Master's Thesis of Philosophy degree

# Anti-inflammatory effect of hUC-MSCs derived CM through inhibition of E-selectin-dependent monocyte recruitment in osteoarthritis chondrocytes

## 골관절염 연골세포에서의 E-selectin 의존성 단핵구 동원 억제를 통한 인간 탯줄유래 중간엽 줄기세포 배양액의 항염증 효과

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# Anti-inflammatory effect of hUC-MSCs derived CM through inhibition of E-selectin-dependent monocyte recruitment in osteoarthritis chondrocytes

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# Abstract

**Introduction:** Osteoarthritis (OA), which affects more than 80% of the elderly over the age of 70, is one of the most common diseases in modern society. Currently, there are no drugs on the market that address fundamental biological causes other than the prescription of NSAIDS drugs for pain and inflammation control and the administration of corticosteroids. Recent studies have highlighted the importance of immune cells in the development and progression of osteoarthritis. Macrophages, particularly M1 cells that have been activated, produce cytokines that play an important role in provoking osteoarthritis inflammation. Recently, Mesenchymal Stem Cells (MSCs) have drawn attention for their trophic and immunomodulatory properties that facilitate tissue regeneration and repair. This study confirmed that chondrocyte cells exposed to an IL-1β environment expressed increased levels of E-selectin signal through NGS analysis, which suggests that they mobilize the monocytes expressing counter receptor to the IL-1β lesion environment. Monocytes are capable of polarization into M1 macrophages which release inflammatory substances when exposed to various inflammatory cytokines found in the body of patients with osteoarthritis. The aim of this study was to confirm the anti-

inflammatory effect of conditioned medium(CM) obtained from cultivating human umbilical cord(hUC)-derived MSCs by assessing its inhibitory effect on the recruitment of monocytes mediated by overexpressed E-selectin in OA chondrocytes.

Methods: We separated cartilage cells from seven chronic osteoarthritis patients undergoing knee replacement surgery, and hUC-MSC separation was accomplished by obtaining umbilical cords from mothers undergoing cesarean sections. The CM that was obtained by treating the separated hUC-MSC with IL-1β was then applied to chondrocyte cells exposed to an IL-1β environment. Experimental groups were conducted in control, IL-1β, and CM groups to determine the effect of CM on the IL-1β lesion environment and to examine the expression of inflammatory and anti-inflammatory cytokines, matrix enzymes, and matrix enzyme inhibitors at the gene and protein levels of chondrocytes. Furthermore, the anti-inflammatory properties of hUC-MSC CM were evaluated by examining the degree of adhesion of chondrocyte E-selectin-dependent monocytes, which were co-cultured directly with three groups of chondrocytes for one hour.

Results: The results indicate that matrix degradative enzymes such

as MMP1, MMP3, MMP13 and ADAMTS4 expression decreased by 7.4%, 31.6%, 21.8%, and 44.9% respectively and matrix prevention factors such as TIMP1 and TIMP3 expression increased by 128.9% and 68.4% respectively when chondrocytes exposed to an IL-1β environment were treated with hUC-MSC CM. Based on the MLDEG analysis method, 126 pro-inflammatory genes and 238 anti-inflammatory genes were found to be differentially expressed in chondrocytes between groups receiving IL-1β and CM. Further analysis was conducted using combined p-value <0.02, combined logFC>1, along with existing prior knowledge, resulting in the identification of five infectious genes (CXCL10, SELE, CAGE1, MYB, and IP6K3) and one anti-inflammatory gene (PTPRF). The SELE gene was selected as the final gene for further investigation, as it is linked to monocyte recruitment and is associated with OA progression. Measurement of E-selectin expression in chondrocytes in each group revealed that hUC-MSC CM showed a reduction of 21.8% at the protein level compared to chondrocytes treated with IL-1β. Following confirmation of the degree of binding to monocytes according to the amount of E-selectin expressed in chondrocytes in each group, the binding to monocytes in the CM group decreased by 53.9% on average compared to the IL-1β group. The expression of E-selectin in chondrocytes was further reduced through blockade

used which decreased by 42.1% in the IL-1β group and 41.8% in the CM group in comparison to the groups that did not use blockade. The interaction between monocytes and E-selectin has been confirmed.

**Conclusion:** hUC-MSC CM reduces the likelihood of E-selectin expression in chondrocytes, and inhibits recruitment of monocytes to the lesion site, indicating its potential for use as an alternative treatment in the current market without drugs capable of treating the underlying biological cause.

**Keyword:** hUC-MSC; Chondrocyte; Osteoarthritis; Inflammation; Monocyte; Macrophage

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# Table of Contents

# List of Tables

# List of Figures

# List of Abbreviations

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs

CM, conditioned medium

COX-2, Cyclooxygenase-2

hUC-MSC, human umbilical cord mesenchymal stem cell

I$\kappa$B, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

IL, interleukin

MMP, matrix metalloproteinase

NF-$\kappa$B, nuclear factor kappa-light-chain-enhance of activated B cells

OA, osteoarthritis

TIMP, tissue inhibitor of metalloproteinase

TNF-$\alpha$, tumor necrosis factor-$\alpha$

# Chapter 1. Introduction

## 1.1. Study Background

Osteoarthritis (OA) is a chronic disorder of the musculoskeletal system that affects the joints, including the knees and hips. OA is a growing threat to public health in an aging society(1). OA can occur as a result of changes that occur in joint tissue as we age. Increased senescent chondrocytes for instance result in an increase in the production of matrix metalloproteinase(MMPs) and cytokines in the joints(2). OA is a pathological process mediated by pro-inflammatory cytokines, particularly IL-1β and TNF-$\alpha$, which exert an influence on both the quality and quantity of the cartilage extracellular matrix (ECM). IL-1β and TNF-α trigger the release of MMP-1, MMP-3, and MMP-13, which inhibit collagen type II and aggrecan expression in chondrocytes(3-5). The typical OA patient has severe joint pain, stiffness, and reduced mobility, which adversely affects their productivity and quality of life, not to mention their financial situation(6). With an aging population around the world, OA poses a significant challenge for the medical establishment due to its high prevalence with age (7). Knee osteoarthritis currently has limited treatment options. There are a number of conventional treatments

available for osteoarthritis, such as nonsteroidal anti-inflammatory drugs (NSAIDs), pain-relieving drugs, platelet-rich plasma (PRP), hyaluronic acid, or corticosteroid-based intra-articular injections, and knee arthroscopic surgery. The underlying biological causes of osteoarthritis, however, cannot be treated with any of the treatment options listed above(8, 9).

Currently available evidence suggests that concurrent inhibition of several pro-inflammatory cytokines may be more effective in the treatment of OA(10). There has been an increase in research on the anti-inflammatory abilities of mesenchymal stem cells. In addition to release anti-inflammatory factors, such as IL1RA, a variety of trophic mediators can be secreted and exert paracrine effects on other cell types(10, 11). MSCs also suppress inflammation by decreasing monocyte activation(10).

Despite a lack of understanding of the disease's pathogenesis, an increasing number of studies demonstrate that monocyte migration plays an important role in perpetuating inflammation in the synovium(12). Through the synovial fluid, synovial monocytes cause inflammatory edema, thickening of the synovium lining cells, and apoptosis of chondrocytes(13). Inflammation and cartilage destruction may occur as a result of excessive transmigration of monocytes and abnormal activation of

monocytes(13). Earlier studies have indicated that in OA synovium, monocyte adhesion molecules such as ICAM-1 and VCAM-1 are more abundant. Increased expression of these molecules may indicate the development of osteoarthritis, since monocyte adhesion molecules stimulate monocyte recruitment and infiltration, triggering further inflammation in the synovium(14-16). The downregulation of adhesion molecules in synovial fluid has therefore been proposed as a means of suppressing the inflammatory activity associated with OA while improving symptoms(17, 18). In OA patients, the expression of E-selectin, one of the monocyte adhesion molecules, is also increased in cartilage and synovium, along with VCAM-1 and ICAM-1, which is also associated with better monocyte recruitment(19-22). From the previous study, it has been established that there is a prolonged increase in E-selectin expression as a result of co-cultivating monocytes and endothelial cells(20) and it is expected that more monocytes can be recruited due to the prolonged increase in E-selectin expression as a result. Thus, it can be seen that an increased expression of E-selectin leads to an increase in monocyte recruitment which ultimately causes a vicious cycle. In spite of this, there have been very few studies regarding the role of E-selectin in synovium fluid.

Next-Generation-Sequencing (NGS) platforms have made it possible to analyze functional gene ontology classifications using RNA-sequencing (RNA-seq), allowing for the annotation and quantification of a large number of genes in one run(23). This study utilized NGS analysis methods using MLDEG to identify new therapeutic target molecules related to OA progression using UC-MSC-derived CM treated chondrocytes to approach more fundamental biological treatments.

## 1.2. Purpose of Research

The purposes of the study were aimed to examine the role of anti-inflammatory paracrine effect of hUC-MSCs derived CM on chondrocytes in OA. The hypothesis was that hUC-MSC-derived CM would have a paracrine anti-inflammatory effect on chondrocytes in OA by inhibiting pro-inflammatory cytokines and, therefore, provide a more fundamental biological treatment for OA.

# Chapter 2. Materials and Methods

## 2.1 Tissue samples

Informed consent was obtained from all patients from whom tissue specimens were harvested as required by our institutional review board (IRB No. 06-2012-78). The study protocol was approved by the review board and implemented according to approved guidelines.

### 2.1.1 Isolation and cultivation of hUC-MSCs

UCs were prepared via cesarean section after full-term delivery. Each UC was washed twice in Dulbecco's phosphate-buffered saline (DPBS; Welgene, Daegu, Korea) to remove blood products before use. Once the UC length and weight have been measured and calculated, each UC was cut into pieces with a thickness of 2-4 mm using surgical scissors. We aligned 1g slices of UCs per 150 pi dish and allowed them to provide a firm attachment to the bottom of the dish for 1 h in a 5% $CO_2$ incubator with humidified air at 37℃. Into the culture dishes, the culture medium, which consisted of low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) and antibiotic-antimycotic solution (100 U/ml penicillin, 100 $\mu$g/ml streptomycin,

and 0.25 μg/ml amphotericin B, Welgene), was gently poured. Non-adherent cells were removed by changing the culture medium every 2-3 days. Cells were detached when they reached 80% confluency by incubating them for 3 minutes with trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 0.53 mM EDTA; Welgene)and leftover tissue was filtered out by using a 100 μm cell strainer (SPL Life Sciences, Pocheon, Korea). After centrifuging at 500g for five minutes at 20℃, the cells were replated at 3,333 cells/$cm^2$, which is passage 1 (p1).

### 2.1.2 Isolation and cultivation of primary chondrocytes

The chondrocytes were isolated from OA patients who underwent total knee arthroplasty (TKA) performed at Seoul Metropolitan Government-Seoul National University Boramae Medical Center. Articular cartilage was harvested from femoral condyles of 7 patients (7 women; mean age: 67.4±5.8 years). Depending on the macroscopic condition of the cartilage, distal and/or posterior femoral condyles were used for chondrocyte isolation. To prevent drying of the bone-cartilage tissue and to wash away fat tissue attached to the bone, it was transferred to a petri dish filled with DPBS. In order to extract cartilage from the

surface of the bone cuts, No. 10 blades were used, and the weight of the obtained cartilage was calculated and measured. Minced cartilage into 1x1mm pieces, then put collagenase type II in a serum-free high-glucose DMEM at 0.6% and digested for 6 hours with gentle agitation. Centrifugation of the sample at 500g for 3 minutes, 20℃ was performed after equal volumes of DPBS were added to it. Resuspended the pellet with 20 mL of DPBS and filtered through a 100 $\mu$m cell strainer. Centrifugation for 3 minutes at 500g, 20℃ for the last time and resuspended the pellet with complete growth media and counted total isolated cell number. Seeded isolated cells at a concentration of $1x10^4$ cells/$cm^2$ and replaced culture medium 1 or 2 days after the initial seeding. The culture medium was changed every three days and cells were split at a ratio of 1:3 when it reached 80% of confluency.

## 2.2 Preparation of hUC-MSC conditioned medium (UC-CM)

Isolated hUC-MSCs from UC were sub-cultivated to p10 and seeded in T-225 flasks using complete media at $2.5x10^4$/$cm^2$. After 24 hours of incubation in a 5% $CO_2$ incubator with humidified air at 37℃, cells were washed with DPBS, followed by serum free low-glucose DMEM, and then incubated for 24 hours with 45 mL of serum free low-glucose DMEM which included 10 ng/mL of IL-1β.

Following incubation, the cells were washed twice with DPBS and once with serum free low glucose DMEM, followed by 45 mL of serum free low glucose DMEM with 1% AA and incubation for an additional 24 hours. Harvested the conditioned medium and centrifuged at 1000g of 25℃ for 5 minutes to remove the debris in the conditioned medium and stored only the upper layer liquid at −80℃.

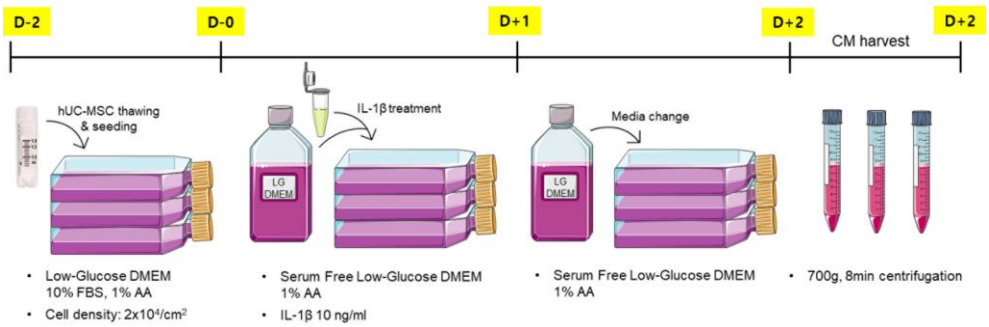



Figure 1. Schematic representation of the CM generated from hUC-MSCs

## 2.3 Treatment of primary chondrocytes

Chondrocytes were seeded at $2x10^4/cm^2$. For mRNA gene expression analysis, 24 well plates were used and $3.8x10^4$ cells/well were seeded. For western blotting, 6 well plates were used and $1.92x10^5$ cells/well were seeded. At the time of use, all cells had levels of viability over 95%.

After seeding the cells into three groups of Control, IL-1$\beta$, and CM, and incubated for 24 hours in a 5% CO2 incubator with

humidified air at 37℃. Medium change for each group was conducted as follows: control group was given serum free low-glucose DMEM, while the IL-1βand CM groups were given serum free low-glucose DMEM supplemented with recombinant IL-1β at 10 ng/ml and incubated for 6 hours. Following 6 hours, all groups of medium were suctioned out, and then treated as follows: control group received fresh serum free low-glucose DMEM, whereas the IL-1β group was treated with recombinant IL-1β at 10 ng/mL in serum free low-glucose DMEM, and the CM group was loaded with pre-produced conditioned medium and incubated for 18 hours.

For mRNA gene expression analysis, all medium from 24 well plate was suctioned out, and after washing with DPBS once, 100㎕ of XPRB buffer was added to begin cell lysis. Western blots were prepared by removing the supernatant and adding a 2x SDS buffer according to the cell confluency for protein preparation.

## 2.4 Cell viability assay

Viability tests were conducted in 96-well plates and $8x10^3$ cells/well/100 ㎕ were seeded and the same cell treatment method was applied. 10㎕ of EZ-Cytox (Daeil Lab Service, Seoul, Korea) was added to each well, and the plates were incubated for total 3 hours at 37℃ in the dark. An absorption measurement was then

performed at 450nm using a microplate reader. The data were collected at three different times; 1hr, 2hr, and 3hr.

## 2.5 U937 cultures

Human monocytic leukemia cell line U937 cells (KCLB No. 21593.1; Korean Cell Line Bank [KCLB], Seoul, Korea) were maintained in suspension culture in RPMI-1640 supplemented with 10% bovine serum (FBS; HyClone) and antibiotic-antimycotic solution (100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml amphotericin B, Welgene) at 37℃ in a humidified atmosphere of 5% $CO_2$.

## 2.6 Cell adhesion assay

Chondrocytes ($1.96x10^4$) were treated with IL-1$\beta$ (10 ng/mL) for 6h, then added hUC-MSC CM for another 18 h. U937 cells ($1x10^5$) were labeled with Dil and then, labeled cells were added to chondrocytes and incubated for 1 h. nonadherent cells were removed from the wells. The numbers of adherent U937 cells were counted in randomly chosen fields at x50 or x100 magnification in fluorescent microscopy.

## 2.7 Gene expression analysis

eCube Tissue RNA mini kit (PhileKorea, Korea) was used to

extract total RNA and quantified using a Nano-Drop ND-100 spectrophotometer (NanoDrop, Wilmington, DE, USA). The cDNA was synthesized using a commercially available kit (SuperiorScript Ⅱ Reverse Transcriptase; Enzynomics, Daejeon, Korea) and then stored at -20℃ till use. Following genes were assessed, 1) Pro-inflammatory cytokines including interleukin-1$\beta$ (IL-1$\beta$), cyclooxygenase-2 (Cox-2), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) 2) Inflammasome-mediated cytokine NOD-like receptor pyrin domain-containning protein 3, cryopyrin (NLRP3) 3) Cystein protease Caspase-1 4) Degradative enzymes and their inhibitors, matrix metalloproteinase-1, -3, and -13 (MMP-1, -3, -13), tissue inhibitor of metalloproteinase-1, -2, and -3 (TIMP-1, -2, and -3), a disintegrin and metalloproteinase with thrombospondin motifs-4, and -5 (ADAMTS-4 and -5) 5) Anti-inflammatory cytokines, interleukin-10, and -13 (IL-10 and -13) 6) Selectin-E (E-selectin). To perform real-time PCR utilizing a LightCycler 480 (Roche Applied Science, Mannheim, Germany), TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Daejeon, Korea) were used. Real-time PCRs were performed in a final volume of 18$\mu$l containing 9 $\mu$l 2X premix (Enzynomics, Daejeon, Korea), and 1 $\mu$l cDNA as the template, 0.36 $\mu$l primer, 7.64 $\mu$l D.W. using the following program: 95℃ for 15 minutes,50

cycles at 95℃ for 10seconds, 60℃ for 15 seconds, 72℃ for 30 seconds, and a final step at 72℃ for 5 minutes. Gene expression was normalized by GAPDH. The sequence of primers for each specific primer is shown in Table 1.

Table 1. The primer sequences used in the Real-Time RT-PCR analysis

| Gene | Primer sequence (5'-3') | Ta (℃) |
|---|---|---|
| GAPDH | F: ACATCCCTCAGACACCATG<br>R: TGTACTTGAGGTCAATGAAGGG | 60 |
| MMP-1 | F: GATCTCTTCATTTGGCCATC<br>R: CTCCAGTATTTGTCCTCTAC | 60 |
| MMP-3 | F: GATCTCTTCATTTGGCCATC<br>R: CTCCAGTATTTGTCCTCTAC | 60 |
| MMP-13 | F: CCCAACCCTAAACATCCAAAAAC<br>R: CTTCCCCTACCCCGCACTTCT | 60 |
| TIMP-1 | F: GCATCTCTGGCCTCTGGCAT<br>R: AGCCCTTATAACCAGGTCCGAGT | 60 |
| TIMP-2 | F: CTGGACGTTGGAGGAAAGAA<br>R: GTCGAGAAACTCCTGCTTGG | 60 |
| TIMP-3 | F: ATGCCCCATGTGCAGTACATCC<br>R: ATCATAGACGCGACCTGTCAGCA | 60 |
| ADAMTS-4 | F: TGTCAGCTTGGTGGTGACTC<br>R: AAGCCCCATTCAAACTGATG | 60 |
| ADAMTS-5 | F: TGTGGAAAGGGGAGAATCTG<br>R: TGCATATTTGGGAACCCATT | 60 |
| NLRP3 | F: ATCTCACGCACCTTTACC<br>R: AAGAAGGCTCAAAGACGAC | 60 |
| Caspase-1 | F: GTGGGAAGAAACACTCTGAGC<br>R: GGAAGAGCAGAAAGCGATAAAATC | 60 |
| TNF-α | F: GACTGACAAGCCTGTAGCCCATGTTGTAGCA<br>R: GCAATGATCCCAAAGTAGACCTGCCCAGA | 60 |
| COX-2 | F: TCAGCCACGCAGCAAATCCT<br>R: GTGATCTGGATGTCAGCACG | 60 |
| IL-10 | F: ACCAAGACCCAGACATCAAG<br>R: GAGGTACAATAAGGTTTCT | 60 |
| IL-13 | F: GTACTGTGCAGCCCTGGAAT<br>R: TTTACAAAGGGGCCACCTC | 60 |
| SELE | F: TGTGTGGGTCTGGGTAGGAACCC<br>R: GTGAAGGTCCAGCAGGGGAATGG | 60 |

## 2.8 Western blot

Cells were lysed with 2X SDS buffer. Protein samples were electrophoresed on SDS-polyacrylamide gels and transferred onto PVDF membranes (Merck Millipore, Billerica MA, USA). The membrane was blocked with 5% skim milk for 1 h, and incubated with the primary antibody overnight at 4℃. Membranes were then washed in PBS-T and fluorescent secondary antibody was added for 1 h at room temperature (RT). Membranes were extensively washed and then chemiluminescent signal was detected using D-Plus™ ECL Pico System (Donginbio, Seoul, Korea) with LAS-4000 mini imager (Fujifilm, Tokyo, Japan).

## 2.9 RNA-sequencing analysis

The raw sequence reads were first trimmed and quality-controlled using TrimGalore(24) (version 0.6.7). The trimmed reads were then mapped to the human reference genome GRCh38/hg38 using HISAT2(25) (version 2.2.1). The mapped reads were assembled and quantified using StringTie(26) (version 2.1.7). The differential expression analysis was conducted using DESeq2(27) (version 1.26.0), edgeR(28) (version 3.28.1), Limma(29) (version 3.42.2), and EBSeq(30) (version 1.26.0). The differential expression results were merged using MLDEG(31) to

get the final differentially expressed genes (DEGs). The significance threshold was *Adjusted P-value* < 0.05 and *log fold change* > 1 for MIX samples versus IL-1β samples. The identified 6 candidate potential biomarker genes are comprised of 5 up-regulated genes and 1 down-regulated genes. The target protein E-Selectin is selected as the final potential biomarker gene that plays a key role in the anti-inflammatory effect of the CM in Osteoarthritis. Gene ontology and pathway enrichment analysis of the DEGs was conducted using EnrichR(32).

# Chapter 3 Results

## 3.1 Conditioned media derived from hUC-MSC attenuate IL-1$\beta$ activity in chondrocytes

For all experiments, chondrocyte isolated from the patient tissue sample was seeded with cells at between passage 3 and 5, and the seeding density was fixed at $2x10^4$ cells/$cm^2$. Control group (Figure 1A), which did not receive any IL-1βstimulation, showed a typical morphology of healthy chondrocytes, seeded in high-glucose DMEM containing 10% FBS and 1% AA, maintained for 24 hours, then changed to serum-free low-glucose DMEM for 24 hours. However, when IL-1β at a concentration of 10 ng/ml was added to the cells (Figure 1B), adhere cells on the plate bottom changed into a round shape, as well as becoming thinner and longer than the control cells. However, there was very little evidence of rounded cells remaining in the group that was treated with CM for 18 hours after 6 hours of IL-1β treatment (Figure 1C), morphologies were very resembled those of the group receiving control treatment. The thinning and lengthening qualities disappeared, and the cells returned to being short, chubby-shaped.

Viability assays were conducted to confirm the relationship between morphological change and viability of the cells using the WST-1 assay (Figure 1D). They were measured three times at 1-

hour intervals, and the pattern was similar at all three points. The IL-1β group showed a statistically significant increase in viability relative to the control group, while the CM group showed an increase relative to the IL-1$\beta$ group.

The effects of IL-1β on chondrocytes were confirmed at the mRNA level. A total of 14 gene expressions were assessed in chondrocytes obtained from four different patients. We assessed the following genes: TNF-$\alpha$, the pro-inflammatory cytokine(33), COX2, an enzyme induced during inflammation, IL10 and IL13, anti-inflammatory cytokines, MMP-1, MMP-3, MMP-13, well known ECM degradative enzymes, TIMP-1, TIMP-2, TIMP-3, which act as inhibitors for these MMPs(33), ADAMTS-4, ADAMTS-5, which are involved in aggrecan degradation, IL-1$\beta$, which plays a central role in OA cartilage loss(34), Caspase-1, which converts IL-1$\beta$ into its active form, NLRP3, which is an inflammasome (Figure 2A-2L). All four batches of the CM group showed a statistically significant reduction in MMP-1 and MMP-3 compared to the IL-1$\beta$ group (Figure 2A and 2B). As for MMP-13, the numerical value in the CM group decreased in all four batches, with the statistical significance in two batches confirmed. (Figure 2C). In regard to TIMP, which can inhibit the effects of these MMPs (33), two batches of TIMP3 have shown statistically significant improvements

from the CM group as compared to the IL-1$\beta$ group (Figure 2D-2F). Aggrecanases, such as disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5, are involved in aggrecan degradation(35). Several ADAMTSs are expressed in cartilage in addition to ADAMTS-4 and -5, however, it is unknown if they are essential for joint health or if they contribute to OA pathogenesis(35). A reduction in ADAMTS-4 and -5 levels by hUC-MSC CM was not confirmed in this study (Figure 2G and 2H).

NLRP3 produces proinflammatory cytokines and degradative enzymes, such as IL-1$\beta$, TNF-$\alpha$ and MMP-3, which lead to cartilage breakdown and synovial inflammation(36). Yet, NLRP3 was not affected by hUC-MSC CM (Figure 2I). Caspase-1, which convert IL-1$\beta$ to its active form(34), decreased to the degree of statistical significance in one batch by hUC-MSC CM (Figure 2J).

An inflammatory cytokine, TNF-$\alpha$, is secreted in early osteoarthritis and is produced by activated synoviocytes, chondrocytes, and monocytes(37). A significant decrease in TNF-$\alpha$ levels was observed in all batches due to hUC-MSC CM treatment (Figure 2K). COX-2 promotes the production of prostalandins, which are a mediator of inflammation, pain, and fever and are the target enzymatic activity for NSAIDS(38). There was a decrease in hUC-MSC CM in all batches, but significance was only

confirmed in two batches (Figure 2L). The effect of hUC-MSC CM on IL-10 and IL-13, which have anti-inflammatory properties, was not observed (Figure 2M and 2N).

A validation of MMP-1, MMP-3, MMP-13, TIMP-1, TIMP-3, and ADAMTS-4 protein expression levels was conducted by western blotting (Figure 3). With respect to MMP-1, it was confirmed that the IL-1$\beta$ group increased by 3195% over the control group and decreased by 47% over the CM group(Figure 3B). For MMP-3, more than 1734% increase in IL-1$\beta$ group was confirmed and 47% decrease in CM group (Figure 3C). In case of MMP-13, 79% increase in IL-1$\beta$ group and 21% decrease in CM group were confirmed (Figure 3D). A moderate rising trend was seen in the mean mRNA values for ADAMTS-4, but the amount of protein expression increased 67% in the IL-1$\beta$ group and decreased 45% in the CM group (Figure 3E). TIMP-1, on the other hand, increased by 129% in the CM group compared to the IL-1$\beta$ group (Figure 3F), and TIMP-3 increased by 68% in the CM group compared to the IL-1$\beta$ group. All groups were normalized to $\beta$-actin (Figure 3G).

It may be suggested that hUC-MSC CM exerts an anti-inflammatory effect by inhibiting matrix degradative enzymes and by increasing the expression of matrix prevention related matrix

factors to achieve a balance between the two.

Figure 2. Effects of hUC-MSC CM on the viability and phenotype of inflamed chondrocytes

The same numbers ($2x10^4/cm^2$) of chondrocytes were seeded in (A)control, (B)IL-1$\beta$, and (C)CM. (A) the morphology was maintained with a short overall length and a sharp tip. (B) the remainder of the cell, except for the nucleus, shows thinned and stretched appearance. (C) displays a recovery following exposure to the hUC-MSC-derived CM, which is similar to that of the control group. Much of the morphology recovered from the thin, elongated form to a shorter, thicker, and pointed form. (D) WST-1 assay of cell viability of chondrocytes treated with IL-1$\beta$ and CM. Results are presented as the mean ± standard deviation; n=4. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001.

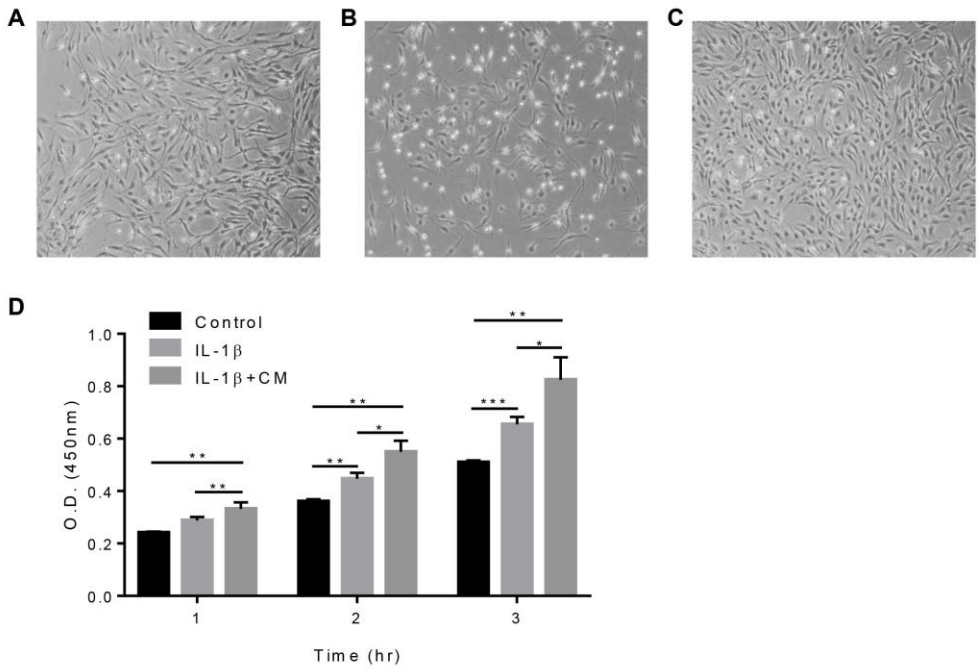
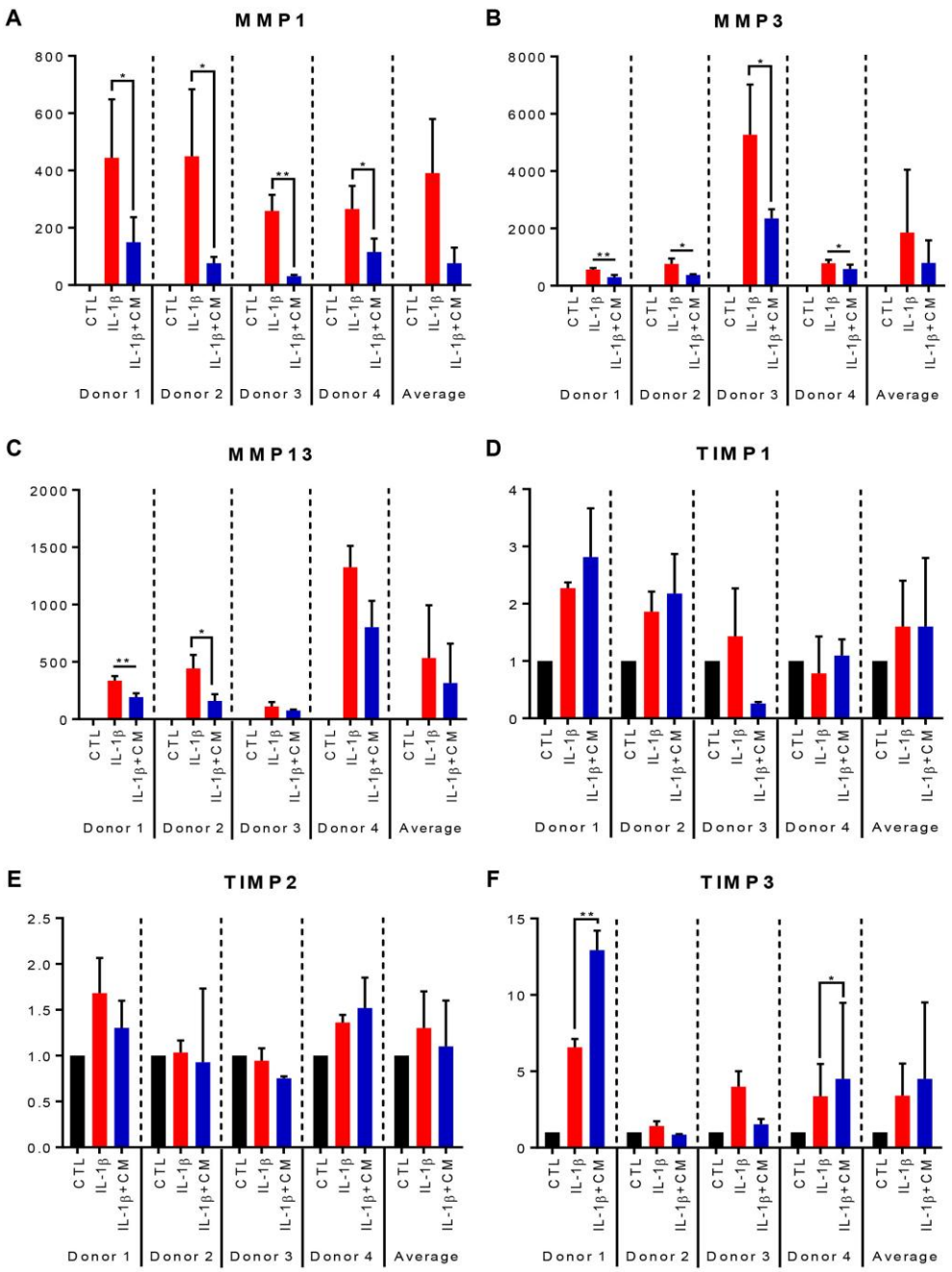
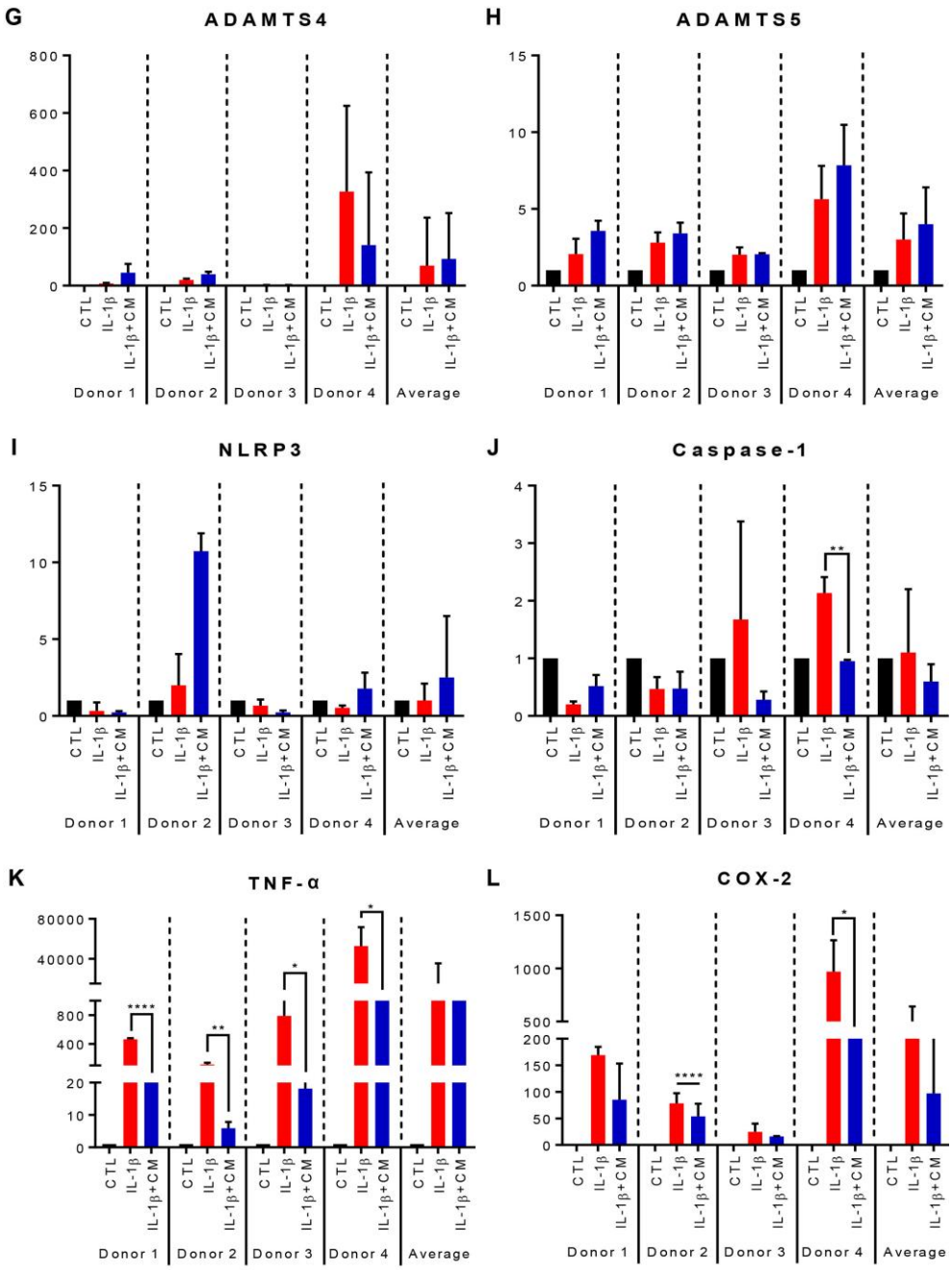


Figure 3. Regulation of matrix proteins in inflamed chondrocytes by hUC-MSC CM

(A-C) Results of MMP genes; (D-F) Results of TIMP genes; (G, H) Results of ADAMTS genes; (I) Results of inflammasome gene; (J) Result of Caspase-1 gene; (K) Result of TNF-$\alpha$ gene; (L) Result of COX-2 gene; (M, N) Result of IL-10 and 13 genes. Results are presented as the mean ± standard deviation; n=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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ADAMTS4
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ADAMTS5
I
NLRP3
J
Caspase-1
K
TNF-α
L
COX-2
CTL
IL-1β
IL-1β+CM
Donor 1
Donor 2
Donor 3
Donor 4
Average

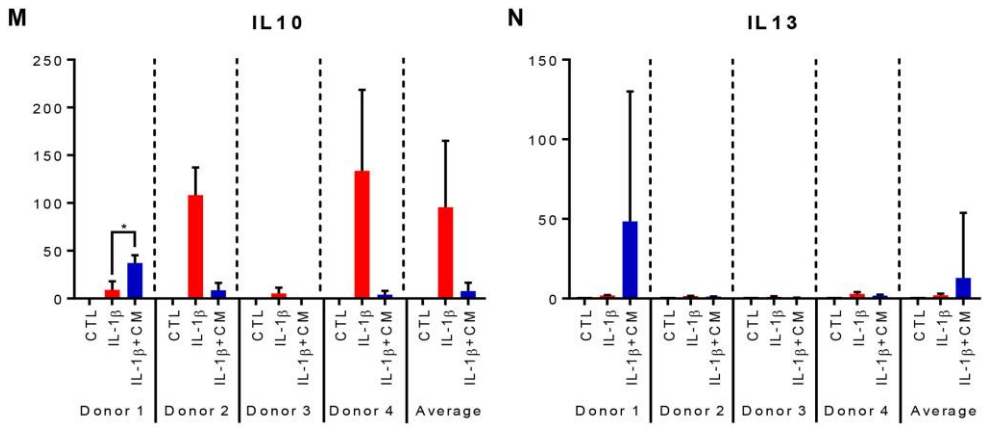
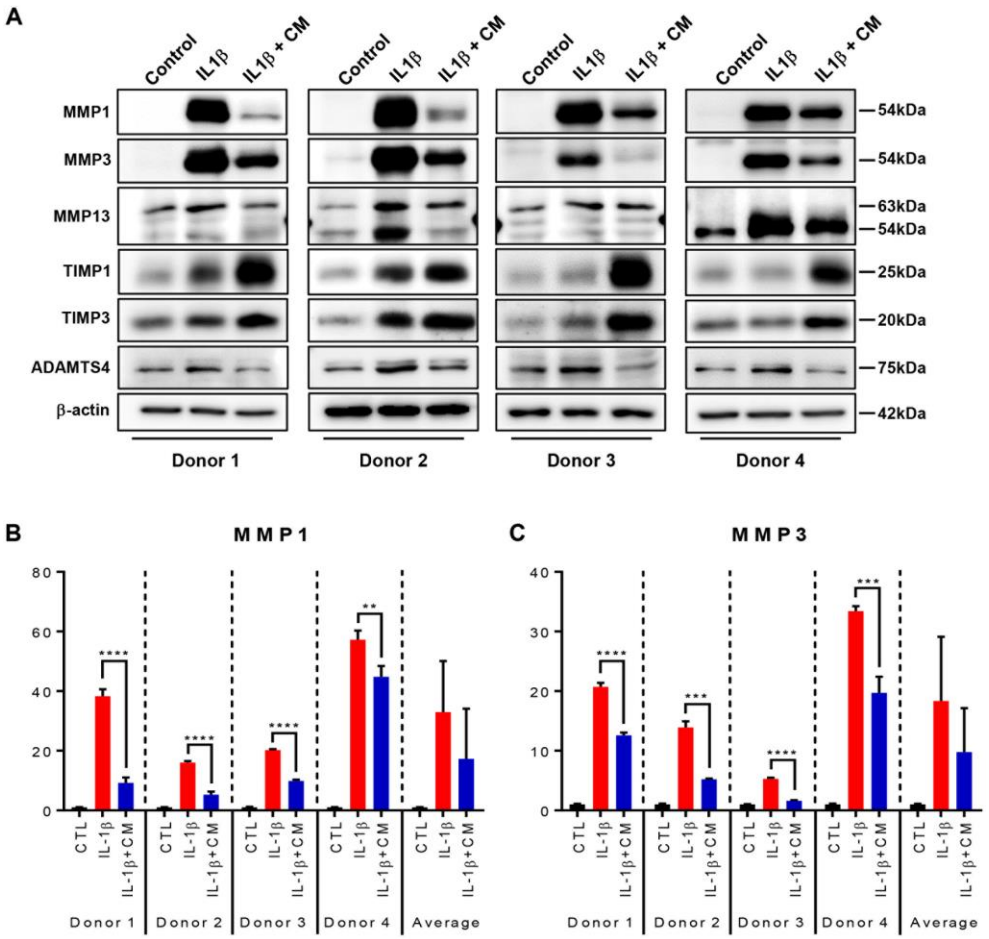
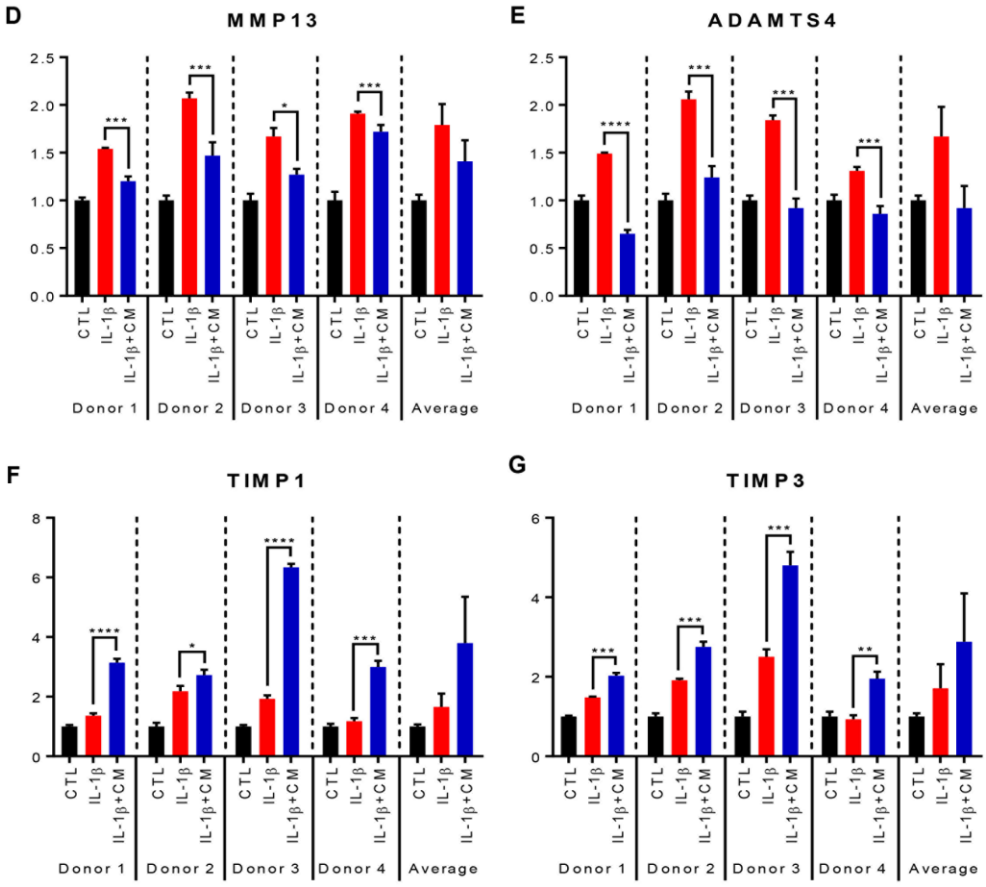

M
IL10
250
200
150
100
50
0
*
CTL
IL-1β
IL-1β+CM
Donor 1
Donor 2
Donor 3
Donor 4
Average
N
IL13
150
100
50
0

Figure 4. hUC-MSC CM changes matrix degrading enzymes and inhibitors in chondrocytes

(A) Western blot quantified protein expression of MMP-1, -3, and -13, TIMP-1, -3, ADAMTS- 4. β-actin expression was used as control for equal protein loading. (B-F) Quantification of band intensities of expressed proteins, n=4. Quantification of each protein bands detected on the western blot was performed using Image J software, normalized against β-actin. Results are presented as the mean ± standard deviation; n=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



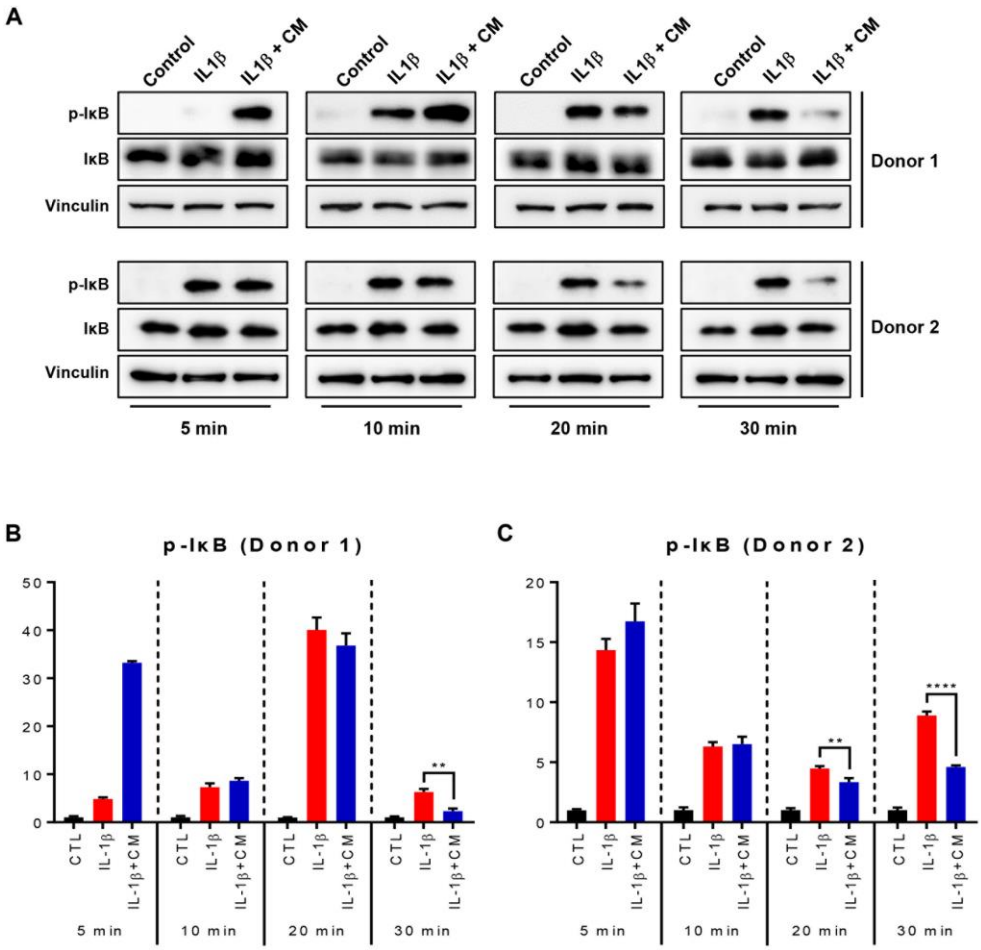

D
MMP13
E
ADAMTS4
F
TIMP1
G
TIMP3
CTL
IL-1β
IL-1β+CM
Donor 1
Donor 2
Donor 3
Donor 4
Average

## 3.2 hUC-MSC CM suppresses IL-1β stimulated inflammatory responses through inhibition of NF-kB signaling pathway

In arthritis, NF-kB is one of the cytokine-induced transcription factors that regulates the expression of MMPs, COX-2, and contributes to inflammation and cartilage degeneration (39, 40). In order to determine the mechanisms through which hUC-MSC CM inhibits inflammatory responses in IL-1β stimulated chondrocytes by activating IkB. It was conducted using two batches of chondrocytes in total. The samples were collected 5 minutes, 10 minutes, 20 minutes, and 30 minutes after the hUC-MSC CM treatment moment. All samples were collected using 2X SDS sample buffer at the harvest time point. We adjusted the amount of 2X SDS buffer used according to the confluency of each group and boiled it at 97℃ for 7 minutes immediately following harvest. The samples were stored at −20℃ until needed.

The results indicate that in both batches, IL-1β significantly increased IkB phosphorylation, a process which can be significantly attenuated by hUC-MSC CM after 30 minutes.

Figure 5. IL-1$\beta$ induces IkB signaling in human OA chondrocytes
Chondrocytes ($1.925x10^5$) were stimulated IL-1$\beta$ (10 ng/ml)for indicated time intervals. Cell lysates were collected. (A-C) The levels phosphorylated IkB were determined by Western blotting. Quantification of each protein bands detected on the western blot was performed using Image J software, normalized against vinculin. Results are presented as the mean ± standard deviation; n=2. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



## 3.3 RNA-seq of UC-MSC derived CM treated human chondrocytes in environments containing IL-1β

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RNA-sequencing analysis was performed using chondrocytes isolated from three patient samples to determine what changes in genetic expression occur when exposed to the IL-1β environment and how that changes when UC-MSCs derived CM is exposed to the chondrocytes in the IL-1β environment.

The MLDEG framework for DEG analysis proceeded with the following workflow (Figure 5A). An initial analysis was conducted with gene expression data obtained from three groups of chondrocyte samples. In order to analyze the obtained expression data, two strategies were employed: the DEG analysis method, which combines the four analysis tools (Deseq2, EdgeR, Limma, and EBSeq), and the condition-specific PPI network construction method. The data were merged and put through a machine learning algorithm. The combined DEGs data obtained after applying the machine learning algorithm was subsequently filtered further. Filtering conditions were as follows. Combined p-value <0.02, combined logFC>1, and prior knowledge. Following final filtering, there were a total of 6 final candidate genes (Table 2), and SELE (E-selectin), which is a major receptor involved in the recruitment of monocytes(21), showed stable results across three or more

chondrocyte batches, was selected for final target gene selection through an *in vitro* test.

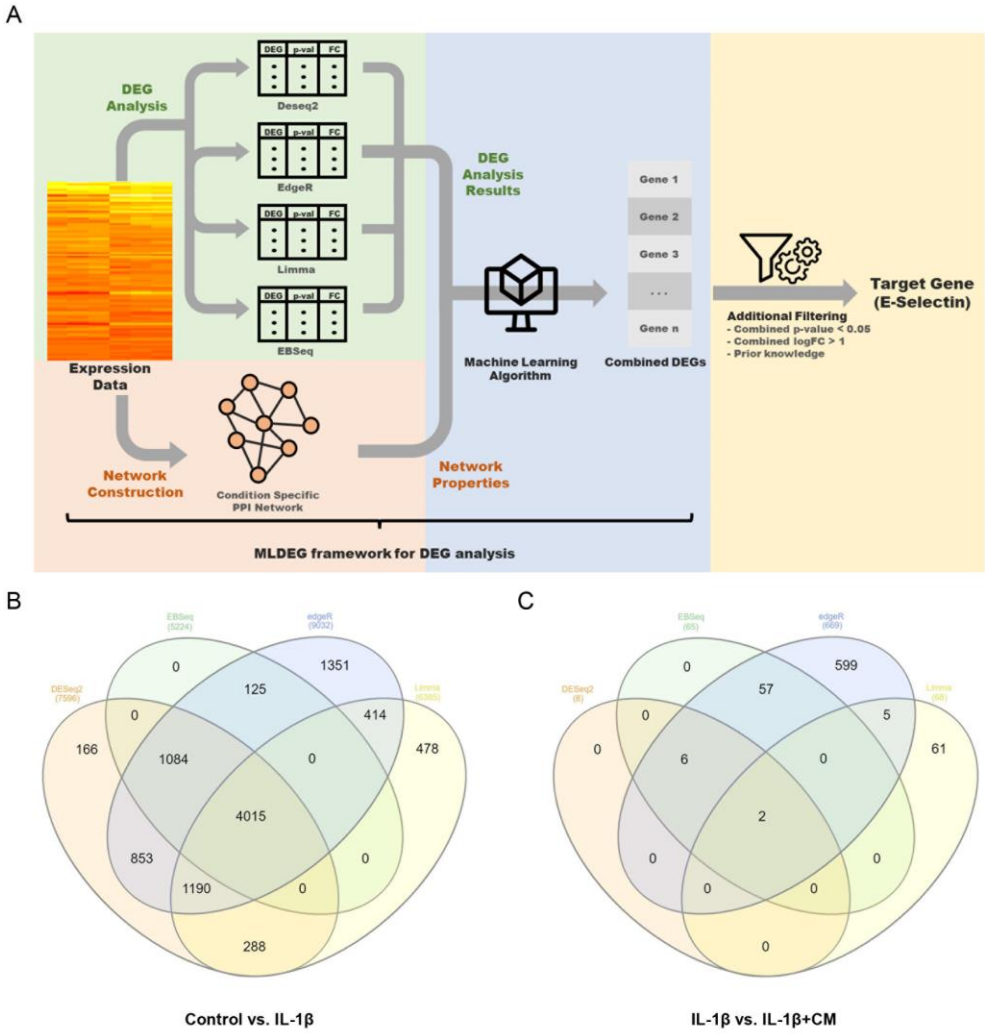
A comparison was made between the results of the four analysis tools (Deseq2, EdgeR, Limma, and EBSeq) applied in the DEG analysis process (Figure 5B and 5C). There is a large degree of discrepancy between the results. It is, therefore, comparing overlapping genes between different methods is not sufficient to provide an accurate assessment. To determine significant target genes computationally, we applied a bioinformatics approach that exploits the power of machine learning.

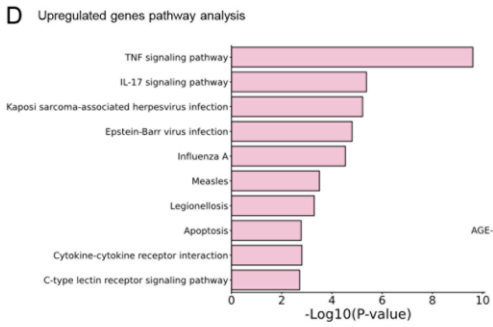
Data from the KEGG pathways analysis was used as a positive control (Figure 5D and 5E). In view of the fact that it is well established that an increase in IL-1$\beta$ induces an increase in TNF-$\alpha$ expression in articular cells such as chondrocytes(34), the fact that the TNF signaling pathway increased the most in the IL-1$\beta$ environment compared to the control, and it decreased the most during CM treatment, suggests that the analysis method was carried out appropriately.

An overall number of 4,598 genes were identified as pro-inflammatory, with 4472 genes exhibiting increased expression in the IL-1$\beta$ group compared to the control group, and 126 genes exhibiting decreased expression in the CM group compared to the

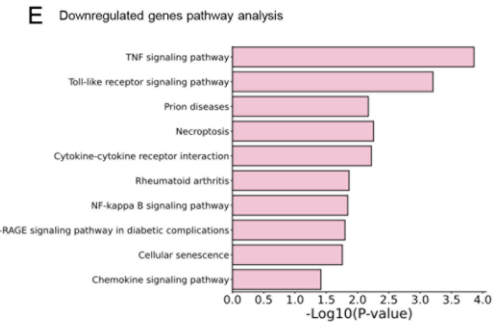
IL-1$\beta$ group. Further, a total of 3,480 genes that can be classified as anti-inflammatory genes were identified, with 3242 genes showing decreased expression in the IL-1$\beta$ group compared to the control group and 238 genes showing increased expression in the CM group compared to the IL-1$\beta$ group. The number of genes was narrowed later substituting above mentioned specific filtering conditions, five genes with pro-inflammatory properties and one gene with anti-inflammatory properties were classified (Table 2).

Figure 6. MLDEG: a machine learning approach to identify DEGs
(A) The overall workflow used to select the target gene E-selectin. (B-C) 4 DEG analysis methods comparison. The differential expression analysis using 4 different methods. The results do not agree with each other to a large degree. Simply selecting overlapping genes of the different methods cannot give accurate results. In this paper, we used a bioinformatics method that takes advantage of the power of machine learning to computationally select significant target genes. (D-E) KEGG pathway enrichment analysis from MLDEG result.






**Control vs. IL-1β**




**IL-1β vs. IL-1β+CM**

Table 2. Analysis of the RNA sequences in UC-MSC derived CM treated human chondrocytes in environments containing IL-1β

**Pro-inflammatory candidate genes:** upregulated in response to inflammation and downregulated in response to CM

| Gene Symbol | Control vs. IL-1β (Up) | | | IL-1β vs. IL-1β+CM (Down) | | |
|---|---|---|---|---|---|---|
| | Combined p-value | Combined logFC | Raw Change Ratio | Combined p-value | Combined logFC | Raw Change Ratio |
| CXCL10 | 0.000 | 9.663 | 811-fold increase | 0.017 | -1.139 | 2.2-fold decrease |
| **SELE** | **0.000** | **9.470** | **710-fold increase** | **0.000** | **-1.620** | **3.1-fold decrease** |
| CAGE1 | 0.000 | 4.454 | 22-fold increase | 0.019 | -3.267 | 9.6-fold decrease |
| MYB | 0.000 | 4.031 | 16-fold increase | 0.004 | -1.689 | 3.2-fold decrease |
| IP6K3 | 0.000 | 3.935 | 15-fold increase | 0.010 | -1.623 | 3.1-fold decrease |

**Anti-inflammatory candidate genes:** downregulated in response to inflammation and upregulated in response to CM

| Gene Symbol | Control vs. IL-1β (Down) | | | IL-1β vs. IL-1β+CM (Up) | | |
|---|---|---|---|---|---|---|
| | Combined p-value | Combined logFC | Raw Change Ratio | Combined p-value | Combined logFC | Raw Change Ratio |
| PTPRF | 0.000 | -1.437 | 2.7-fold decrease | 0.001 | 1.136 | 2.2-fold increase |

## 3.4 Effect of hUC-MSC CM on candidate gene expression in chondrocytes

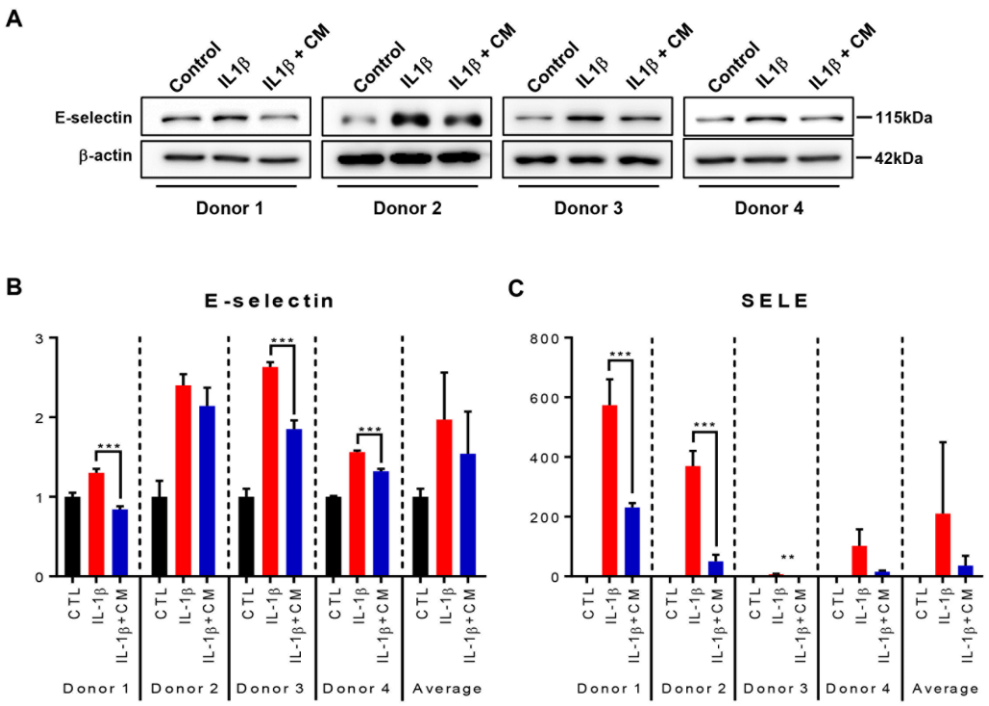
Among the six candidate genes obtained through the MLDEG, E-selectin, which was selected following additional in vitro verification, plays a pivotal role in monocyte recruitment, and their expression is transcriptionally induced by inflammatory cytokines, including IL-1 and TNF(20, 21). E-selectin expression is increased in cartilage and synovium of OA patients and is also associated with the infiltration of monocytes by adhesive attraction in inflamed knee joints of patients with active OA(19).

In all four donor batches, the expression of E-selectin was seen to increase with IL-1β treatment and to decrease with hUC-MSC CM treatment, both at the mRNA and protein levels (Figure 6). The mRNA of E-selectin induced 210-fold increases in the IL-1β group in four batches and decreased by 5.8-fold in the hUC-MSC CM group (Figure 6C). On the protein level, it was found that the levels of E-selectin increased by an average of 2-fold in the IL-1β group and decreased by 1.3-fold following hUC-MSC CM treatment (Figure 6B). The results of this study can be compared with the results of previous studies. Sandell et al. conducted a microarray study in 2008 that demonstrated E-selectin levels in human chondrocytes increased 65-fold after being treated with IL-

1β, significance of the results of this study was confirmed.

Figure 7. In vitro analysis of candidate gene expression using hUC-MSC CM in chondrocytes

(A) The Western blot analysis demonstrated a significant difference in the E-selectin expression between chondrocyte exposed to IL-1β and chondrocyte treated with CM after exposure to IL-1β environment. The expression of β-actin was used as a control for equal protein loading. (B) Quantification of E-selectin protein bands detected on the western blot was performed using Image J software, normalized against β-actin. (C) Quantitative real-time PCR analysis of SELE gene expression in chondrocytes. Results are presented as the mean ± standard deviation; n=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



## 3.5 Effect of hUC-MSC CM on monocyte-chondrocyte interaction via E-selectin

Selectins are proteins composed of a lectin domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail (41). According to established research, lectin and EGF domains are essential for mediating interactions with ligands (41). It has been reported that a reduction in the number of SCR domains correlates with a reduction in the affinity of E-selectin for hematopoietic cells (41). In a study published by Aleisa in 2019, the binding ability of E-selectin proteins containing SCRs of different lengths was analyzed and tested. As the length of the SCR increased, the number of cells that could be captured increased, as did the force to bind the cell. Following the results of these previous studies, we planned an experiment taking into account the fact that when E-selectin is shaded from chondrocytes, it is not known at which sites it will be shaded. The experiment was started with membrane E-selectin and monocytes before moving on to solid E-selectin. Prior to focusing on soluble E-selectin, experiments were conducted on membrane E-selectin and monocyte adhesion. By comparing the binding of E-selectin and monocyte according to the presence and absence of IL-1β and

hUC-MSCM treatment, the efficacy of hUC-MSCM was first assessed.

The group without Dil staining was used as a negative control (Figure 7A). On average, in the group exposed to IL-1$\beta$, chondrocytes and monocytes bound 7.8-fold greater than in the control group, and this binding was reduced by about two fold in the group treated with hUC-MSC CM (Figure 7B). Given that E-selectin expression is associated with leukocyte infiltration, as more E-selectin is found in an IL-1$\beta$ enriched environment such as shown in this result, the more monocytes will infiltrate into the inflammatory site with a higher probability, as demonstrated by this result, resulting in an increase in inflammation. Accordingly, it can be seen that hUc-MSC CM reduces the expression of E-selectin, inhibits its binding to monocytes, thus contributing to the reduction of inflammation.

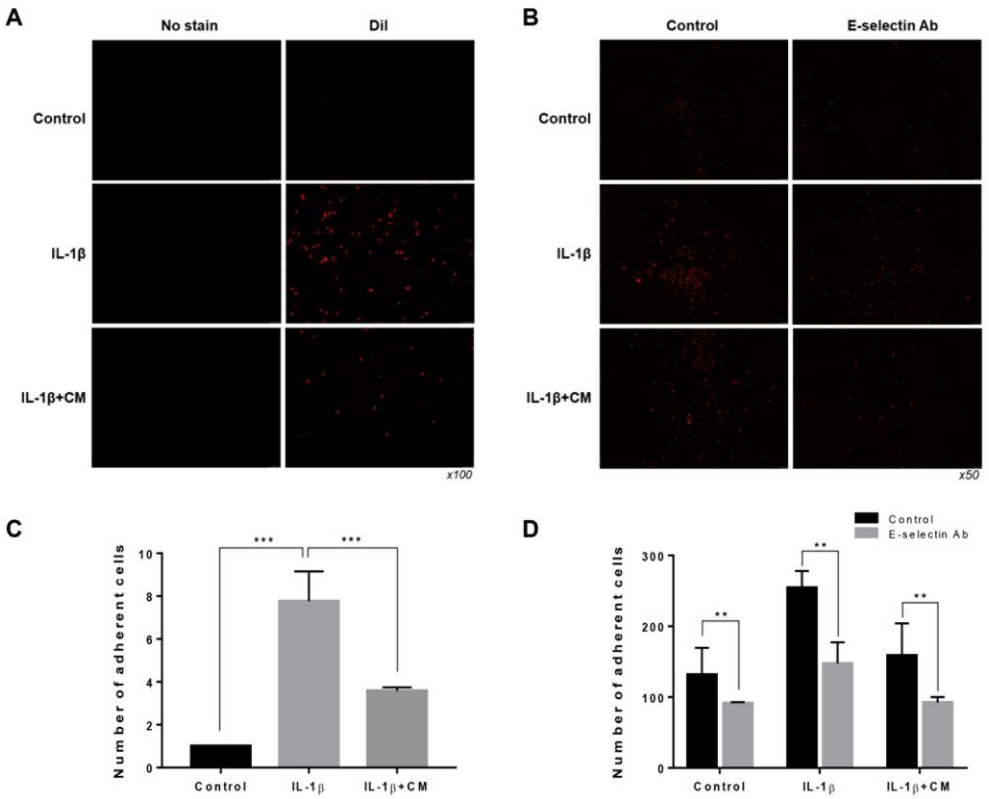
In order to confirm whether monocytes bind to chondrocyte-expressed E-selectin, E-selectin expressed in the chondrocytes was blocked, and then the binding of monocytes was examined (Figure 8A). The blockade procedure was conducted for one hour before binding with monocytes. Blockade treatment significantly reduced the number of monocytes binding to E-selectin in all groups (Figure 8B). Accordingly, it has been

confirmed that monocyte adhesion was mediated by chondrocyte-expressed E-selectin. It is interesting to note that, despite the use of blockade, the greatest amount of binding was observed in the IL-1$\beta$ group and this was reduced to a level equivalent to the control group.

Figure 8. Monocytes bind to chondrocyte-expressed E-selectin

Dil-stained monocytes were bound for an hour with chondrocytes in three different conditions. (A) $1.96x10^4$ chondrocytes were seeded per well, and $1x10^5$ monocytes were Dil-stained and bound to the chondrocytes. As a negative control, monocytes without Dil staining were used. (C) The quantification analysis of chondrocytes and bound monocytes was conducted using the Image J software. (B) Control groups were not pre-processed with blockade and bound to monocytes. (D) Quantitative analysis of chondrocytes and conjugated monocytes according to the use of blockades was performed using image J software.

Results are presented as the mean ± standard deviation; n=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



# Chapter 4 Discussion

The most important findings of the study are that CM derived from hUC-MSC reduced OA progression-related matrix degradative-related factors and increased OA prevention-related matrix factors. Furthermore, it was confirmed that hUC-MSC CM inhibited the expression of chondrocyte-derived E-selectin, thereby reducing the possibility of contact between monocytes and chondrocyte-derived E-selectin.

According to their structure and substrates, MMPs can be categorized into several types, including collagenase (MMP-1, MMP-13), gelatinase (MMP-2, MMP-9), stromelysin (MMP-3), metalloelastase (MMP-12), matrilysin (MMP-7), and membrane-type matrix metalloproteinase (MT-MMPs) (33). The results of this study indicate that the expression of MMP-1, MMP-3, and MMP-13 was significantly reduced by hUC-MSC CM. Considering the fact that collagen is the most abundant structural macromolecule in cartilage and that it makes up approximately 60% of cartilage's dry weight(42), the fact that MMP-1 and MMP-13 belonging to the collagenase category were significantly reduced by hUC-MSC CM is undoubtedly a remarkable result. In particular, the result that MMP-13, which degrades Type Ⅱ collagen, which comprises 90 to 95% of all collagen (42), decreased suggests that hUC-MSC CM

could be an excellent therapeutic agent for alleviating OA progression. Furthermore, considering that one of the causes of bone destruction in osteoarthritis is an imbalance of MMPs and their inhibitor, TIMP, the result of significant increase in TIMP-1 and TIMP-3 by hUC-MSC CM suggests that it could be used as a therapeutic agent in dealing with the biological cause of the disease. Therefore, it may be concluded that hUC-MSC CM exhibits anti-inflammatory effects by reducing the expression of factors associated with cartilage degradation.

NGS analysis conducted in this study to examine the anti-inflammatory effect of hUC-MSC CM was significantly simplified by using MLDEG to derive and verify more accurate OA-related candidate genes. Prior to machine learning analysis, four independent statistical analysis tools were employed, and since different analysis methods were applied to each tool, passing the filter of four tools alone represents a highly reliable discovery of candidates. Additionally, the MLDEG used in this study has already been validated due to the publication of the study in 2021 (31). As a result, only six genes were filtered through MLDEG, thereby increasing the efficiency of the in vitro validation. In several chondrocyte batches, we verified that the expression of E-selectin was increased by IL-1$\beta$ and decreased by hUC-MSC CM, the

same results as the NGS analysis, indicating a similar trend has been reported in the Sandell paper published in 2009 (43). Additional experiments will have to be conducted, however, to identify the quantity of soluble E-selectin released by chondrocytes in supernatants.

In this study, hUC-MSC CMs were obtained from hUC-MSCs that had been treated with IL-1$\beta$, creating a similar OA environment where high concentrations of IL-1$\beta$ are present. Despite not being reported in the results, higher concentrations of growth factor and anti-inflammatory cytokine were released from IL-1$\beta$ stimulated hUC-MSCs when compared to hUC-MSCs in the absence of IL-1$\beta$ stimulation. It is possible to interpret and compare these results with previous studies in other human tissues. For example, previous studies, such as Broekman (2016), Chen (2015), and Fan (2012), confirmed better therapeutic effects in hMSCs pre-treated with inflammatory substances such as IL-1$\beta$ or TNF-$\alpha$. In view of this, it has been proposed that soluble factors released by hUC-MSC following IL-1$\beta$ stimulation regulate E-selectin expression in chondrocytes exposed to IL-1$\beta$.

In our future work, we plan to observe soluble E-selectin in supernatants shed from chondrocytes. In HUVEC cultures, expression of E-selectin is absent at basal levels but is rapidly

(2h) induced by TNF. Surface expression is transient, it declines to 10% of peak levels within 24 hours(44). In light of this, it is predicted that the quantity of E-selectin expressed in chondrocytes will be significantly lower than soluble E-selectin in supernatant. At the same time, the concentration of E-selectin present in shedding and soluble form will be measured as well as the degree of chondrocyte activation, ultimately allowing change in chondrocytes to be noticed. As an additional experiment, the amount of E-selectin present in soluble form will be measured and, at the same time, the degree of monocyte activation and subsequent changes in chondrocytes will be determined.

# Chapter 5 Conclusion

hUC-MSC significantly alleviated chondrocyte damage caused by IL-1$\beta$ -induced inflammation. hUC-MSC CM resulted in a significant reduction in pro-inflammatory cytokines such as TNF-$\alpha$ and COX-2 and matrix degradative enzymes such as MMP-1, MMP-3, MMP-13, and an increase in matrix prevention factors such as TIMP-1, TIMP-3. E-selectin expression in chondrocytes was significantly reduced in the group of hUC-MSC CM, which led to reduced monocyte adhesion. This study demonstrated that hUC-MSC CM exert chondroprotective effects. The paracrine mechanism of hUC-MSC may be responsible for the protection of chondrocytes against IL-1$\beta$ exposure. Therefore, the current study offers a potential method for attenuating osteoarthritis by taking a fundamentally biological approach

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# 국문초록

# 골관절염 연골세포의 E-selectin 의존성 단핵구 동원 억제를 통한 인간 탯줄유래 중간엽 줄기세포 배양액의 항염증 효과

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**배경:** 70세 이상 인구의 80% 이상에 영향을 미치는 골관절염은 흔한 질병 중 하나이다. 하지만 현재 통증과 염증 조절을 위한 NSAIDS 약물처방 및 corticosteroid 투여 외에 근본적인 생물학적 원인을 다루는 약물은 현 시장에 없는 상태이다. 골관절염의 발달과 진행에 대한 면역세포의 관여는 최근 연구에서 강조되었다. 특히 활성화된 M1 대식세포는 골관절염의 염증과정을 증폭시키는 사이토카인을 분비한다. 본 연구에서는 NGS분석을 통하여 IL-1$\beta$ 환경에 노출된 연골세포의 E-selectin 발현이 증가됨을 확인하였으며, 이는 라이간드를 발현하는 주변 단핵구를 IL-1$\beta$ 병변 환경으로 동원한다. 단핵구는 골관절염 환자의 체내 존재하는 IL-1$\beta$ 및 여러 염증성 사이토카인들에 의하여 염증성 물질을 방출하는 M1 대식세포로 분극화 될 수 있다. 본 연구는 면역억제 세포로서 항염증 인자 방출을 통해 염증을 감소시키고 단핵구의 활성도를 감소시킬 수 있는 인간 탯줄(human

Umbilical Cord, hUC) 유래 중간엽 줄기세포(Mesenchymal Stem Cell, MSC)를 배양한 배양액(Conditioned Medium, CM)이 골관절염 염증상황에서 연골세포의 E-selectin의 발현을 감소시켜 E-selectin 의존성 단핵구 모집을 억제한다는 가설과 함께 골관절염 진행 억제능력을 평가하였다.

**방법:** 슬관절 치환술을 시행한 총 7명의 만성 골관절염 환자에서 연골세포를 분리하였으며 hUC-MSC 분리 위하여 제왕절개 시술을 받는 산모에게서 탯줄을 분리, 수령하였다. 분리한 hUC-MSC에 IL-1β 자극을 준 후 얻어낸 CM을 IL-1β 환경에 노출 되어있는 연골세포에 처리해 주었다. 최종 실험그룹은 컨트롤, IL-1β, CM 그룹으로 진행되었으며 IL-1β병변 환경에 미치는 CM의 영향을 확인하기 위하여 연골세포의 유전자와 단백질 수준에서 염증성 및 항염증성 사이토카인, 기질 효소 및 이들 효소의 억제제의 발현을 조사하였다. 후에 MLDEG 분석 방법을 통하여 IL-1β 환경에 노출된 연골세포와 IL-1β 환경에 노출된 후 hUC-MSC CM을 처리해준 연골세포 비교 시 발현 정도에 차이를 나타내는 유전자를 비교 분석하였다. 결과, 전염증성 후보 유전자 5개, 항염증성 후보 유전자 1개를 얻었다. 최종적으로 단핵구 모집에 관여하는 SELE 유전자를 선택, IL-1β 환경에 노출된 연골세포와 IL-1β 환경에 노출된 후 hUC-MSC CM에 추가 노출된 연골세포 에서의 E-selectin 발현 정도를 비교하였다. 또한 컨트롤, IL-1β, CM 3개 그룹의 연골세포와 단핵구를 1시간 direct co-culture하여 연골세포 발현 E-selectin 의존성 단핵구의 부착 정도를 확인하여 hUC-MSC CM의 항염증 효과를 평가하였다.

**결과:** IL-1β 환경에 노출된 연골세포에 hUC-MSC CM 처리시 기질분해

효소인 MMP1, MMP3, MMP13, ADAMTS4 단백질 발현이 차례로 47.4%, 31.6%, 21.8%, 그리고 44.9% 감소, 기질 분해 방지 인자인 TIMP1, TIMP3 단백질 발현이 각각 128.9%, 그리고 68.4% 증가함을 확인하였다. MLDEG 분석 방법을 동원하여 IL-1β 와 CM 그룹의 연골세포에서 발현 차이를 보이는 총 126개의 전염증성 유전자와 238개의 항염증성 유전자를 발견하였다. 후에 combined p-value <0.02, combined logFC>1 그리고 기존의 사전 지식을 필터링 조건으로 설정하여 추가 분석한 결과 전염증성 유전자 5개(CXCL10, SELE, CAGE1, MYB, and IP6K3), 항염증성 유전자 1개(PTPRF)를 최종적으로 얻었다. 단핵구 모집에 관련성이 있어 OA 진행에 영향을 미치는 SELE 유전자를 추가 실험을 위한 최종 유전자로 선발하였다. 각 환경에 따른 연골세포에서의 E-selectin 발현양을 측정한 결과 hUC-MSC CM에 의한 E-selectin발현이 IL-1β그룹의 연골세포 대비 단백질 수준에서 21.8% 감소됨을 확인하였다. 각 환경의 연골세포에서 발현되는 E-selectin양에 따른 단핵구와의 결합 정도를 확인한 결과, IL-1β그룹의 연골세포 대비 CM그룹에서 단핵구와의 결합이 평균 53.9% 감소하였다. 더 나아가 연골세포의 E-selectin 발현을 blockade사용하여 차단 후 단핵구와 결합시킨 결과 blockade 사용하지 않은 그룹 대비 IL-1β그룹에서 42.1%, CM그룹에서 41.8% 감소함을 확인하여 단핵구가 E-selectin과 결합함을 확인하였다.

**요약:** OA 발병으로 인하여 고농도의 IL-1β환경에 노출된 연골세포에서의 E-selectin 발현은 염증성 물질 방출가능한 M1 대식세포로 분극화가 가능한 주변 단핵구를 병변환경으로 동원하게 된다. hUC-MSC CM은 연골

세포의 E-selectin 발현 가능성을 감소시켜 병변환경으로 주변 단핵구의 모집을 차단할 수 있는 효과를 나타내어 생물학적 원인을 다루는 약물이 없는 현재 시장에서 대체치료법으로의 사용 가능성을 보여준다.

**Keyword:** 탯줄유래 중간엽 줄기세포; 연골세포; 골관절염; 염증; 단핵구; 대식세포

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