL-8 secretion: chondrocytes = 77.85 ± 3.93 pg/mL, chondrocytes + MSC= 51.98 ± 14.72 pg/mL, and chondrocytes + eMSC= 120.52 ± 23.33 pg/mL. Genes with higher expression levels are shown in red, whereas genes with lower expression levels are shown in green. Genes with average expression levels are shown in black. 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= 6-9 of 3 independent experiments.

Table 2.4: IL-1 stimulated chondrocyte gene expression changes after 48hrs in co-culture with free MSC or eMSC treatment. Fold regulation compared to IL-1 stimulated chondrocytes with no treatment. Values represent the average gene fold regulation normalized to non-stimulated chondrocyte gene expression. Only values with a >2 fold change are shown.

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

Downregulated		
Gene	**MSC**	**eMSC**
CXCL8	-5.87	-2.34
CSF2	/	-3.9
CXCL5	/	-2.08

IL-1/TNF stimulated chondrocytes (FIG 2.4) treated with MSC had differential gene expression when compared to IL-1 stimulated chondrocytes by up-regulating CCL2, CCL20, CCL5, CSF2, CSF3, CXCL5, IL1B, IL1A, IL4, PF4, CXCL12, IL6, ADAMTS1, BMP2, and BMP4 and only down-regulating CXCL8 (Table 2.5) eMSC treated chondrocytes also exhibited pro-inflammatory cytokine and ECM remodeling gene up-regulation such as CCL20, CCL5, CSF3, CXCL5, IL1B, PF4, CXCL12, IL6, ADAMTS13, MMP1, and MMP13; however, pro-chondrogenic genes BMP2, BMP4, SOX9, and ACAN were also up-regulated with this treatment

(Table 2.5). Interestingly, assessment of IL-8 secretion levels resulted in significantly lower levels for MSC treated chondrocytes, but significantly higher levels for eMSC treated chondrocytes when compared to IL-1/TNF stimulated chondrocytes (FIG 2.4D) reflecting the results observed in the gene expression analysis.

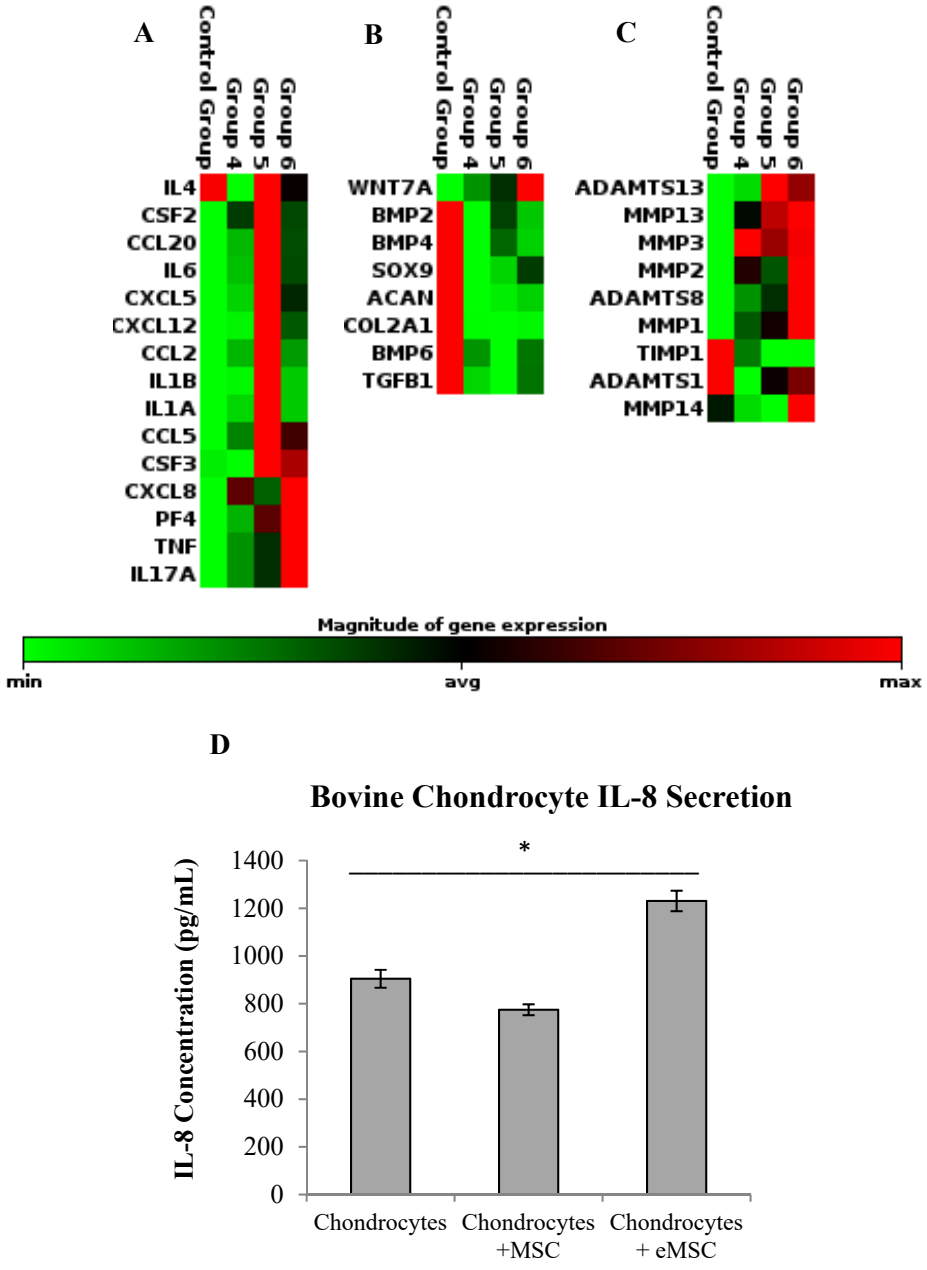

Figure 2.4: Differential gene expression panel of IL-1/TNF stimulated chondrocytes treated with MSC or eMSC. A) cytokines and chemokines, B) chondrogenic genes C) matrix remodeling

proteins, D) IL-8 secretion. Control group = basal chondrocytes, Group 4= IL-/TNF1 stimulated chondrocytes, Group 5= IL-1/TNF + MSC, Group 6=IL-1/TNF stimulated chondrocytes + eMSC. IL-8 secretion: chondrocytes = 904.52 ± 37.65 pg/mL, chondrocytes + MSC= 774.71 ± 22.87 pg/mL, and chondrocytes + eMSC= 1230.66 ± 42.86 pg/mL. Genes with higher expression levels are shown in red, whereas genes with lower expression levels are shown in green. Genes with average expression levels are shown in black. 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= 6-9 of 3 independent experiments.

Table 2.5: IL-1/TNF stimulated chondrocyte gene expression changes after 48hrs in co-culture with free MSC or eMSC treatment. Fold regulation compared to IL-1/TNF stimulated chondrocytes with no treatment. Values represent the average gene fold regulation normalized to non-stimulated chondrocyte gene expression. Only values with a >2 fold change are shown.

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

Gene	MSC	eMSC
CXCL8	-2.19	/

2.3.3 PGE_2 Secretion by MSC and eMSC

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 highligted the role of secreted factors such as

PGE_2 on the anti-inflammatory and immunomodulatory properties of MSC and eMSC in co-culutre with macrohages and following LPS exposure [12, 22, 30-33]. Therefore, PGE_2 secretion was quantifiedafter 48hrs in co-culture with chondrocytes exposed to IL-1 or IL-1/TNF. We determined that MSC and eMSC both secrete high levels of PGE2 when stimulated with either IL-1 or IL-1/TNF (FIG 2.5).

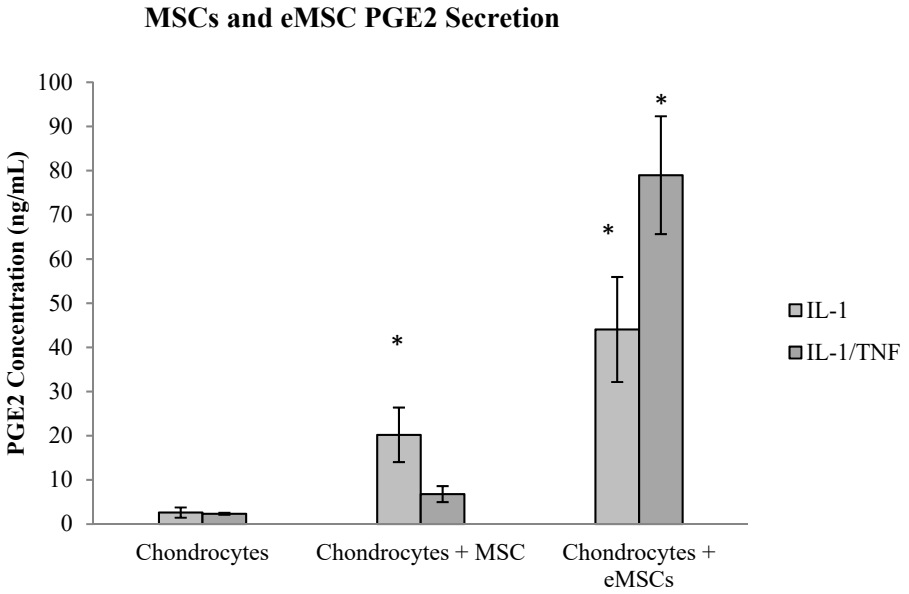

Figure 2.5: PGE2 secretion by MSC or eMSC in co-culture with stimulated bovine chondrocytes. MSC and eMSC secrete significant levels of PGE2 when stimulated with IL-1 or IL-1/TNF compared to stimulated chondrocytes. PGE_2 secretion: IL-1 stimulated chondrocytes= 2.61 ± 1.16 ng/mL, IL-1/TNF stimulated 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.

2.3.4 Prostaglandin E_2 (PGE_2) Dose Response

Recent studies have identified PGE_2 as a key mediator of the anti-inflammatory effect of MSC in attenuating inflammation in osteoarthritic chondrocytes and other joint cells [12]. However, the role of PGE_2 in OA is not entirely understood as it has been shown to have both catabolic and anabolic effects [34]. Therefore, after demonstrating that MSC and eMSC produce PGE_2, we conducted studies to test the effect of exogenous PGE_2 on stimulated chondrocytes

(FIG 2.6). In both conditions, IL-1 and IL-1/TNF stimulated chondrocytes, PGE_2 did not attenuate IL-8 levels and in most conditions, it exacerbated the inflammatory response by promoting IL-8 secretion. These results reflect the same observed trend with MSC or eMSC treatment of stimulated chondrocytes (FIG 2.3-2.4).

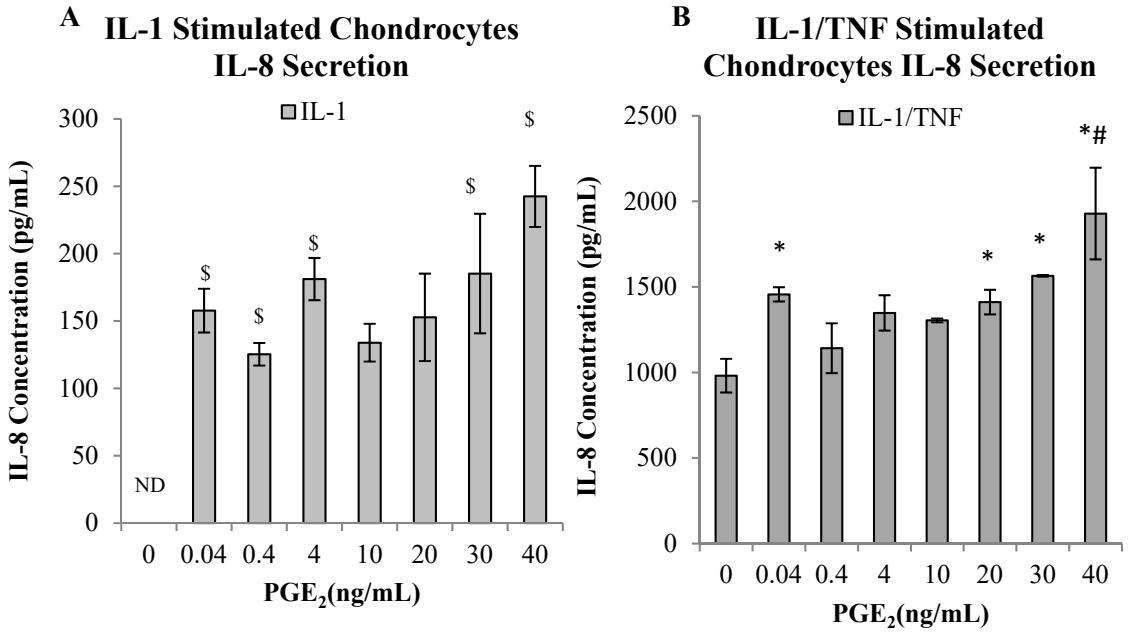

Figure 2.6: IL-8 secretion of IL-1 and IL-1/TNF stimulated chondrocytes doses with exogenous PGE_2. A) IL-1 stimulated chondrocytes and B) IL-1/TNF stimulated chondrocytes. Bar graphs represent the mean ± SEM.

2.3.5 Characterization of IL-1 and IL-1/TNF Stimulated Macrophages

Thus far, our results indicate that MSC treatment for IL-1 and IL-1/TNF stimulated chondrocytes does not attenuate inflammation or reverse the production of ECM remodeling proteins. In fact, many pro-inflammatory cytokines and matrix metalloproteinases genes were upregulated in the presence of MSC treatment. This phenomenon could be caused by the high PGE_2 secretion levels by MSCs as very low concentrations of PGE_2 have been shown to decrease pro-inflammatory gene expression and have chondroprotective activity [35]. However, we included similar concentrations of PGE_2 in the dose response study (0.04 ng/mL) and an increase in IL-8 was still observed. Yet, several *in vivo* studies have reported a decrease in pro-

inflammatory cytokine secretion and an increase in collagen and proteoglycan production after intra-articular injection of MSC [17]. As mentioned before, MSC do not engraft in the cartilage defects, but can be found in the synovial membrane shortly after injection. There MSC could be exerting their immunomodulatory function by attenuating synovial macrophage inflammatory activity which has been characterized as key driver of chronic inflammation in OA [19]. 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 [31]. Therefore, we hypothesized that in OA the main target for MSC immunomodulatory action may be the macrophages and not the chondrocytes. However, our M1 macrophage model used lipopolysaccharide (LPS) stimulation which is a model of bacterial-induced inflammation and is not necessarily representative of the inflammation that occurs in OA joints. Therefore, to better replicate the inflammatory environment in an OA joint, IL-1 +TNF-α were used as inflammatory stimuli for M1 pro-inflammatory macrophages.

By using key inflammatory mediators of OA as pro-inflammatory stimulus for M1 macrophages, we intended to investigate their response to the same factors that we and others have used to stimulate monolayer chondrocytes and MSC. To test this hypothesis, we first characterized the effect of IL-1 and IL-1/TNF stimulation on M1 peripheral blood derived macrophages by measuring IL-8 secretion after 48hrs. Stimulated macrophages produced high levels of IL-8 when stimulated with IL-1/TNF, but not with IL-1 alone (FIG 2.7).

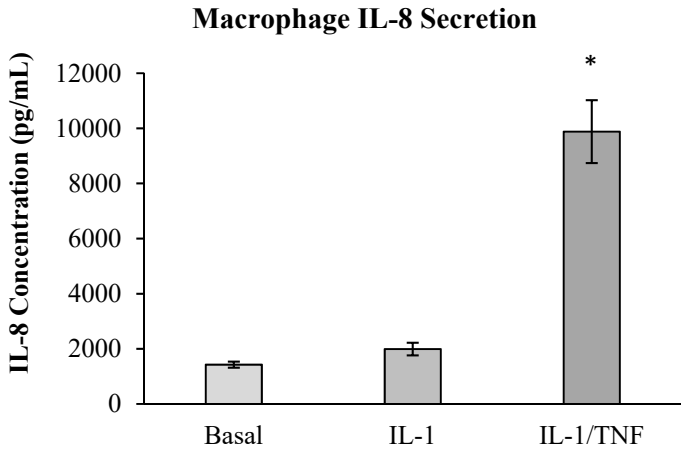

Figure 2.7: Peripheral blood-derived macrophage response to OA stimuli. M1 macrophages secrete a plethora of cytokines and chemokines including IL-8. M1 macrophages respond poorly to IL-1 stimulus alone; however IL-1/TNF-α stimulus induces a significant pro-inflammatory response in the macrophages. However, IL-6 secretion was not promoted with either stimulus. LPS (data not shown) was utilized as a positive control for inflammatory response. Bar graphs represent the mean ± SEM of n=6 of 2 independent experiments.

The immunomodulatory effects of PGE_2 on pro-inflammatory (M1) macrophages stimulated with LPS has been extensively studied by our group and others. PGE_2 immunomodulation is characterized by a dose-dependent decrease in M1 markers such as inducible nitric oxide synthase (iNOS) expression and TNF-α secretion and an increase in M2 markers such as CD206 expression and IL-10 secretion [22, 31, 32, 36, 37]. After testing the macrophage response to IL-1 and IL-1/TNF stimuli, the effects of PGE_2 on OA stimulated chondrocytes were teased out with an exogenous PGE_2dose response. Macrophages responded to increasing concentrations of PGE_2 by secreting more IL-8 (FIG 2.8A). In addition to measuring IL-8, the secretion levels of chondroprotective [38] IL-10 were measured (FIG 2.8B). 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 where complex *in*

vivo environments comprising multiple divergent stimuli could result in intermediate phenotypes with both pro- and anti-inflammatory characteristics [39, 40].

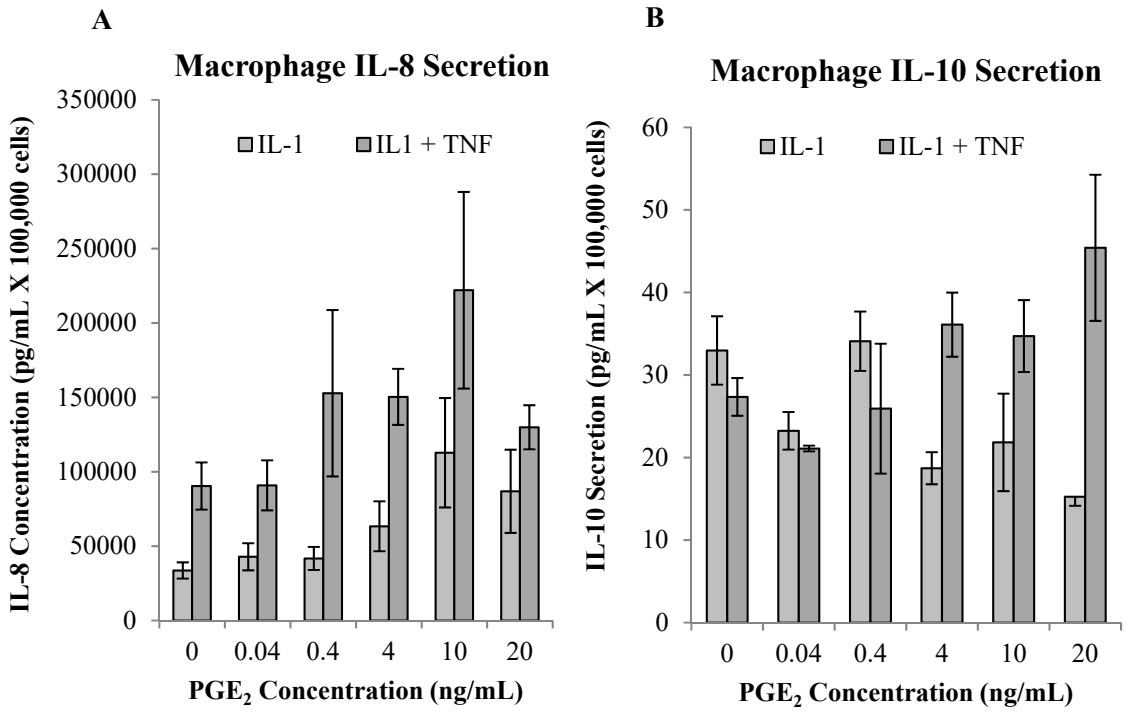

Figure 2.8: OA stimulated peripheral blood-derived macrophages response to PGE_2. IL-1 or IL-1/TNF stimulated macrophages were treated with different PGE_2 concentrations 0-20 ng/mL and their A) IL-8 secretion and B) IL-10 secretion was measured by ELISA. Bar graphs represent the mean ± SEM of n=6-9 replicates of 2-3 independent experiments.

2.3.6 Mixed Culture Studies with Chondrocytes, Macrophages and MSC

After characterizing both the individual macrophage and chondrocytes responses to OA stimuli, we proceeded to test the cell interactions between chondrocytes, macrophages, and MSC after stimulation with IL-1 and IL-1/TNF utilizing a mixed culture setup. Bovine IL-8 secretion was measured after 48 hours in culture to tease out the chondrocyte inflammatory response. Remarkably, when macrophages were introduced in the culture, IL-8 secretion was significantly down regulated for the IL-1/TNF stimulated chondrocytes and the IL-1 stimulated chondrocytes followed a similar trend (FIG 2.9). As previously postulated, our results suggest that the targets for MSC immunomodulatory action are the macrophages. Once macrophage promoted

inflammation is attenuated, the inflammatory state in chondrocytes can also be mitigated. Our results emphasize the importance of developing more comprehensive *in vitro* systems to test therapeutic interventions for OA and suggest that unlike DEX, MSC may be used as an OA therapy only when synovial cells are highly inflamed.

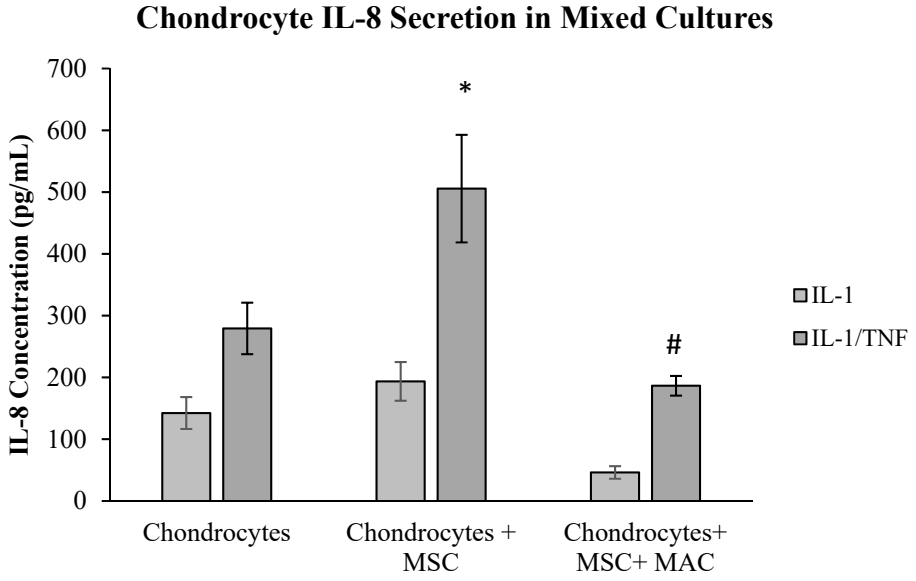

Figure 2.9: Mixed culture studies IL-1 or IL-1/TNF stimulated chondrocytes, macrophages and MSC were cultured together for 48 hours. Bovine IL-8 patterns reveal a significant attenuation of inflammation in the IL-1/TNF mixed culture when compared to the chondrocyte + MSC condition. IL-1 stimulated cells follow a similar pattern. Bar graphs represent the mean ± SEM of n=6-9 replicates of 2-3 independent experiments.

2.4 Conclusions

The present study established a proof-of-concept *in vitro* inflammatory OA model composed of the predominant OA contributing cell types, chondrocytes and macrophages. Using this model we were able to promote cell responses that reflect the pathophysiology of OA. In addition, by challenging the *in vitro* system with DEX, a well characterized glucocorticoid we were able to validate our model by showing similar responses to osteoarthritic chondrocytes. However, when a MSC treatment was used to attenuate chondrocyte inflammation, we discovered that the co-culture of MSC or eMSC with IL-1 and IL-1/TNF stimulated chondrocytes results in

increased inflammation and promotion of extracellular matrix catabolism. In addition, it was evident that high concentrations of PGE_2, a key mediator of MSC immunomodulatory function, further exacerbated chondrocyte inflammation. Therefore, we tested if the addition of macrophages, which perpetuate inflammation in osteoarthritic joints and respond to PGE_2 by switching to an anti-inflammatory phenotype, will promote the decrease of inflammation. Indeed, the interaction of MSC, macrophages, and chondrocytes resulted in lower secretion of pro-inflammatory cytokines by chondrocytes (FIG 2.10). These results may be useful in understanding the discrepancy in the literature regarding MSC efficacy as an OA treatment.

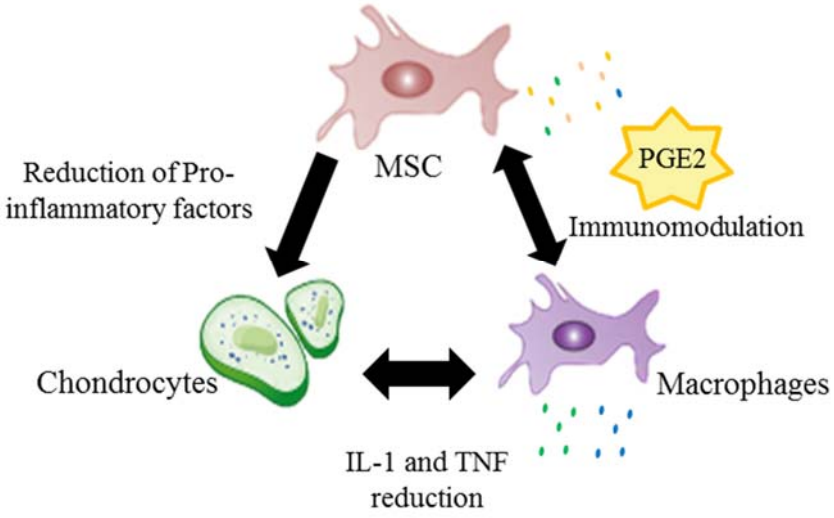

Figure 2.10: Complex cellular interactions. Attenuation of macrophage inflammation by MSCs results in attenuation of chondrocyte inflammation which was previously promoted when co-cultured with MSCs alone.

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CHAPTER 3: MULTILAYER STACKABLE TISSUE CULTURE PLATFORM FOR 3D CO-CULTURE

Note: This chapter is reproduced from the following manuscript written by **Ileana Marrero - Berrios**:

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3.1 Introduction

The study of single cell populations *in vitro* allows investigators to independently manipulate individual components of the cellular environment, in the absence of confounding cellular interactions [1]. However, by simplifying the cellular environment, the physiological relevance of the observed results might become compromised because single cell population analysis excludes the effects of paracrine signaling, cell-cell interactions with other cell types in the vicinity, cell-extracellular matrix interactions, and 3D architecture provided by their native tissue [1, 2]. Some of these issues have been addressed using conditioned media treatments, mixed cultures, and co-culture studies with compartmentalized cells.

While conditioned media treatments can sometimes recapitulate soluble cell-produced factors effect on other target cells, often bio-active molecules have short half-lives [3]. In addition, conditioned media introduces a unidirectional interaction of the producing cell to the target cell, ignoring the reciprocal signaling needed in many systems. Therefore, other approaches have been explored, such as mixed cell cultures or co-cultures systems using transwells. Although mixed cell cultures can provide valuable information regarding cell-cell paracrine and juxtacrine interactions and have been used for many applications from wound healing[4] to mimicking the

blood brain barrier [5], microenvironments may still need to be recapitulated with cell physical separation as the interacting cells exist in different tissue compartments. Thus, co-cultures such as the ones achieved by transwells- where one cell type is seeded in a permeable insert placed on top of a tissue culture well and another is seeded in the bottom of the well- can help tease out paracrine cell signaling [6]. These devices allow bidirectional cell communication and the accumulation of high concentration levels of a secreted factor produced in the vicinity of a cell to induce a response in the target cell, unlike conditioned media [6, 7]. However, it is important to note that standard transwells are characteristically different from tissue culture well with respect to both substrate and surface area.

In addition, when more than 2 cell components are necessary to develop a more comprehensive system that can mimic tissues or organs [2], regular transwell co-cultures do not suffice. Several strategies to overcome this limitation have been developed including the use of hydrogels in transwells [8], hydrogel constructs, stackable monolayer cell sheets [9, 10], paper-based stackable layers [11-15] and hydrogel-mesh layers [16]. With these types of *in vitro* systems, cell signaling, cell-cell and cell-substrate interactions can be studied in 3D space, which has been shown to better reflect cell responses and produce more reliable systems for drug testing etc. However, there are several drawbacks to these as well. Transwells are designed for the culture of only 2 cell types at a time without introducing mixed cultures and the distance between the layers cannot be controlled. The stackable monolayer cell sheets require specialized tools and multiple steps for their fabrication and eventually merge with each other making cell isolation from a single layer impossible. The hydrogel constructs, paper-based stackable layers and mesh layers all introduce cells embedded in a hydrogel which can migrate from one layer to the other, an outcome which might be undesirable in certain models [15] and researchers face challenges in cell isolation and imaging due to light scattering produced by the support materials [16].

In complex diseases such as osteoarthritis (OA) catabolic and inflammatory processes orchestrated by multiple cell types in the joint lead to the eventual destruction of articular cartilage [17]. Although the molecular mechanisms of OA initiation and progression are still poorly understood, many of these processes are mediated by the secretion of soluble factors that affect cellular function perpetuating a chronic state of inflammation [18, 19]. The development of more comprehensive *in vitro* models that replicate the paracrine cell signaling in OA is crucial for understanding the pathophysiology of the disease and develop new treatments. Motivated by the need of a platform that can, 1) support the growth of multiple cell types, 2) be modified with surface coatings, 3) control the spatial distribution of cell subsets, 4) be stacked into 3D constructs to form tissue-like assemblies, 5) de-stacked for further cell analysis, 6) be compatible with microscopy and, 6) allow for ease of manipulation without the need of further training or extra tools, among other requirements, we developed a stackable tissue culture insert and characterized its biocompatibility, ease of use, and potential for tissue culture applications. We also compared our inserts with standard tissue culture plastic as well as transwell inserts and determined that while each successfully supported growth of all cell types investigated, significant variability exists among all three, including the "gold standards", tissue culture plastic and transwell inserts. In addition, our easily fabricated and assembled device could be designed to successfully culture at least 8 cell types within a minimum distance of 0.8mm of each layer. Each of the cell components in the tri-culture system that we evaluated was viable and functional. Furthermore, we detected synergistic effects when 3 cell types were cultured together. This demonstrates the need to more fully interrogate *in vitro* culture systems and our stackable insert can provide a tool to fill the current technological void to do so.

3.2 Materials and Methods

Poly-L-Lysine (PLL), FITC-labeled PLL, DMSO, ethanol, and acetone, were purchased from Millipore Sigma (Burlington, MA). Whatman Nucleopore track-etched polycarbonate membranes (GE Healthcare) were purchased from VWR (Philadelphia, PA). Acrylic sheets (1/32" or 1/16" W) were purchased from McMaster-Carr (Elmhurst, IL). High glucose Dulbecco's Modified Eagle Medium (hgDMEM), Minimum Essential Medium- alpha (MEM-α), Advanced RPMI 1640 Medium, fetal bovine serum (FBS) (Gibco), L-glutamine (Gibco), penicillin/streptomycin (P/S) (Gibco), basic fibroblast growth factor (bFGF) (Gibco), Trypsin- EDTA (0.25%) with phenol red (Gibco), phosphate buffer saline 1X pH 7.4 (Gibco) calcein AM (Molecular Probes), ethidium homodimer (Molecular Probes) and Hoechst 33342 (Invitrogen) were purchased from Thermo Fisher Scientific (Waltham, MA).

3.2.1 Stackable Insert Fabrication

Acrylic support rings (dimensions) were designed using SolidWorks design software and cut using a laser cutter (Epilog, Golden, CO). Supports were sonicated for 30 min in ultrapure water to eliminate any residual adhesive protectant and then air dried. To assemble the stackable inserts, polycarbonate microporous (0.4 μm pore size) membranes (GE Healthcare) were attached to the acrylic support by chemical solvation utilizing acetone (Sigma) and left to bind for 5 min. The inserts underwent a series of sterilization and surface modification processes consisting of 15 min submersion in 70% ethanol, air drying in a biological safety cabinet for ~30min, plasma bombardment to modify surface charge (600mTorr, 100W power, 60 seconds) using a plasma generator (ENI, Rome, Italy) sterilized using ultraviolet light (15min/side) and coated with poly-L-lysine (PLL) to facilitate cell attachment as described below.

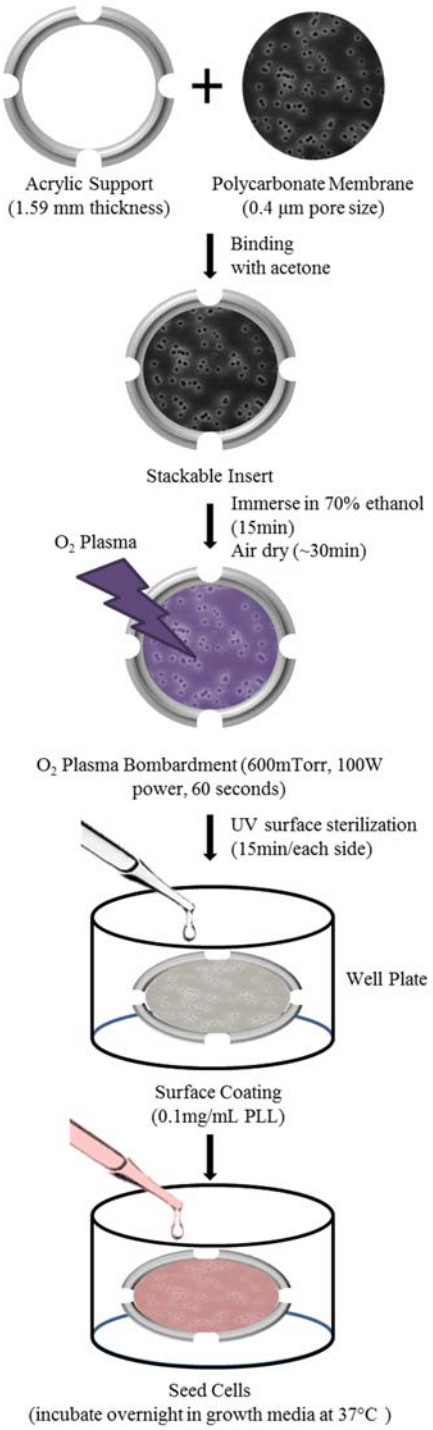


Figure 3.1. Schematic representation of the stackable insert fabrication and preparation for cell culture. A laser cut acrylic support was chemically bound to a polycarbonate membrane, sterilized, plasma treated, and coated with PLL to facilitate cell attachment.

3.2.2 O_2 Diffusion Modeling

The effect of multi-layer arrangement on oxygen (O_2) distribution, dissolution in media, and utilization by cells on different layers was modelled using COMSOL4.3a (Comsol Inc., Burlington, MA). The modeling was used to understand relationship between number of layers, number of cells on each layer, and depth at which O_2 concentration reaches hypoxic levels to the cells. For the simulation, a stack of 3 layers was used with a layer thickness of 0.1 mm and $5.0X10^4$ on each. These layers are arranged at 2, 4, 6 mm from the bottom, inside the well of a 12-well tissue culture plate. A cross section of the cylindrical well of the 12 well-plate with a radius (r) of 11 mm and filled up to a height (h) of 10 mm with a cell culture media was used as a modeling geometry. Then, the O_2 consumption reaction rate "R" (mol/M^3.Second) of each layer with cell density "Cd" was calculated using Michaelis-Menten kinetics and the following parameters: 1) three layers with cultured chondrocytes ($5.0X10^4$ to $4.0X10^5$ cells per layer), 2) cell culture media with glucose concentration of 2.5g/L (25mM) up to a height of 10 mm, and 3) maximum O_2 consumption rate (OCR_{max}) of 6.25 x 10^{-17} mol/cell/second. The cell density Cd ($1.315x10^{10}$ to $10.53x10^{10}$ cell/m^3) was calculated by taking the ratio of cells on one layer and the volume of culture media in the well ($\pi r^2 h$).

$$R = \frac{OCR_{max} C_{O2}}{C_{half} + CO_2} Cd$$

Equation 3.1. Oxygen reaction rate for each layer (stack) of multi-stack culture system using Michaelis-Menten kinetics.

3.2.3 Surface Coating

To modify the cell growth surface, the stackable inserts were individually transferred to the wells of a 12-well multiwell tissue culture plate (Falcon) and coated by adding FIT-C labeled or regular Poly-L-Lysine (PLL) solution at 0.1 mg/mL (5mg PLL in 50 mL sterile water) for 5 min. After coating, the PLL solution was aspirated, the inserts rinsed twice with sterile water and then allowed to dry for at least 2 hours before introducing cells or media [1]. To verify the addition of

surface coating, membranes coated with FIT-C labeled PLL were imaged using an inverted fluorescent microscope (Olympus IX81). Inserts were visualized using SlideBook software version 5.0 (Intelligent Imaging Innovations, Denver, CO, USA) image analysis software.

3.2.4 Cell Culture and Maintenance

All cells were maintained in a humidified 37°C incubator with 5% CO_2 and cultured in their respective media until used for experiments.

3.2.4.1 Chondrocyte culture

C28/I2 human chondrocyte cell line was obtained as a gift from Mary Goldring's Laboratory at the Hospital for Special Surgery Research Institute (New York, NY) and was cultured using hgDMEM supplemented with 10% FBS, 1% P/S, and 2mM L-glutamine [2]. C28/I2 cells were thawed at passage 11, cultured at $1.6X10^4$ cells/cm^2 until 90% confluent and passaged every 4-5 days until needed for experiments. Cells at passage 13-20 were used for this study.

3.2.4.2 Mesenchymal Stromal Cells culture

Human bone morrow-derived mesenchymal stromal cells (MSCs) were purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine, Temple, TX) and cultured following established protocols [3]. MSCs were thawed at passage 2-3 and seeded on a 175cm2 flask at $1.714x10^3$ cells/cm^2 on MEM-α containing no deoxy- or ribonucleosides and supplemented with 10% FBS, 2mM L-glutamine, 1% P/S and 1ng/mL bFGF. Cells were grown to 70% confluence, detached with trypsin and seeded in 225cm^2 flasks for further expansion until passage 4-5.

3.2.4.3 Macrophage culture

Human macrophages were obtained following protocols previously described by Gray et al [4]. Briefly, human peripheral blood (New York Blood Center) was fractionated utilizing density

gradient centrifugation (Ficoll-Paque Premium, 1.077 g/mL, GE Healthcare, Piscataway, NJ). The buffy coat was gently collected and washed twice with 1X PBS and the mononuclear cells were isolated by enriching the $CD14^+$ cell population, using magnetic bead cell sorting according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Monocytes were seeded at $1X10^7$ cells/175 cm^2 flasks in Advanced RPMI supplemented with 10% FBS, 1% P/S, 4mM L-glutamine, and 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce differentiation into proinflammatory macrophages (M1 macrophages). After 7 days of culture, the differentiated cells were detached with trypsin-EDTA (TE 0.25%) and cryopreserved at passage 1 in fully supplemented Advanced RPMI containing 10% DMSO. Passage 1 macrophages were used for our studies by quickly thawing and culturing them in Advanced RPMI the night prior an experiment to allow cell attachment as described below.

3.2.5 Biocompatibility of stackable tissue culture insert

Chondrocytes, macrophages, and MSC were seeded in the stackable insert, tissue culture plastic (TCPS) wells, or commercially available tissue culture inserts (transwells,) to characterize the cell attachment, viability, and functional secretion of different cell types with this device in comparison with commercially available *in vitro* growth substrates. For this, cells were seeded at a concentration of $2.5X10^4$ cells/cm^2 in wells containing the stackable insert with PLL coating (1.82 cm^2 of growth area) or in the wells of a 12-well tissue culture plate (3.8 cm^2 growth area) and transwells (0.9 cm^2 growth area) as controls. The cells were incubated overnight in their respective media at 37°C, 5% CO_2 to let them attach. After overnight incubation, the cells were washed once with 1X PBS and their media was replaced with hgDMEM supplemented with 10% FBS, 1% P/S, and 2mM L-glutamine. Then the cells were returned to the incubator and left in culture for 48hours.

To perform tri-culture studies, after the cells were washed with 1X PBS the stackable inserts with different cells were transferred to a new 12-well plate using tweezers and arranged in a stack (3 stackable inserts/well) as described in Table 2. Stacked cells were cultured in 2mL of fresh supplemented hgDMEM and returned to the incubator for 48hours in culture.

3.2.6 Viability and Cell Attachment

After 48hours the cell culture supernatant was gently collected, stored in the -80°C freezer for further analysis, and the media was replaced with fully supplemented hg DMEM containing calcein-AM, which is converted to the green fluorescent calcein by active esterases in live cells, ethidium homodimer-1, which binds to the DNA of dead cells emitting red fluorescence, and Hoechst 3332 which also binds to nuclear DNA emitting blue fluorescence. Cells were returned to the incubator for 20 min with staining media and then washed with fresh media (3X) and imaged using an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan). LIVE/DEAD cells were visualized using SlideBook software version 5.0 (Intelligent Imaging Innovations, Denver, CO, USA) image analysis software. To determine cell viability (%), live cell, dead cells, and nuclei were counted using Fiji ImageJ software.

Alternatively, to perform image-based cell counting after 48 hours of culture in the tri-culture system, supernatants were gently aspirated and replaced with 4% (w/v) paraformaldehyde (PF) for 20 min at room temperature to fix the cells. Enough 1X phosphate buffered saline (PBS) (Life Technologies) was added to each well to achieve 1% (w/v) PF. The fixed cells were stored at 4°C until further use. Fixed cells were transitioned to room temperature, washed 3X with PBS for 5 min, and incubated with 300µL of Hoechst stain (Molecular Probes) diluted 1:5000 in PBS for 30 min in the dark. Cells were then washed twice with PBS and maintained in 300µL of PBS for imaging. Stained nuclei were visualized at 4X using an inverted fluorescent microscope and counted using Fiji ImageJ software to determine cell attachment number.

3.2.7 Cytokine measurement

Cell culture supernatants collected from the cells in different growth substrates were thawed and analyzed using enzyme linked immunosorbent assays (ELISA) for interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α (Biolegend, San Diego, CA, USA), according to the manufacturer's instructions. Absorbances were recorded using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA).

3.2.8 Statistical analysis

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 (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis with a significance level of $\alpha = 0.05$ in Kaleida-Graph software version 4.1 (Synergy Software, Reading, PA, USA).

3.3 Results

3.3.1 O_2 modeling

As first step in determining the ultimate efficacy of our device design, we modelled oxygen diffusion, as O_2 transport could be a limiting factor for tissue culture. Therefore, the effect of a multi-layer arrangement on O_2 distribution, dissolution in media, and utilization by cells on different layers was modelled using COMSOL4.3a. The O_2 concentration along the vertical axis of a well in a 12 well plate is shown in FIG 3.2.

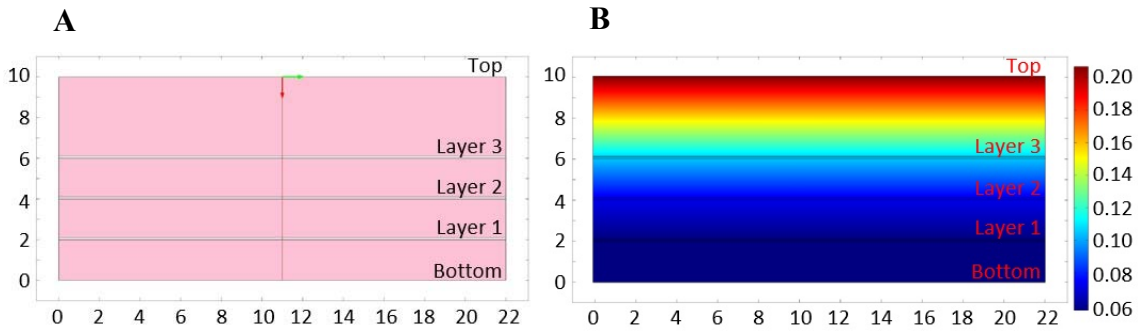


Figure 3.2. Modeling geometry. A) Cross section of a well filled with media up to 10 mm and consisting three cell layers. B) O_2 concentration distribution in the well with 3 stackable inserts.

COMSOL modeling in FIG 3.3 shows that the concentration of O_2 from the top to bottom of a well is decreased with increasing numbers of cells on each layer. O_2 concentration from the top to the bottom in a well with 3 layers and 5.0 $X10^4$ cells (blue line with $1.31x10^{10}$ cell/m^3) on each layer (which we ultimately used in our studies) is in the range distant from hypoxic conditions and each layer experiences a minute variation in O_2 concentration. However, as the cell density increases not only is the O_2 concentration at the top and bottom layers different, but also the O_2 concentration differential experienced by an upper layer compared to the layer below increases. Based on this model we can predict that if the density of cells on each layer remains constant, then the O_2 concentration will decrease from the top to the bottom as the number of layers increase, and we can design our cultures with respect to both cell number/layer and total cell layers to prevent O_2 limitation variables. For example, using a cell seeding density of $5.0X10^4$, a maximum of 8 layers can be accommodated in a well without affecting cell viability.

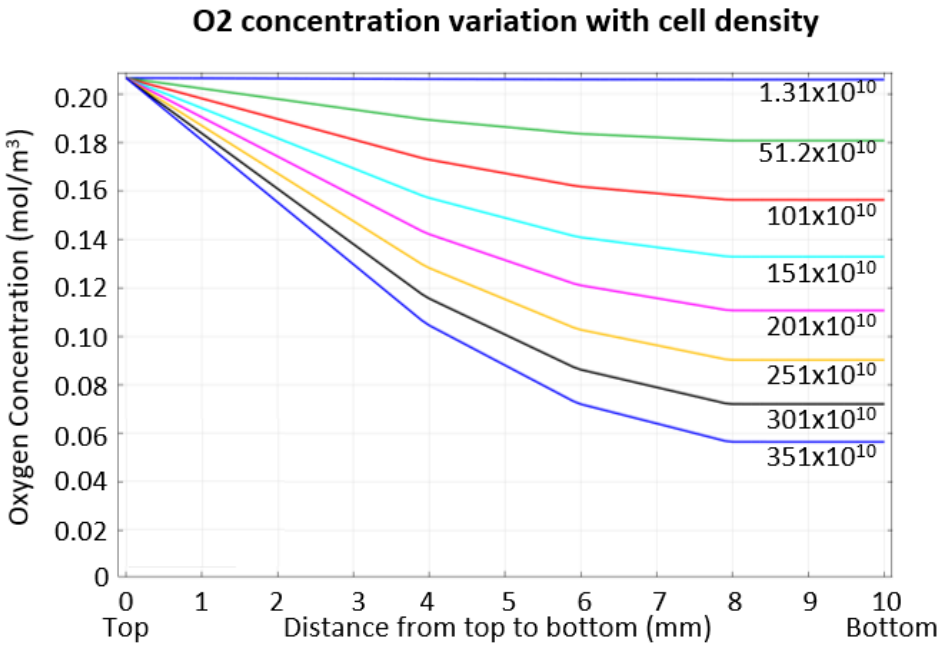


Figure 3.3. Graph showing the O_2 concentration along the vertical axis of the well indicated by red color line in Figure 2. Lines with different color indicates the density of cells on each layer. O_2 concentration for 3 stacks with 50,000 cells on each used in our experiment is indicated at top with blue line graph.

3.3.2 Membrane coating testing and characterization

Having modeling the parameter for optimal culture design, as a next step, the compatibility of our stackable membranes to be coated with functional molecules to promote cell attachment and function was tested. As depicted in Figure 3.4, membranes coated with FITC PLL (FIG 3.4) emitted a relatively uniform fluorescent signal and the fibers of the membrane can be clearly seen.

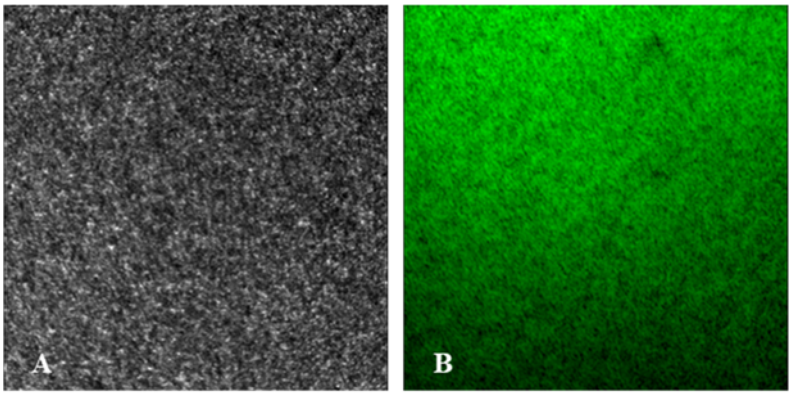


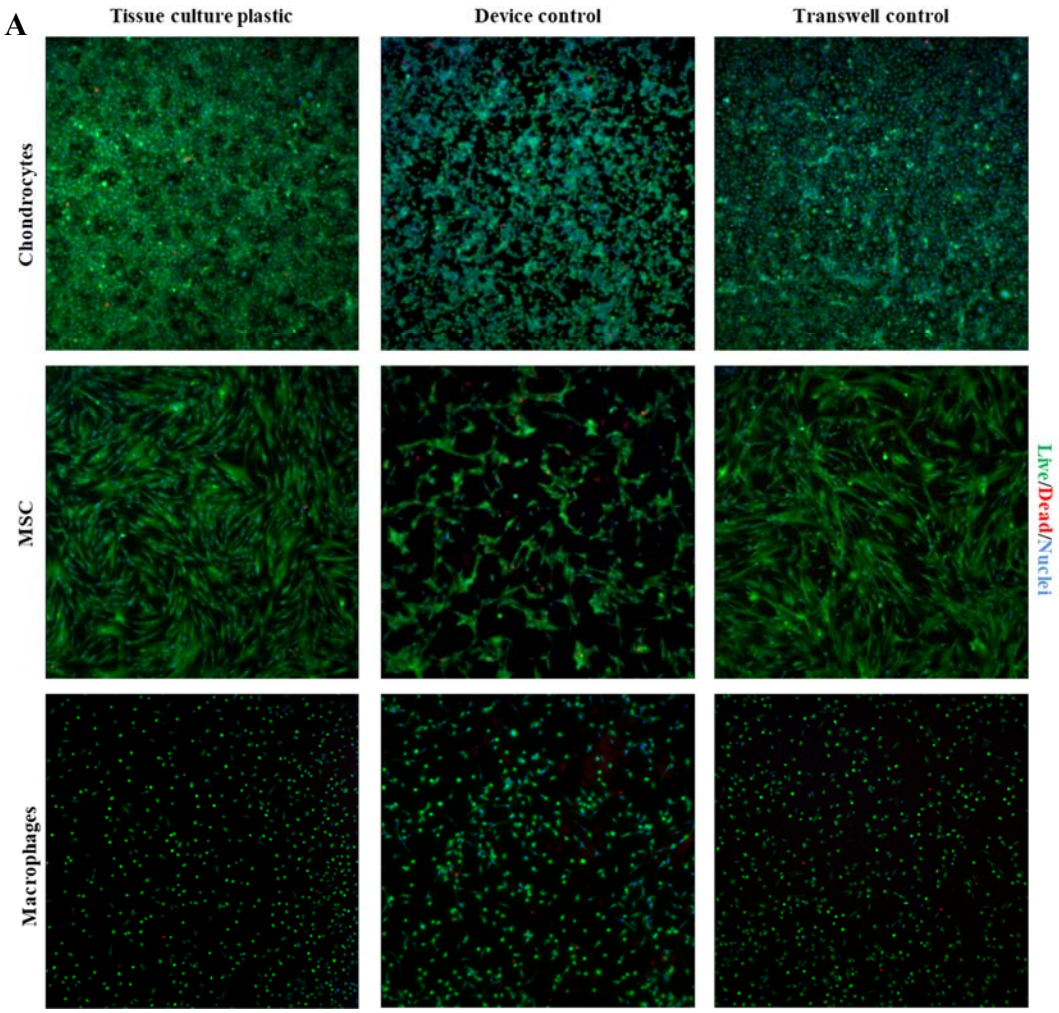
Figure 3.4. FITC-PLL coating. Membranes were imaged after PLL surface coating using phase contrast (A) and fluorescent microscopy (B). The presence of PLL coating is indicated as bright green fluorescence in image B.

3.3.3 Biocompatibility of stackable tissue culture insert

3.3.3.1 Cell viability

Our next set of studies was designed to utilize our device to investigate the viability and function of 3 distinct cell types, chondrocytes, macrophages, and MSCs, cultured alone in the stackable insert. Cells were seeded at a seeding density of $2.5x10^4$ cells/cm^2 in the stackable inserts to evaluate the effect of the growth substrate (PLL-coated microporous polycarbonate membrane) on cell viability (LIVE/DEAD assay) and function and compare it with commercially available products. All studies were conducted using the same seeding density for all 3 cell types to minimize changes in cell autocrine and paracrine signal transduction due to changes in the initial seeding density [1]. LIVE/DEAD fluorescent cells were visualized and quantified after

48hours in culture. Overall, it was observed that all cell types are capable of attaching and surviving on the stackable tissue culture insert for at least 48hours. We observed high cell viability independently of the substrate as shown in figure 3.5. This demonstrates that although in general the stackable insert can sustain cell viability after *in vitro* culture of cells from different linages.



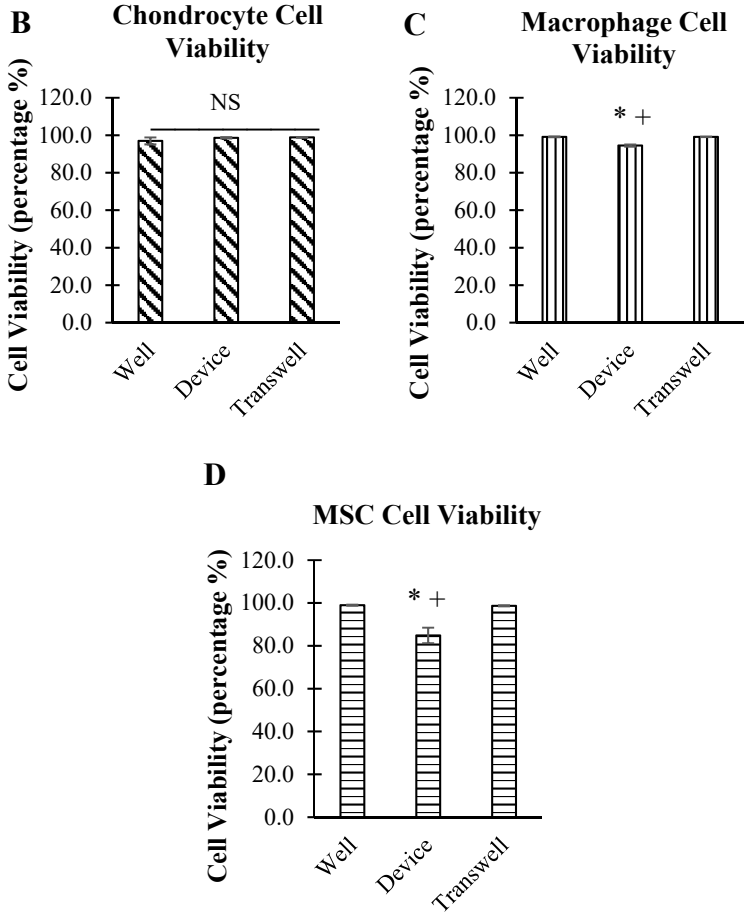


Figure 3.5. Cell viability comparison. (A) Representative images of cells seeded in the stackable tissue culture insert, TCPS well, or transwell inserts. Percentage of viable chondrocytes (B), macrophages (C), and MSC (D) in all growth substrates. The minimum viability values were 98%, 94% and 84% for chondrocytes, macrophages, and MSC, respectively when seeded in the stackable insert. Bar graphs represent the average percent viability (%) ± SEM of n= 8-24 images for at least 2 independent experiments ($P < 0.05$ as compared to Well (*), Device (#), or Transwell (+) by ANOVA with Fisher's LSD post hoc test). NS= not significant.

3.3.3.2 Cell Functional Secretion

Since one of our main interests by developing a stackable tissue culture insert is to study cell paracrine interactions, it was important to characterize the effects on cell secretory function. We first examined interleukin (IL)-8, a chemokine which recruits neutrophils to an injury site [2]. IL-8 is secreted by a variety of cell types including chondrocytes, pro-inflammatory macrophages and MSCs [3-5]. We compared secretion levels on all cell types with standard tissue culture and transwells. In our studies we found that IL-8 was secreted by all cell types. However,

macrophages had the highest secretion levels when cultured on all substrates, followed by chondrocytes and then MSC (FIG 3.6). Interestingly, IL-8 secretion values were comparable between the cells cultured on TCP and the stackable inserts, while cells seeded on transwell inserts had significantly lower secretion levels.

IL- 6 secretion was also measured, since this pleiotropic cytokine has been found to be constitutively secreted by chondrocytes (in low levels) and MSC, and secreted by macrophages when exposed to inflammatory stimuli [4, 6]. As indicated by FIG 3.6, IL-6 total secretion levels vary depending on the cell type, where MSC secreted more IL-6 when cultured on all substrates, followed by chondrocytes, and no secretion was detected in the macrophage supernatants. In addition, there was no significant difference in the level of IL-6 secreted by each cell type when comparing the stackable insert and transwell inserts.

Finally, tumor necrosis factor (TNF-) α levels were assessed by ELISA. TNF-α is a well described pro-inflammatory cytokine secreted by osteoarthritic chondrocytes and classically activated macrophages in the presence of inflammatory stimuli such as lipopolysaccharide (LPS). High levels of TNF- α could also indicate endotoxin contamination generated during the stackable insert fabrication and sterilization process [7]. However, the presence of TNF- α was not detected in any of the cell types for all substrates (data not shown). Collectively, these studies suggest that cytokine secretion can be substrate dependent, regardless of whether the substrates represent traditional “gold standards” such as TCP and transwells, or our novel stackable insert, further supporting the need for multi-culture substrate uniformity when developing multi-culture *in vitro* systems.

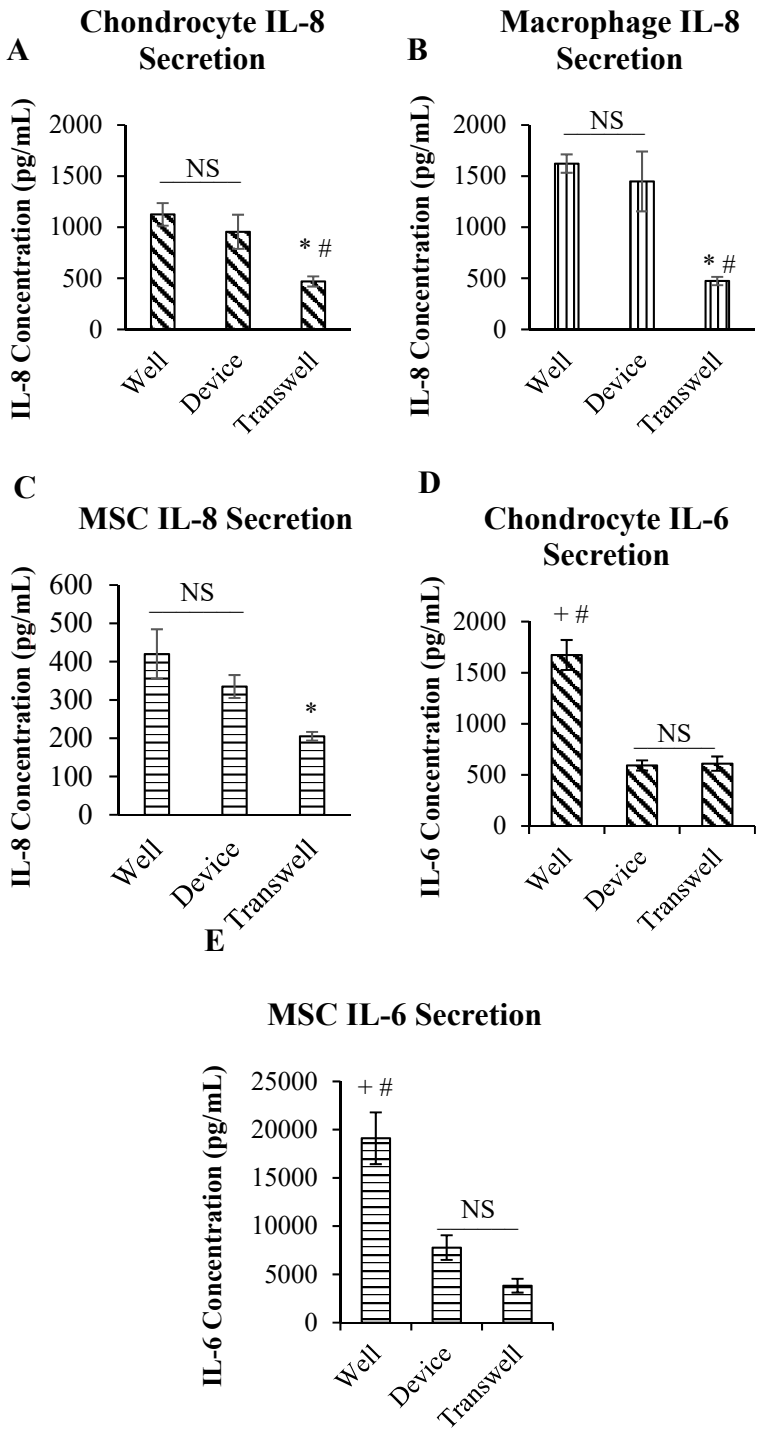


Figure 3.6. Cell cytokine secretion in different growth surfaces. IL-8 and IL-6 levels for A/D) chondrocytes, B) macrophages, and C/E) MSCs were measured from the cell culture supernatants after 48hours of culture. TNF- α was not detected in any of the cell types and IL-6 was not detected in the macrophage cultures. Bar graphs represent the average cell secretion ± SEM (pg/mL) of 3 independent experiments ($P < 0.05$ as compared to Well (*), Device (#), or Transwell (+) by ANOVA with Fisher's LSD post hoc test). NS= not significant.

3.3.4 Multi-culture Studies

Thus far our studies indicate that individual cell types can remain viable and maintain functional secretion in the stackable tissue culture inserts. In addition, the O_2 diffusion model indicated that cells will have adequate O_2 supply through the insert stack for a wide range of cultured cells per insert in a three insert per well configuration FIG 3.2-3.3. Therefore, studies were next designed to test the efficacy of our custom inserts in a tri-culture system, including chondrocytes, macrophages, and MSC in different spatial configurations as indicated in Table 3.2. Cell attachment and functional secretion influenced by paracrine signaling were assessed after 48 hours in culture.

Table 3.2. Tri-culture studies stack configurations. Stackable inserts seeded with chondrocytes, macrophages, or MSC were arranged in a well following six different configurations and cultured for 48hours.

Configuration	1	2	3	4	5	6
Top	Macrophages	MSC	Macrophages	Chondrocytes	Chondrocytes	MSC
Middle	MSC	Macrophages	Chondrocytes	MSC	Macrophages	Chondrocytes
Bottom	Chondrocytes	Chondrocytes	MSC	Macrophages	MSC	Macrophages

3.3.4.1 Cell attachment

After seeding the cells in the stackable insert and letting them attach overnight, the cells were placed in stacks containing 3 layers changing the spatial configuration of each individual cell type (configurations 1-6, Table 2). Overall, no statistically significant differences in cell attachment number were identified depending on the stack placement for chondrocytes and MSCs. However, macrophages had significantly lower cell counts in configuration #1 when compared to configuration # 3 and #4 (FIG 3.7B). Overall, the combined total cell numbers remained consistent across all spatial configurations (FIG 3.7D).

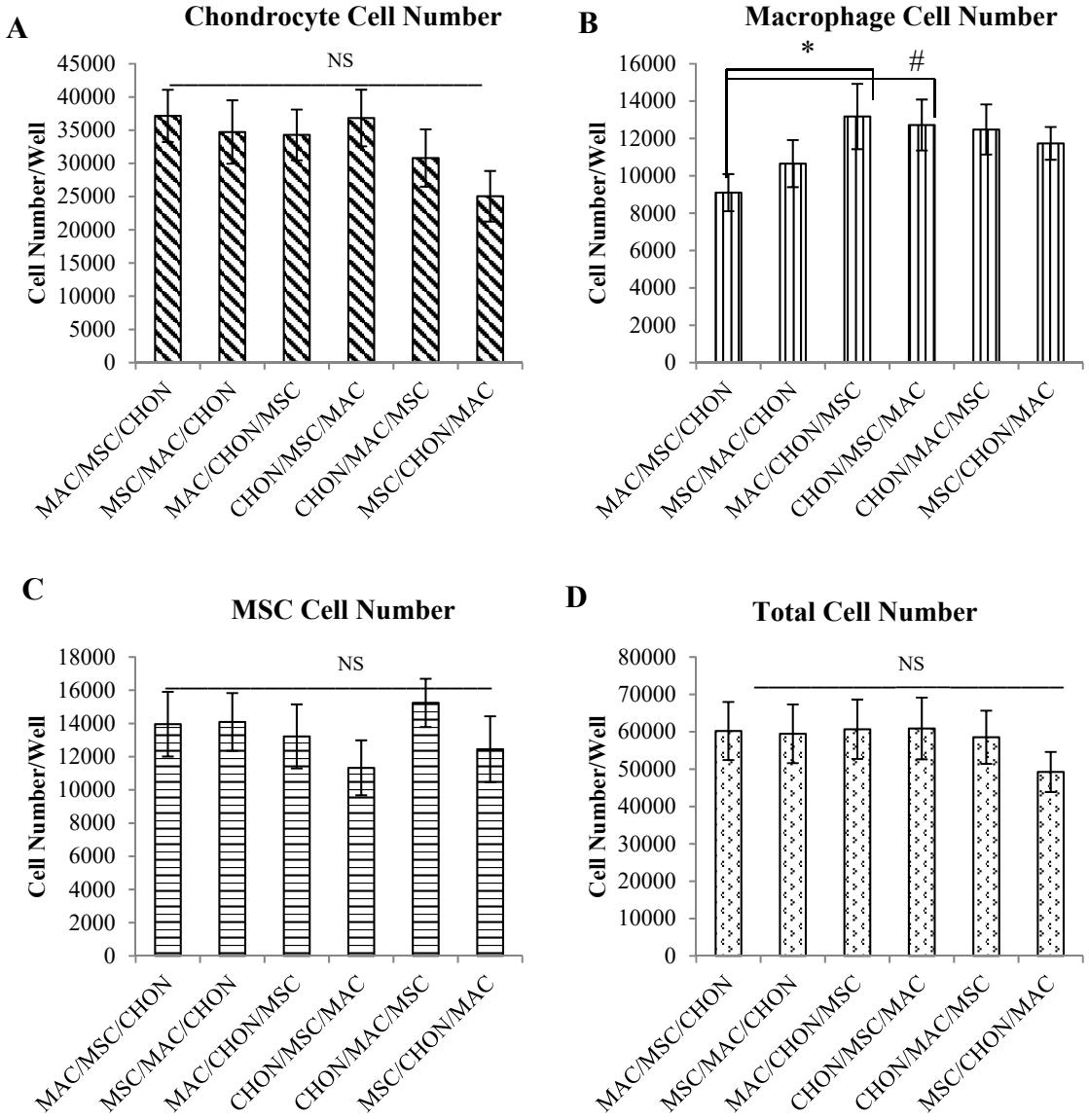


Figure 3.7. Different cell types cultured for 48hours in multiple stack configurations. X-axis indicates the configuration type and the location of the cells (Top/Middle/Bottom). A) chondrocyte cell counts, B) macrophage cell counts, C) MSC cell counts, and D) total cell counts of different culture configurations. CHON= chondrocytes, MAC= macrophages and MSC= mesenchymal stromal cells. Bar graphs represent the average cell counts per well ± SEM of n= 6-12 samples from at least 3 independent experiments ($P < 0.05$ as compared to configuration #3 (*) or configuration #4 (#) by ANOVA with Fisher's LSD post hoc test). NS= not significant.

3.3.4.2 Cell Functional Secretion

Similarly, we observed some variation (although not statistically significant) in total secretion levels of IL-8 (FIG 3.8A) and IL-6 (FIG 3.8B) for the tri-culture configurations when

comparing the order of culture stacks. In addition, the average total IL-8 levels (3,431.64 pg/mL) produced by the tri-culture configurations was in the same order of magnitude when compared to the total IL-8 secretion levels (2,738 pg/mL) produced by chondrocytes, macrophages, and MSC cultured separately in individual stackable inserts. TNF-α was not detected in any of the configurations.

Remarkably, the average total IL-6 levels (25,653.52 pg/mL) produced by the tri-cultures were 3 times higher than the sum of total IL-6 secretion s (8,369.25 pg/mL) produced by individual cells cultured separately in the stackable inserts. This demonstrates a dramatic synergistic and specific effect when 3 cell types are cultured together in a tri-culture configuration.

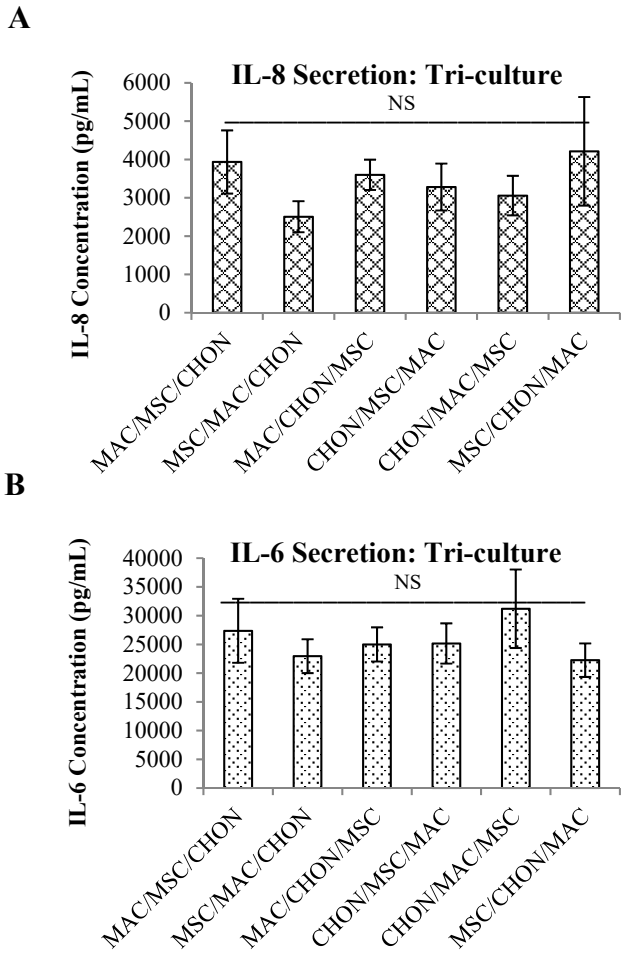


Figure 3.8. Total cell cytokine secretion in different tri-culture configurations. A) IL-8 and B) IL-6 were measured from the cell culture supernatants after 48hours of culture. While IL-8 secretion levels in tri-culture were relatively similar to the addition of the individual cell type secretion, the IL-6 tri-culture secretion was in average 3 times higher than the combined individual cell secretions. X-axis indicates the configuration type and the location of the cells (Top/Middle/Bottom). CHO= chondrocytes, MAC= macrophages and MSC= mesenchymal stromal cells. Bar graphs represent the average total cell secretion ± SEM (pg/mL) of n= 6-11 samples from at least 3 independent experiments. NS= not significant.

Table 3.3. Changes in cell functional secretion due to changes in culture configuration. In tri-culture configurations the IL-6 secretion levels are at least 3 times higher than the IL-6 sum of individual cell culture secretions (single chondrocytes, macrophages, and MSC) showing a synergistic effect. However, IL-8 secretion levels in tri-culture are similar to the IL-8 sum of individual cultures.

Single Device Culture	IL-6 (pg/mL)	IL-8 (pg/mL)
Chondrocytes (CHON)	592.49	955.16
Macrophages (MAC)	0	1,447.72
Mesenchymal stromal cells (MSC)	7,776.75	335.13
Addition of cell secretion averages	**8,369.25**	**2,738.00**

Tri-culture Configurations	IL-6 (pg/mL)	IL-8 (pg/mL)
MAC/MSC/CHON	27,365.57	3,934.75
MSC/MAC/CHON	22,948.77	2,505.54
MAC/CHON/MSC	24,993.99	3,598.81
CHON/MSC/MAC	25,155.43	3,280.13
CHON/MAC/MSC	31,202.14	3,057.31
MSC/CHON/MAC	22,255.24	4,213.28
Average secretion	**25,653.52**	**3,431.64**

3.4 Discussion and Conclusions

In these studies, we describe the development of a microporous membrane based stackable insert for cell culture comprised of a polycarbonate membrane attached to an acrylic support which is easy to fabricate and to handle. The microporous membrane surface can be chemically modified and coated with polymers to support the attachment of a wide array of cell types. The stackable insert surface allows for cells to adhere and proliferate while the small pore size, but high pore density, permits the diffusion of O_2 and signaling molecules between stacks

enabling paracrine cell interactions. In addition, the separation between each insert layer can be controlled by varying the thickness of the acrylic support in between the stacks, which allows for the precise placement of cells at distances that better mimic their *in vivo* niches. With this system, different cell types can be seeded separately and then co-cultured together in different layers or at different times allowing for the spatial-temporal manipulation of the culture system to characterize cell physiological responses and even mimic disease phenotypes such as OA.

Initial studies focused in characterizing the stackable insert's biocompatibility, ease of use, and potential for tissue culture applications by examining the cell viability and functional secretion of 3 different cell types present in articular joints, chondrocytes, macrophages and MSC. Each was seeded on a stackable insert and compared to 2 commercially available products, tissue culture multi-well plates and transwells. Our studies showed that after 48hours in culture, all cell types exhibited high cell viability on all substrates. However, all cell types had distinct cytokine secretions depending on the growth substrate. These results highlight the inherent differences with common tissue culture substrates and their effects on cellular function.

Although the stackable inserts, multi-well plates, and transwells are made of different materials, it is common practice to perform co-culture studies with mixed growth substrates. Generally, co-culture studies using commercially available tissue culture inserts (transwells) are performed by seeding one cell type in tissue culture treated polystyrene (TCPS) multiwell-plates and another cell type in transwells [1]. Transwell inserts have a smaller effective growth area than their corresponding well-plates and use a variety of growth substrates such as polyethylene terephthalate (PET), polycarbonate (PC), or ECM coated polytetrafluoroethylene (PTFE) membranes, which are selected depending on the desired application[2]. The miss-match in cell growth substrates could introduce additional variability to the system. The cell-material interactions are dependent on multiple factors such as material composition [3], surface

topography [4-6], surface charge [6-8], pore size [9], pore density [10], surface wettability [6, 11], availability of functional groups [7, 12, 13], protein adsorption [7, 14, 15] etc., which affect how cells attach, spread, proliferate and function [5]. For example, HEK 293 cells have been shown to preferentially attach to a PC surface over PET or PTFE surfaces in commercially available tissue culture inserts[16]. It has also been documented that growth surface differences can affect cell phenotypes. In a study performed by Rostam et at., monocyte-derived macrophages differentiated into pro-inflammatory or anti-inflammatory macrophages in response to PS altered surface chemistry [17]. In this study, they hypothesized that changes in macrophage polarization could be due to interaction with different proteins adsorbed into the modified PS surface. Indeed, different physicochemical procedures to make these materials compatible with cell culture can introduce a variety of functional groups and/or surface etching that can favor the adsorption of a variety of proteins present in culture media serum or promote the cells to secrete ECM, thereby differentially affecting the ability of cells to attach, spread and grow [8, 18, 19]. In our studies we observed changes in cell density and morphology for the MSCs seeded in the stackable insert. This could be due to the cell-growth surface interactions produced by our insert fabrication protocol and might indicate that cell-specific surface modifications might be needed in future studies.

Using radiofrequency or microwave plasmas for tissue culture surface modifications is the current industry standard [20]. Therefore, the TCPS well-plates and the PET transwells used for this study are most likely modified with plasma treatment like the stackable insert; however, the plasma composition and exact procedures used in a commercial setting are unknown as each company has its own process, making a direct product comparison challenging. As evidenced by our studies, even commercially available products stimulate differential cell secretion indicating that there is not necessarily a “gold standard”. Therefore, even though the stackable insert also induced changes in cell secretion, it provided the advantage of conducting more controlled

experiments by eliminating confounding variables due to variances in growth substrates [16] and changes of effective growth area [21] when used to culture multiple cells together.

To test the stackable insert in a multi-culture experimental setting, chondrocytes, macrophages, and MSC were seeded on the stackable insert in separate wells and then stacked together in different spatial configurations. After 48hours in culture, the stacks were separated, and the cells layers were analyzed for attachment. Generally, no significant differences were observed in cell numbers at the bottom and middle of the stack indicating that cells remained attached even in deeper layers that were covered by more cell-containing layers, which has been shown to be a limiting factor in other 3D systems due to poor O_2 and nutrient diffusion to lower layers [11, 14]. This phenomenon was originally predicted by our O_2 diffusion model indicating that at the utilized seeding density and height of the stack, enough O_2 will be diffusing to all layers inside the well, therefore, not negatively affecting cell viability. Surprisingly, we did observe significant differences in cell attachment for macrophages in configuration #1, where they are in the top layer. However, configuration #3 also had macrophages placed in the top layer and it did not have lower macrophage cell attachment. 3D culture systems have consistently shown better cell viability, attachment and proliferation in the upper layers due to better access to O_2 and other nutrients [22]. However, our results show conflicting data for certain configurations. We hypothesize that these differences in cell attachment are due to a “neighboring cell” effect rather than nutrient availability. As cell signaling between neighboring cells can lead to changes in cellular physiology such as proliferation, differentiation, migration, tissue repair, etc; this specific culture configuration promoted macrophage detachment or death over the 48hours culture period.

To further probe changes in cell secretory function, the secretion levels of IL-6, IL-8, and TNF-α were measured as high levels of these cytokines have been shown to induce cell

detachment, apoptosis and/or necrosis in other experimental systems [23-25]. Although variable in their total concentration, IL-6, IL-8, and TNF-α (not detected) did not have significant differences among the tested configurations. Interestingly, we observed cytokine specific synergistic cell interactions in all the tri-culture configurations. IL-6 secretion was on average 3 times higher with cells in tri-culture than the combined secretion of the individual cells seeded in the stackable device. While IL-6 showed a synergistic cell interaction, IL-8 total secretion remained an additive effect indicating that this phenomenon is cytokine specific. Using the stackable insert we were able to interrogate paracrine cell interactions while eliminating confounding variables such as cell response to different growth surface composition and the effects of cell-cell interactions in mixed cultures due to the lack of an easily accessible platform to culture multiple cell types in the same well. Further studies with an expanded panel of cytokines and growth factors will be conducted to provide a better understanding of the changes in cellular physiology and paracrine signaling due to changes in cell interactions and spatial arrangement. In addition to extending the cytokine panel, studies testing the cellular response to osteoarthritic inflammatory stimuli will be completed.

One of the major advantages of the stackable tissue culture insert is its versatility. In addition to changes in spatial configuration of different cell types, spatial-temporal interactions among cells could be studied by adding or removing layers from the stack at different time points. More complex *in vitro* systems where cell-ECM interactions need to be considered can be established by modifying the surface of the porous membrane, coating the insert with biomaterials or adding cells embedded in a hydrogel on it. Inflammatory disease models like arthritis could be replicated by stimulating one or multiple cell layers and if cell migration studies are required, an insert with a larger pore size could be fabricated with ease.

3.5 Supporting information

To characterize cell attachment to the different growth surfaces, we quantified Hoechst stained nuclei 48hours after culture, using image-based cell counting. Cell attachment was cell type and growth surface dependent as demonstrated in Table 3.1.1. When comparing the final number of attached cells per growth area in the different substrates, it was evident that chondrocytes attached to all growth surfaces and although cell attachment per growth area was ~20% lower in the stackable tissue culture insert than in TCPS, these differences were not statistically significant (FIG 3.1A). Interestingly, macrophages and MSC had higher cell attachment to transwells in comparison to TCPS or the stackable insert. Approximately, 43% and 97% higher macrophage attachment was observed to the stackable insert and transwells inserts, respectively (FIG 3.9B). In contrast, MSCs had ~41% lower cell attachment to the stackable insert and 44% higher cell attachment to transwells inserts when compared to TCPS (FIG 3.1.1C).

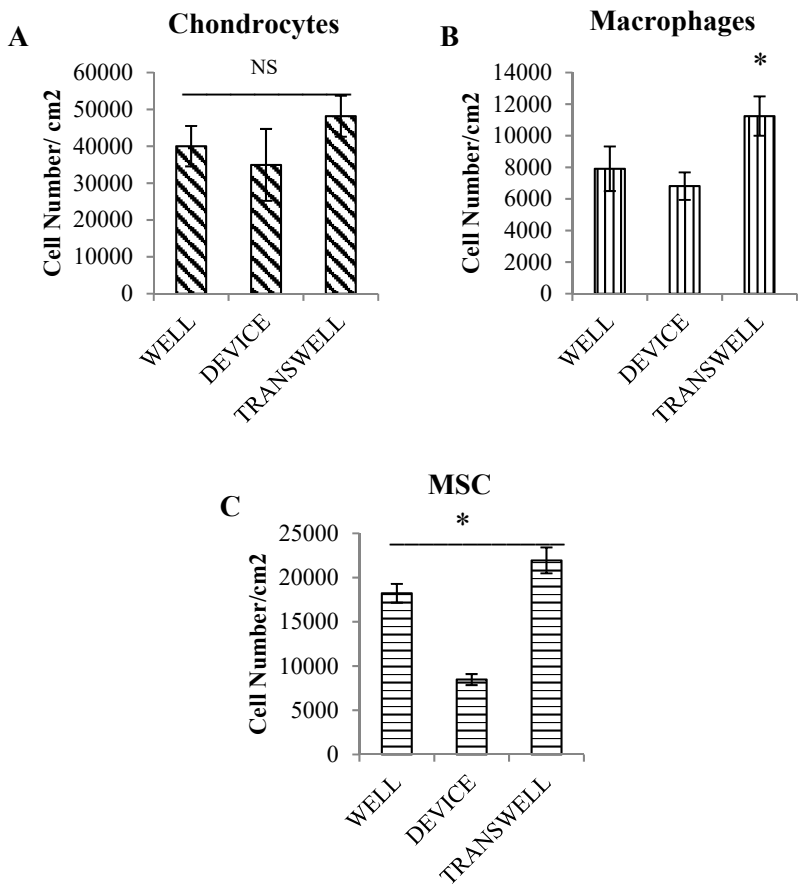


Figure 3.1.1. Total cell counts and attached cells per area of different cell types. Cells were seeded at $2.5X10^4$ cells/cm^2 in TCPS, the stackable inserts, and transwell inserts. Overall, cells showed cell- and substrate- dependent cell attachment to the different growth surfaces. Bar graphs represent the average total cell number per well (A-C) or average cell number per growth area (D-F) ± standard error of mean of n=9-18 samples, 4 independent experiments. * indicates statistically significant differences of a group compared to all others ($p<0.05$)

Table 3.1.1. Comparison of attached cells after 48hours of culture with initial cells seeded. Percentages were calculated based on the total cell counts when compared to the initial number of cells seeded. Data represents the percent average ± SEM of n=9-18 individual samples.

	Percent Attachment (%)		
Growth Surface	**Chondrocytes**	**Macrophages**	**MSC**
Well	160 ± 21 %	32 ± 6 %	73 ± 4 %
Device	140 ± 39 %	25 ± 4 %	34 ± 2 %
Transwell	193 ± 22 %	45 ± 5 %	88 ± 6 %

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CHAPTER 4: CONCLUSIONS

4.1 Conclusions of the Dissertation

The studies completed in this dissertation have led to the establishment of a proof-of concept monolayer chondrocyte and macrophage model of OA to study the effect of potential new treatments on chondrocyte inflammatory, metabolic, and chondrogenic function and to compare these with standard treatment options. In addition, this system can provide us with an important avenue to unravel the therapeutic mechanistic differences among treatments.

Chondrocytes stimulated with low (IL-1) or high (IL-1/TNF) levels of inflammation 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. These gene expression changes occur during disease development when the metabolic balance in articular cartilage is tilted towards catabolism [1]. Although *in vitro* models of chemically stimulated chondrocytes are common, studies generally focus in one disease component (i.e. inflammation, chondrogenesis, or tissue regeneration); therefore, our approach to use a panel of chondrocyte functional genes provides a more comprehensive approach to investigate disease biomarkers, mechanisms of progression, and test the efficacy new therapies. To validate the predictive capability of the *in vitro* system, we treated OA-stimulated chondrocytes with dexamethasone. As expected, we observed a down-regulation of pro-inflammatory cytokine genes and even promotion of pro-chondrogenic genes; which has been previously reported by other studies [2, 3].

Disease modifying treatments such as mesenchymal stromal cell (MSC) therapies interact with the inflammatory joint milieu and respond accordingly by adjusting their secretome to

attenuate inflammation [4]. However, caution must be exerted when selecting *in vitro* systems to evaluate a disease pathophysiology or the therapeutic efficacy of new treatments as simple monoculture cell systems could skew the study conclusions [5, 6]. Our studies reflect this phenomenon by showing that MSC and alginate-encapsulated MSC (eMSC) treatment exacerbate inflammation and promote a catabolic state in OA-stimulated chondrocytes in monocultures. However, when OA-stimulated chondrocytes and macrophages seeded in co-culture were treated with MSC, we observed a drastic decrease in pro-inflammatory cytokine secretion. These results are in agreement with previously reported studies where a decrease of inflammatory markers in the synovium and cartilage has been observed after intra-articular injection of freely migrating MSC [7]. This data highlights the importance of considering multi-cellular interactions when studying complex systems such as the articular joint.

Several *in vitro* systems have been developed to study cell paracrine, juxtacrine, and cell-ECM interactions [8-11]. For example, commercially available tissue culture transwells have been widely used to study cell interactions in co-culture. However, when more than two cell types are needed, researchers have to rely on mixed cell cultures which do not necessarily recapitulate the *in vivo* cell niche. Another approach is to co-culture cell embedded in a biocompatible scaffold with multiple layers; however, cells tend to migrate across layers resulting in mixed cell cultures [12-14]. In addition, cell separation and isolation becomes challenging in this type of setup. Therefore, we developed a stackable tissue culture insert that allows for the culture of different cell types in a well, to study cell paracrine interactions. We demonstrated that this new device can be modified to promote the attachment and growth of cells from different linages, can be stacked with different cell layers at variable distances to recreate a tissue and then easily destacked to analyze individual cell components; providing a versatile tissue culture tool.

To further investigate the interactions among MSC, chondrocytes, and macrophages, we used the stackable insert to establish a tri-culture system. Using this system, we demonstrated that independently of the position of a stack in the well (top, middle, or bottom), the cell attachment is not affected. This was a critical parameter for the development of the stackable insert, since other studies have reported oxygen and nutrient gradients affecting cell function; particularly in the bottom layers [8]. In addition, these results confirm our oxygen diffusion model predictions, where we indicate that at the utilized seeding density a maximum of 8 layers could be stacked in a well without compromising oxygen transport. However, we did observed changes in cell attachment for one tri-culture configuration in the top layer which were mostly likely due to cell paracrine interactions.

Interestingly, the tri-culture system promoted cytokine specific synergistic secretion. In all culture configurations, we observed an approximately 3X increase in IL-6 secretion when compared to monocultures total IL-6 secretion. However, we did not observed the same behavior for other tested cytokines. Using the stackable tissue culture insert enabled us to understand 1) the interaction of all three major OA cells types in our model system, 2) by varying the tri-culture configuration, we were able to gain some insight on the relative interaction of each of the cell types with its neighbors and, 3) by culturing all cell types together we were able to observe cytokine specific synergistic effects that cannot be reproduced with commercially available tissue culture devices.

4.2 Future work and other considerations

4.2.1 Effects of cell ratio and MSC treatment dose

In the present studies, we did not examine the effects of cell ratio, as all cell types were cultured at a 1:1:1 ratio since macrophages and MSC, and MSC and chondrocytes have been previously co-cultured at this ratio showing attenuation of inflammation and pro-chondrogenic function [15, 16]. However, the optimal ratio of MSCs-chondrocytes-macrophages and of chondrocytes-macrophages still needs investigation in further studies.

4.2.2 Expansion of cytokine panel and biochemical analysis

To characterize the *in vitro* system described in Chapter 2 of this dissertation, we performed a bovine RT-PCR gene panel comprised of pro-inflammatory cytokines, ECM remodeling proteins, and chondrogenic genes. However, at the protein level we were able to perform only IL-8 ELISAs due to the lack of well-developed ELISA assays for bovine proteins. We attempted to develop ELISAs with paired antibodies for IL-1, TNF, MCP-1 and IL-6; however, we were not successful. Therefore, other biochemical assays should be used to characterize cell responses such as collagen and proteoglycan content and secretion to the media [17].

4.2.3 The use of primary cells versus cell lines

Primary bovine chondrocytes, peripheral blood-derived macrophages, bone marrow derived MSC and the C28/I2 chondrocyte cell line were used in our studies. The use of each cell type brings several advantages and disadvantages to our experimental setup. First, using primary bovine chondrocytes at low passages and high seeding density maintains their chondrocytic phenotype allowing us to study chondrocyte responses to different stimuli; however, these cells cannot be expanded over a long period of time because they dedifferentiate. In addition, by using primary cells, the cell number that can be used is limited making it necessary to obtain more cells

from different donors. This can result in intra-experimental variability as described by Gray et al. [18]. On the other hand, cell lines are geared towards proliferation, can be maintained with ease, and can be expanded over multiple passages without affecting their function allowing for the establishment of standardized systems. However, their gene expression is not identical to primary chondrocytes, particularly in their ECM metabolism. Now that we have established a tri-culture system using the stackable tissue culture insert and C28/I2 chondrocytes, MSC and macrophages, we will replicate the previous experiments using primary bovine chondrocytes to compare viability and secretion to the C28/I2 cell line, assess inter-species cell interactions, and observe if chondrocyte inflammation is attenuated in this system.

4.2.4 Validate the stackable insert tri-culture as an in vitro model of OA

While the studies we described thus far support the incorporation of our approach in baseline culture systems, to validate our tri-culture system as an *in vitro* model of OA, molecules and drugs with previously known effects on joint tissue will be used. Catabolic mediators such as TNF and IL-1; pro-anabolic factors such as TGF-β and IGF-1; Naproxen, an anti-inflammatory drug; and Dexamethasone a corticosteroid, will be added to the system and their effects will be characterized with biochemical assays. Protein secretion will be monitored by ELISAs.

4.2.5 Introduce other joint cell types and cell-ECM interactions in the stackable insert system

While the systems described in this dissertation are useful to study cellular physiology, the lack of both structural and matrix-effects might limit their ability of making more accurate predictions on treatment efficacy. Therefore, further studies incorporating engineered tissue constructs and stackable inserts will be performed. 3D *in vitro* constructs that have been developed to study OA and/or cartilage defects repair include chondrocytes and/or osteoblasts seeded in homogeneous [19, 20] or spatially-varying matrices [21, 22]. However, no models are currently available that consider all the components of joint tissue including , 1) all of the

composite cell types, 2) the spatial distribution of each of the cell types, 3) ECM composition and its spatial distribution, and 4) native mechanical properties of cartilage tissue components; all of which have been found to have a critical role in promoting OA inflammation [23, 24]. Multi-cellular 3D constructs will be significant in elucidating the pathogenesis of degenerative joint diseases as well as assessing the efficacy of potential therapeutics against the disease by recapitulating the complex signaling occurring in the joint [22, 25]. Therefore, including cells such as synovial fibroblasts and osteoblasts will provide additional cell paracrine and cell-cell interactions that can change the inflammatory milieu in the system. In addition, growth surface modifications that better mimic the molecular and architectural cues that cells experience *in vivo* will provide cell-ECM interactions to make a more comprehensive system. The 3D *in vitro* model will be compared with current models such as engineered cartilage constructs made by our collaborators [17, 26].

4.3 References

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