Small G-protein activation in articular chondrocytes by interleukin-6, interleukin-8, and osteogenic protein-1.

A Thesis

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ABSTRACT

The studies outlined in this honor's thesis were designed to better understand the mechanisms at both the cellular and molecular levels associated with the onset of osteoarthritis. The horse was used as a model organism, and the experiments were performed on normal equine chondrocytes cultured *in vitro.* Interleukin-6 (IL-6) and interleukin-8 (IL-8) are known to have catabolic effects on the cartilage matrix while osteogenic protein-1 (OP-1) is a known anabolic factor. There is no complimentary information regarding the effects of these peptides on small G-protein (Cdc42, Rac, and Rho) activation in articular chondrocytes. The objectives of the present study were to determine the activation status of Cdc42, Rac, and Rho after treatment with IL-6, IL-8, or OP-1. The G-proteins play an important role in the maintenance of the actin cytoskeleton and chondrocyte phenotype, so they are of interest because of their clinical relevance in regards to osteoarthritis. To determine a suitable dose for use in these activation assays, chondrocytes were treated *in vitro* with varying concentrations of IL-6, IL-8, or OP-1, and changes in RNA transcripts of MMP-3, MMP-13, Col2A1, and Aggrecan were quantified using RT-PCR. In the case of IL-6, IL-8, and OP-1, it was determined that a dose of 100ng/mL media stimulated catabolism/anabolism. Chondrocytes were also examined using confocal microscopy following treatment with IL-6, IL-8, or OP-1 to determine if a correlation exists between the normal vs fibroblastic phenotypes and the activation status of the small G-proteins.

Our initial hypothesis was that IL-6 and IL-8 would increase Cdc42 and Rac and decrease Rho activity, while OP-1 would have an opposite effect. Activity of the small G-proteins was determined through an affinity binding assay and subsequent western analysis. To date, our experiments indicate that treatment of the chondrocytes with IL-6 and IL-8 resulted in a decreased activity status of Cdc42 and Rac, which is contrary to the initial hypothesis. Studies to determine the effects of OP-1 and the activation status of Rho in response to all treatments are nearing completion.

INTRODUCTION

Articular Cartilage and Osteoarthritis

Chondrocytes are the resident cells of articular cartilage and are responsible for the generation and maintenance of the collagen and proteoglycan components of the extracellular matrix (ECM).[1] Interactions between chondrocytes and the ECM are fundamental to the maintenance of normal cartilage phenotype. The cell is isolated within the ECM which is neither vascularised nor innervated, and nutrient/waste exchange occurs through diffusion. The chondrocyte rises to clinical prominence in the case of articular cartilage because these properties predispose the tissue to degenerative conditions, the most common being osteoarthritis (OA).[2] Although OA may affect juveniles, it is more associated with the older population such that most people of 70 years of age will have some symptoms of the disease. In fact, the clinical syndrome of osteoarthritis is one of the most common causes of pain and disability in middle-aged and older people.

The phenotypic expression of chondrocytes is in part controlled through regulation or modulation of the actin cytoskeleton. Chondrocytes expressing normal phenotype are generally polyhedral and have a cortical rim of actin while loss of phenotype is associated with a fibroblastic shape and cytosolic actin stress fiber formation. This correlation between the organization of the actin cytoskeleton and chondrocyte phenotype is important for understanding the maintenance of the chondrocyte phenotype and cartilage metabolism. [3]

GTPase proteins

The small G-proteins Cdc42, Rac, and Rho regulate signaling cascades involved in organization of the actin cytoskeleton, cell cycle control, gene expression and cell migration.[4-8] The G-proteins cycle between an active, GTP-bound state, and an inactive, GDP-bound state. Under normal physiological conditions, GTPases are GDP-bound and inactive. Once the cell is stimulated, the GTPase will release GDP and bind GTP. This process is catalyzed by guanine nucleotide exchange factors (GEFs). Once activated, GTPases interact with a variety of effecter proteins to promote cellular responses. GTPases are turned off by GTPase-activating proteins (GAPs) which stimulate the hydrolysis of GTP to GDP. Only in the GTP-bound form can these G-proteins communicate with signal transduction cascades and alter cell morphology. A major role of the GTPases is to interact with cellular target proteins and impact the reorganization and maintenance of the actin cytoskeleton. The G-proteins are associated with specific phenotypic alterations of the actin cytoskeleton such as stress fibers and focal adhesions (Rho), veil-like lamellipodial (Rac), and filopodial microspikes (Cdc42).[4, 5, 7, 9-11]

Because of their role in regulation of the actin cytoskeleton, G-proteins are of particular interest in the understanding of OA because their regulation will ultimately alter cell morphology and phenotypic expression.

Interleukin-6 (IL-8) and Interleukin-8 (IL-8)

The proinflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) are major regulators of the inflammatory response and have been identified as

pathogenic factors in a number of different tissues.[12, 13] Current research suggests that these cytokines are released in response to acute inflammation and exert a biological effect on the cell through cell-surface receptors.[14, 15] In the joint, regulation of these catabolic compounds plays an important role in the progression of osteoarthritis (OA), and understanding their role in chondrocyte physiology is therefore of significant clinical importance.[3, 5, 12, 13, 16-18] The mechanism by which these cytokines affect cartilage and chondrocyte physiology has not yet been extensively studied.

Osteogenic Protein-1 (OP-1)/ Bone morphogenetic protein-7 (BMP-7)

Several studies in a variety of both *in vitro* tissue culture and *in vivo* animal models have examined the anabolic effects of OP-1 on bone formation and cartilage regulation and maintenance. These studies have shown that OP-1 is expressed in normal articular cartilage, and that it is responsible for stimulating matrix synthesis in chondrocytes.[19-22] *In vitro* studies have demonstrated that OP-1 inhibits terminal chondrocyte differentiation. This terminal differentiation would otherwise result in chondrocyte hypertrophy and mineralization, which alters the phenotype of the chondrocyte. Together, these studies suggest that OP-1 preserves the normal chondrocyte phenotype.[23] This action opposes the effects of the interleukins, and the mechanism of this inhibition remains unknown.

Understanding the roles and regulations of these three peptides will further the overall understanding of chondrocyte biology and thereby further elucidate the mechanisms associated with the cellular onset of OA.

METHODS

The Cellular Model

Equine (*Equus equus)* chondrocytes were used as a cellular model for these experiments. Cartilage samples were harvested from donated research horses, and the chondrocytes were isolated from the harvested cartilage as previously described.[24] A digestion medium was used which consisted of 0.075% collagenase in F-12 complete medium (F-12 containing 10% fetal bovine serum (FBS), HEPES buffer, L-glutamine, α-ketoglutaric acid, ascorbic acid, penicillin, and streptomycin). Ten mLs of collagenase medium was added per gram of cartilage, and digestion was carried out for 12 hours at 37^{o}C. The cell suspension was filtered through a sterile funnel containing a base layer of 44 μm mesh and 4 layers of cheesecloth. The cells were pelleted, resuspended in medium, and stored in liquid nitrogen for the experiments described below.

Histology

Chondrocytes were grown in monolayer on cover slips at 50% confluence, treated with IL-6 (100 ng/ml), IL-8 (100 ng/ml), or OP-1 (100 ng/mL) for 0, 2, or 30 minutes, and fixed in 4% paraformaldehyde. Chondrocytes were subsequently incubated with 0.1% Triton and then 7% BSA to block non-specific antibody binding. The actin cytoskeleton was stained with Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA), and the nucleus was identified with To-PRO-3 nucleic acid stain (Invitrogen). The cover slips were mounted with ProLong Gold antifade reagent (Invitrogen), and the cells were visualized using confocal microscopy as previously described.[11]

Dose Response

Chondrocytes from horses 8-18 months old were plated at 80% confluence and grown at 37°C in F-12 complete media with 10% FBS for 48 hrs. The medium was changed on day 2 to defined DMEM/F-12 and mini-ITS (5nM insulin, 2ug/mL Transferrin, 2ng/mL Selenous Acid, 25ug/mL Ascorbic Acid, 420ug/mL BSA, 2.1 ug/mL linoleic acid).[22] On day 2, cells were treated with IL-6 (10, 50, or 100 ng/mL media), IL-8 (10, 50, or 100 ng/mL media), or OP-1 (50, 100, or 150 ng/mL media). This range of doses was examined for IL-6, IL-8 or OP-1 based on the doses examined in previous literature. The medium was exchanged on day 4 with the same treatment. On day 6, the media was removed, the cells were rinsed with PBS, lysed with Trizol, and total RNA was extracted. The IL-6 and IL-8 used for these studies was obtained from R & D Systems, Minneapolis, MN, and the OP-1 was obtained from Stryker Biotech, Hopkinton, MA.

Real-time quantitative PCR assays were then performed to assess changes in transcript levels of the matrix metalloproteinases 3 and 13 (MMP-3 and MMP-13), aggrecan, and collagen type IIb (Col2A1). Total RNA was reverse transcribed and amplified by use of a one-step system with sequence detection software (Applied Biosystems version 2.0, Foster City, CA). The primers and dual-labeled fluorescent probes (6-carboxyfluorescein [6-FAM] as the 5′ label [reporter dye] and tetramethylrhodamine [TAMRA] as the 3′ label [quenching dye]) were designed with Primer Express Software Version 2.0b8a (Applied Biosystems, Foster City, CA) and using equine sequences published in GenBank, sequenced in our laboratory, or obtained from Dr. Alan Nixon (Comparative Orthopaedics Laboratory, Cornell University,

Ithaca, NY). Gene expression of these compounds was assessed relative to the transcript levels of 18S RNA using the 2^{-ddCt} method as previously described.[25]

Affinity Binding Assay

Chondrocytes from horses 8-18 months old were plated at 100% confluence and grown in F-12 complete media with 10% FBS for 48 hrs. The medium was changed on day 2 to defined DMEM/F-12 and mini-ITS. On day 3, cells were treated with IL-6, IL-8, or OP-1 at 100 ng/mL media for 0, 2 or 30 minutes. The cells were rinsed with 1X PBS, and lysed using pGEX lysis buffer (1% Triton, 20 mM HEPES, 51 mM EDTA, 1.0 mM DTT, 15 mM NaCl, 1 mM NaN_3, 1.2 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 0.1 μM GDP, pH 8.0) for the Cdc42/Rac assay or rinsed with 1 X TBS, and lysed with Mg^{2+} lysis buffer (10% glycerol, 5% NP-40, 750 mM NaCl, 50 mM $MgCl_2$, 5 mM EDTA, 125 mM HEPES, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1.0 mM PMSF, pH 7.5) for the Rho assay as described previously.[6, 11, 26] The resultant chondrocyte lysates were utilized for the affinity binding assays as described below.

To generate positive and negative controls, cos 7 cells were transfected as described previously with plasmid DNA using FuGENE 6 according to the manufacturer's direction (Boehringer Mannheim, Mannheim, Germany).[27] Hemagglutinin-tagged pcDNA3 plasmids expressing wild type Cdc42, Rac, or RhoA, constitutively active Cdc42(Q61L), Rac(G12V), or RhoA(G14V), or dominant-negative Cdc42(T17N), Rac(T17N), or RhoA(T19N) were used.

Pulldown Assay for Cdc42 and Rac

To determine Cdc42 and Rac activity, an affinity-binding assay was performed using the cell lysate prepared as outlined above. The downstream target of Cdc42, p21-binding domain (PBD) of p21-activating kinase-1, was expressed in pGEX plasmid in *Escherichia coli*, purified, and coupled to glutathione-agarose beads as previously described.[28, 29]

The chondrocyte lysates were subjected to centrifugation at 5,000 × *g* for 1 minute at 4°C, and the total protein content was quantified using the Bradford method. Equal protein contents (1.0 mg) from each sample were loaded onto PBD beads to selectively retain GTP-Cdc42.[28, 29] The lysate/bead mixture was rocked for 30-45 minutes at 4°C, centrifuged, and rinsed three times with pGEX lysis buffer to remove unbound proteins from the suspension. Western blot loading dye was then added to the final pellet, and the beads were boiled for 5 minutes to release the retained Cdc42 and Rac. Samples were resolved by 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk and then probed with a primary mouse antibody to either Cdc42 or Rac (BD Biosciences, Palo Alto, CA). Secondary antibodies of horseradish peroxidase-coupled sheep anti-mouse were used to detect the primary antibody with enhanced chemiluminescence (ECL) reagent, and the resultant chemiluminecence was quantified using a Chemi-Doc station with Quantity One software (Bio-Rad, Richmond, CA). A ratio of retained active: total (GTP: total (GTP+GDP)) at times 0, 2, and 30 minutes ($[GTP/(GTP+GDP)]t_0$, $[GTP/(GTP+GDP)]t_2$, $[GTP/(GTP+GDP)]t_{30}$) was calculated, and the ratios were normalized to t_0=1.0 for comparison between treatments.

Pulldown Assay for Rho

Glutathione-agarose beads were created and tagged with the Rho binding domain (RBD) of the downstream target, Rhotekin (TRBD) as described.[30, 31] Equal protein contents of 4mg were loaded with the RBD beads (using the same protocol as described for Cdc42 and Rac), the samples were resolved with PAGE, transferred to a PVDF membrane, and a monoclonal antibody to Rho (BD Biosciences, San Jose, CA) was used to detect Rho and a ratio of retained active:total (GTP: total (GTP+GDP)) was calculated.

RESULTS

Histology

Chondrocytes were observed using confocal microscopy to evaluate the actin cytoskeleton after treatment with IL-6, IL-8 or OP-1. Untreated, control chondrocytes demonstrated a defined cortical rim, minimal stress fibers, and a polyhedral shape **[Figure 1]**. With 2 minutes of IL-6 and IL-8 treatment, cells developed cytosolic stress fibers, which remain after 30 minutes of treatment. The increase in stress fibers is indicative of Rho activation.[4] Cells treated with OP-1 at 2 minutes appeared round with a defined cortical rim of actin. The chondrocytes remained in this state after 30 minutes OP-1 treatment **[Figure 1]**.

Dose Response

As outlined above, chondrocytes were treated with IL-6 (10, 50, or 100 ng/mL media), IL-8 (10, 50, or 100 ng/mL media), or OP-1 (50, 100, or 150 ng/mL media).

RNA expression of Collagen type IIb (Col 2A1) **[Figure 2]** was unaffected by treatment with IL-6, IL-8, or OP-1. The catabolic matrix metalloproteases, MMP-13 [**Figure 3]** and MMP-3 **[Figure 4]**, were increased with treatment of IL-6 at 100ng/mL, IL-8 at 50 and 100ng/mL, and OP-1 at 50 ng/mL. Aggrecan **[Figure 5]** expression was increased after treatment with IL-6 at 100 ng/mL and OP-1 at 100 ng/mL. Due to inter-animal variability, more trials are in progress to verify these findings. Based on this preliminary data, a dose of 100ng/mL media was utilized for the affinity binding assays with IL-6, IL-8, or OP-1.

Activation Status of G-Proteins

Cdc42- Cdc42 activity **[Figure 6-7]** was normalized to 1.0 among all no treatment controls for comparison to either IL-6 or IL-8. IL-6 treatment (2 minutes) resulted in a significant decrease in active, GTP-bound Cdc42 compared to the no treatment control (mean 55.9%, range 10.0%-84.3%; p=0.05). After 30 minutes of IL-6 treatment, Cdc42 activity remained significantly decreased (mean 51.3%, range 31.9-79.1%; p=0.05). There was no difference in Cdc42 activity between 2 and 30 minute treatment time points. IL-8 had a similar effect on Cdc42 activation status. There was a significant decrease in Cdc42 activation status after 2 minutes of treatment (mean 56.7%, range 21.0-81.7%; p=.05). There were no significant differences in Cdc42 activity between 2 and 30 minute time points.

Rac- Rac activity **[Figure 8-9]** was normalized to 1.0 among all no treatment controls for comparison to either IL-6 or IL-8. IL-6 treatment (2 minutes) resulted in a significant decrease in active, GTP-bound Rac compared to the no treatment control

(mean 70.9%, range 12.3%-86.4%; p=0.1). After 30 minutes of IL-6 treatment, Rac activity remained significantly decreased (mean 56.3%, range 21.7-64.1%; p=0.1). There was no difference in Rac activity between 2 and 30 minute treatment time points. IL-8 had a similar effect on Rac activation status. There was a significant decrease in Rac activation status after 2 minutes of treatment (mean 53.6%, range 40.3-63.4%; p=0.1). There were no significant differences in Rac activity between 2 and 30 minute time points. There is a significant decrease in GTP-Rac in response to both cytokines at both 2 and 30 minutes.

Rho-RBD pulldowns are in progress for treatments with IL-6, IL-8, and OP-1.

DISCUSSION

The roles of IL-6, IL-8, and OP-1 on cartilage catabolism and anabolism, respectively, have been previously established.[13, 18, 19, 21-23, 32, 33] In the present study, we sought to determine the effects of these cytokines on the activation status of the small G-proteins—Cdc42, Rac, and Rho. The effects of IL-6 and IL-8 in regulation of these small G-proteins were explored as potential molecular mechanisms responsible for the loss of chondrocyte phenotype and degradation of the cartilage matrix. The RhoA subfamily of small G-proteins were investigated due to their previously described effects on actin cytoskeleton organization and thus the regulation of chondrocyte phenotype.[4, 10, 34, 35] Reconciling these results with those of other studies will increase the understanding of these mechanisms and may lead to novel treatments of articular cartilage deterioration and arthritis.

Previous studies in our lab have examined the role of insulin-like growth factor-I (IGF-I), an anabolic compound, on articular chondrocytes. These studies suggest that IGF-I diminishes active, Cdc42 and Rac, and preserves the normal chondrocyte phenotype.[26, 28]

The findings of the present study suggest that both IL-6 and IL-8 diminish the activation status of Cdc42 and Rac. The histological findings demonstrate that after exposure to IL-6 and IL-8, a normal chondrocyte loses its polyhedral shape and assumes a more elongated, fibroblastic morphology with inherent cytosolic stress fibers and loss of the cortical rim of actin. These findings for IL-6 and IL-8 are in agreement with the results of other studies examining the impact of the closely related cytokine, IL-1.[3, 16, 17, 33, 36]

It is difficult to reconcile the results of the Cdc42 and Rac activation studies with the histological results because in the case of both IGF-I and IL-6 and IL-8, the activation status of Cdc42 and Rac are diminished, but there are substantially different histological findings. After treatment with IGF-I, the normal chondrocyte phenotype is preserved, while treatment with IL-6 and IL-8 results in a loss of the normal morphology. It is unclear why diametrically opposing mediators such as IL-6, IL-8, and IGF-I have the same effect on Cdc42 and Rac activation. Since the signaling networks that regulate cellular metabolism following cytokine stimulation are complex and involve a number of converging and diverging pathways, it is probable that these peptides activate parallel networks to generate distinct signaling pathways that are converging at some point at an upstream target of Cdc42 and Rac. Furthermore, it is

likely that the underlying cellular signaling is a very rapid event, and so the timing of the experiments is of utmost importance which might explain the difference between the histological findings and the results of the activation study.[4, 11, 26] Further studies to elucidate the upstream pathways from cell surface receptor to Cdc42 and Rac should provide additional information regarding the role of the small G-proteins in chondrocyte phenotypic control and therefore cartilage matrix regulation.

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FIGURE LEGENDS

Figure 1. Chondrocytes stained with Alexa Fluor 488 phalloidin (blue) to visualize the actin cytoskeleton and To-PRO-3 (red) to visualize nucleic acid with confocal microscopy. Control chondrocytes **[A]** show a defined cortical rim, minimal stress fibers, and tetrahedral shape. IL-6 **[B]** and IL-8 **[C]** treated cells demonstrate a less defined rim and increased number of stress fibers. OP-1 treated cells **[D]** show a more defined rim and rounded appearance.

Figure 2. Chondrocytes were treated with IL-6 (10, 50, or 100 ng/mL), IL-8 (10, 50, or 100 ng/mL), or OP-1 (50, 100, or 150 ng/mL), and Col2b expression levels were quantified relative to the expression of 18S (which is indicative of the relative amount of total RNA). The values were normalized with respect to the no treatment group.

Figure 3. Chondrocytes were treated with IL-6 (10, 50, or 100 ng/mL), IL-8 (10, 50, or 100 ng/mL), or OP-1 (50, 100, or 150 ng/mL), and Col2b expression levels were quantified relative to the expression of 18S (which is indicative of the relative amount of total RNA). The values were normalized with respect to the no treatment group.

Figure 4. Chondrocytes were treated with IL-6 (10, 50, or 100 ng/mL), IL-8 (10, 50, or 100 ng/mL), or OP-1 (50, 100, or 150 ng/mL), and Col2b expression levels were quantified relative to the expression of 18S (which is indicative of the relative amount of total RNA). The values were normalized with respect to the no treatment group.

Figure 5. Chondrocytes were treated with IL-6 (10, 50, or 100 ng/mL), IL-8 (10, 50, or 100 ng/mL), or OP-1 (50, 100, or 150 ng/mL), and Col2b expression levels were quantified relative to the expression of 18S (which is indicative of the relative amount of total RNA). The values were normalized with respect to the no treatment group.

Figure 6. Activation status of Cdc42 in articular chondrocytes detected by Western blot analysis using a monoclonal Cdc42 antibody. The top row represents only active, GTP-bound Cdc42 in no treatment controls (NT) and chondrocytes treated with IL-6 or IL-8 for 2 or 30 minutes. The bottom row represents whole cell lysate (WCL) which contains both GTP and GDP-bound Cdc42 and serves as a loading control for the PBD assay. This Western blot is representative of 4 individual experiments.

Figure 7. The mean ratio of GTP-Cdc42:total Cdc42 from n=4 experiments (+/- SE). Experiments were performed independently and using chondrocytes from three different animals. The no treatment control (NTC) value is set to 1 and all values are expressed as relative to the NTC.

Figure 8. Activation status of Rac in articular chondrocytes detected by Western blot analysis using a monoclonal Rac antibody. In each whole cell lysate (WCL) the expression of total Rac (GTP and GDP bound) was similar, serving as a loading control for the PBD assay.

Figure 9. The mean ratio of GTP-Rac:total Rac from n=3 experiments (+/- SE). Experiments were performed independently and using chondrocytes from three different animals. The no treatment control (NTC) value is set to 1 and all values are expressed as relative to the NTC.

Figure 1

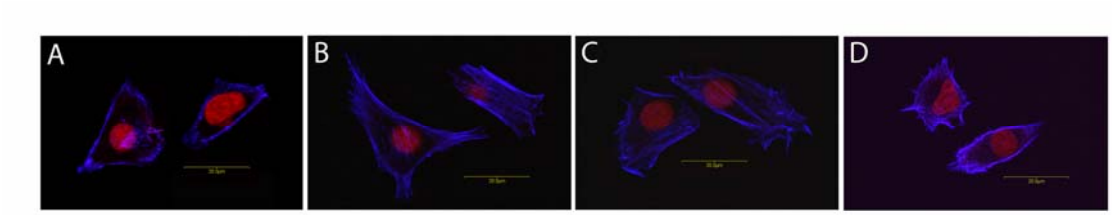
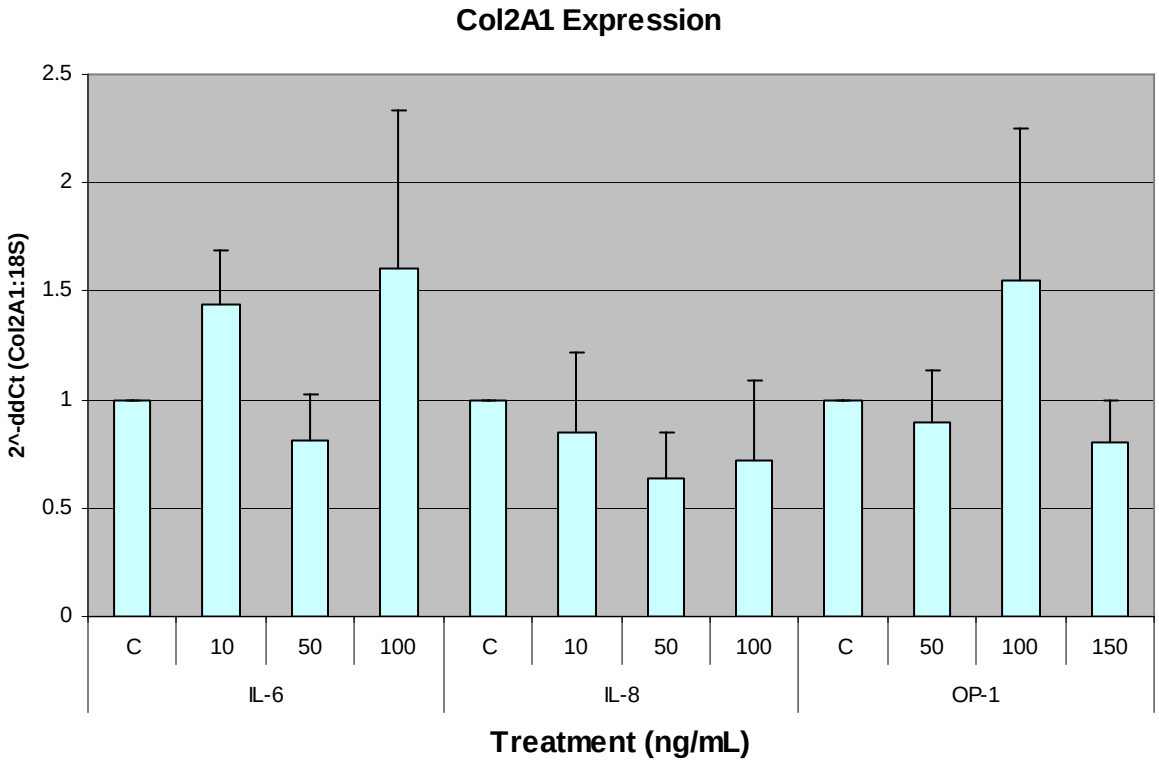


Figure 2


Col2A1 Expression
2^-ddCt (Col2A1:18S)
2.5
2
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1
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10
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100
C
50
100
150
IL-6
IL-8
OP-1
Treatment (ng/mL)

Figure 3

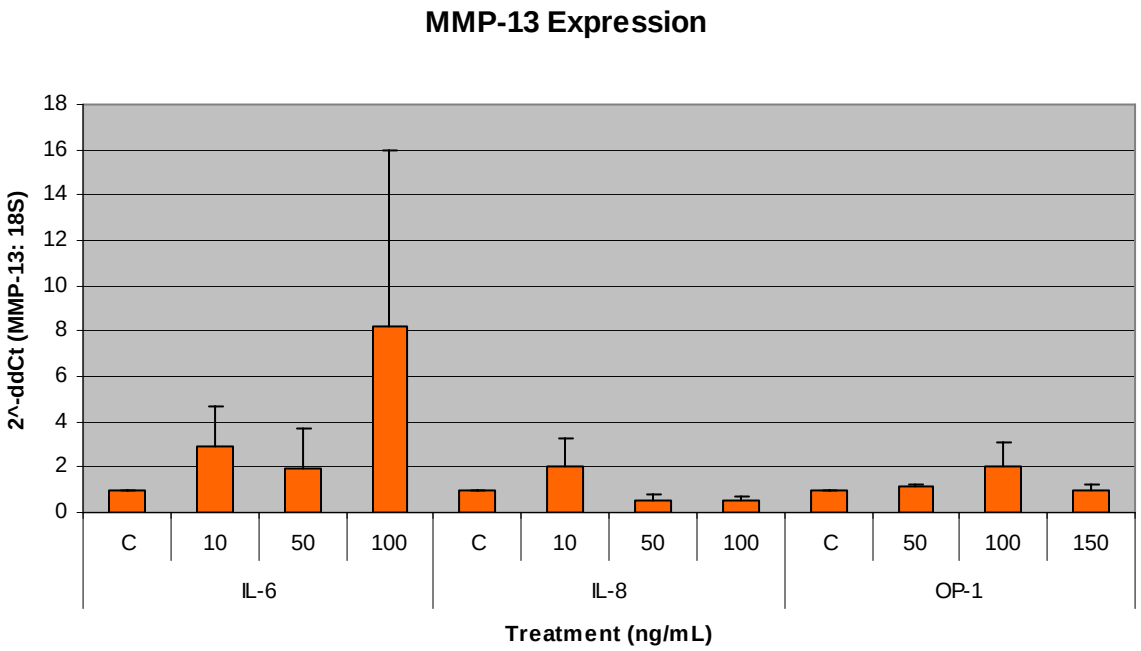

MMP-13 Expression
2^-ddCt (MMP-13: 18S)
18
16
14
12
10
8
6
4
2
0
C
10
50
100
C
10
50
100
C
50
100
150
IL-6
IL-8
OP-1
Treatment (ng/mL)

Figure 4

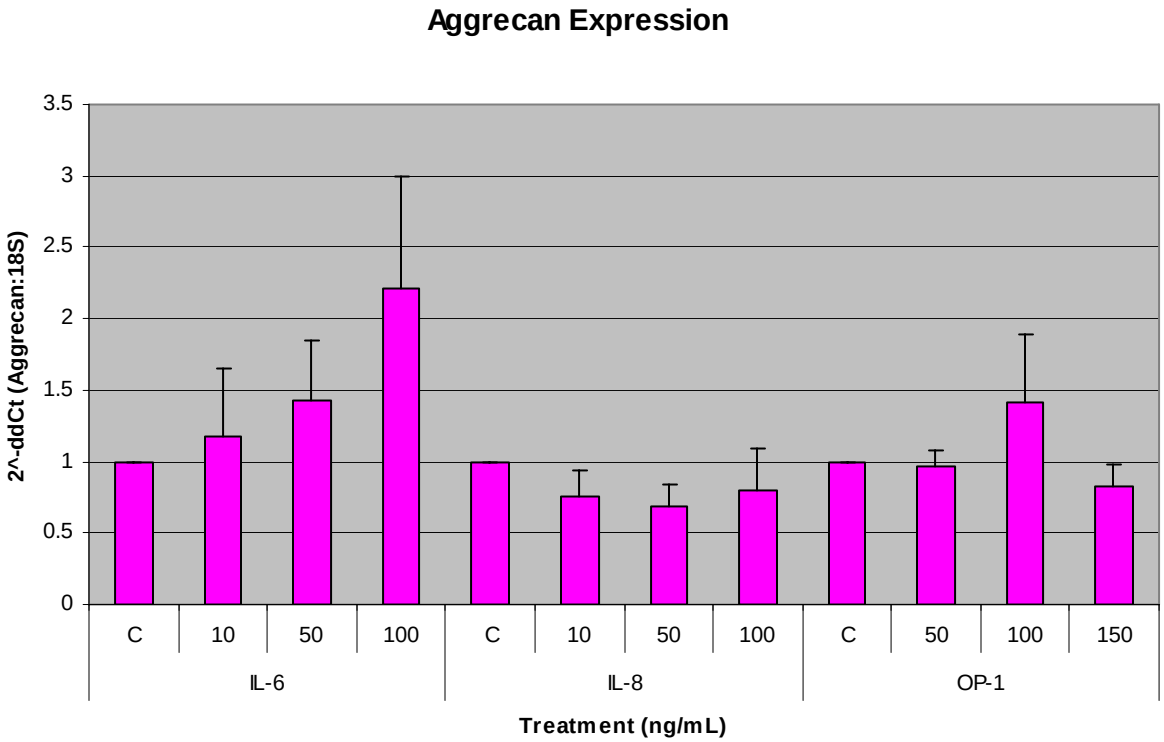

Aggrecan Expression
2^-ddCt (Aggrecan:18S)
3.5
3
2.5
2
1.5
1
0.5
0
C
10
50
100
C
10
50
100
C
50
100
150
IL-6
IL-8
OP-1
Treatment (ng/mL)

Figure 5

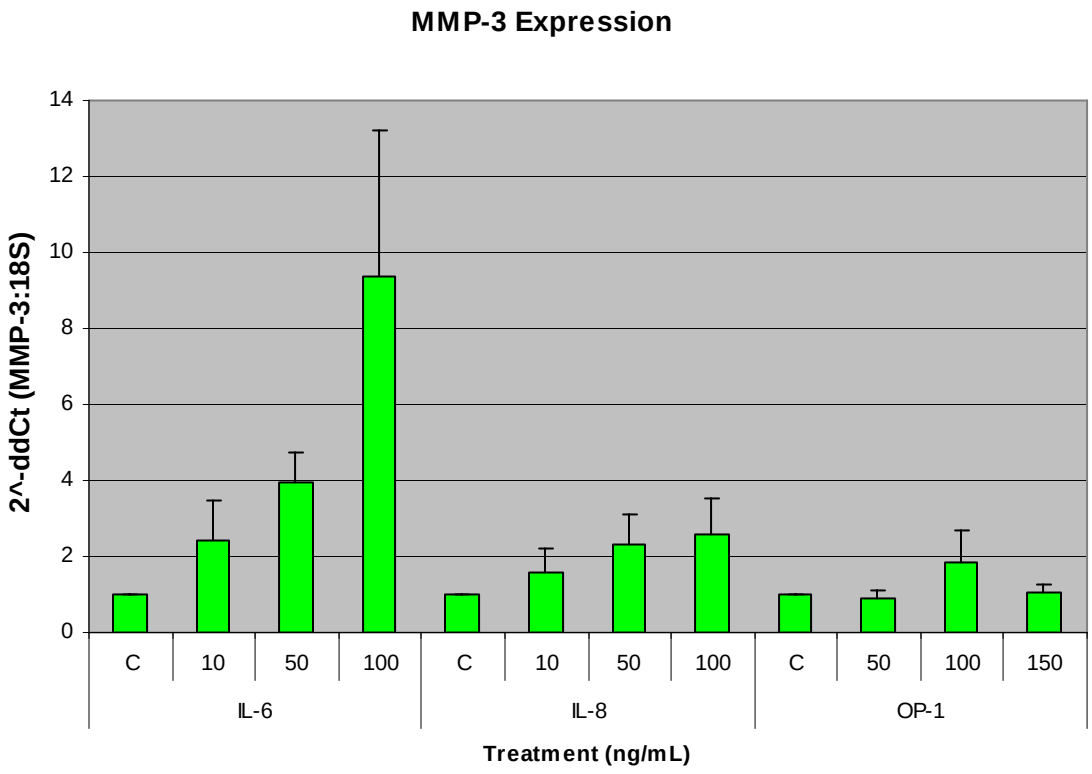

MMP-3 Expression
2^-ddCt (MMP-3:18S)
14
12
10
8
6
4
2
0
C
10
50
100
C
10
50
100
C
50
100
150
IL-6
IL-8
OP-1
Treatment (ng/mL)

Figure 6

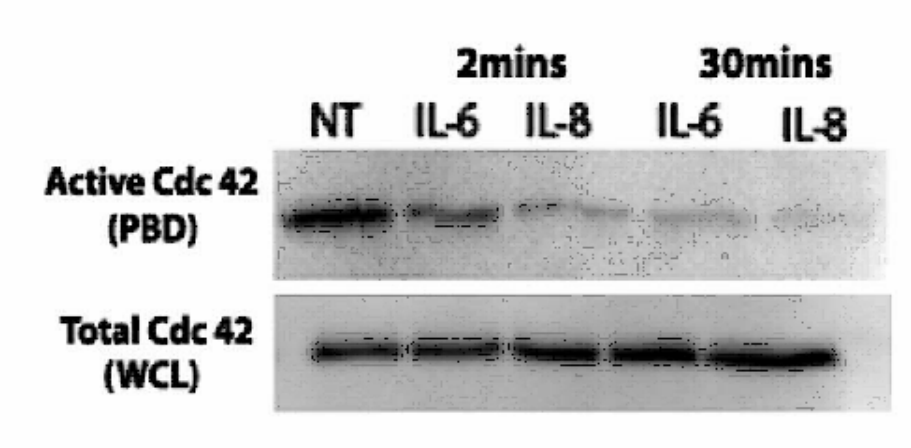

2mins
30mins
NT
IL-6
IL-8
IL-6
IL-8
Active Cdc 42
(PBD)
Total Cdc 42
(WCL)

Figure 7

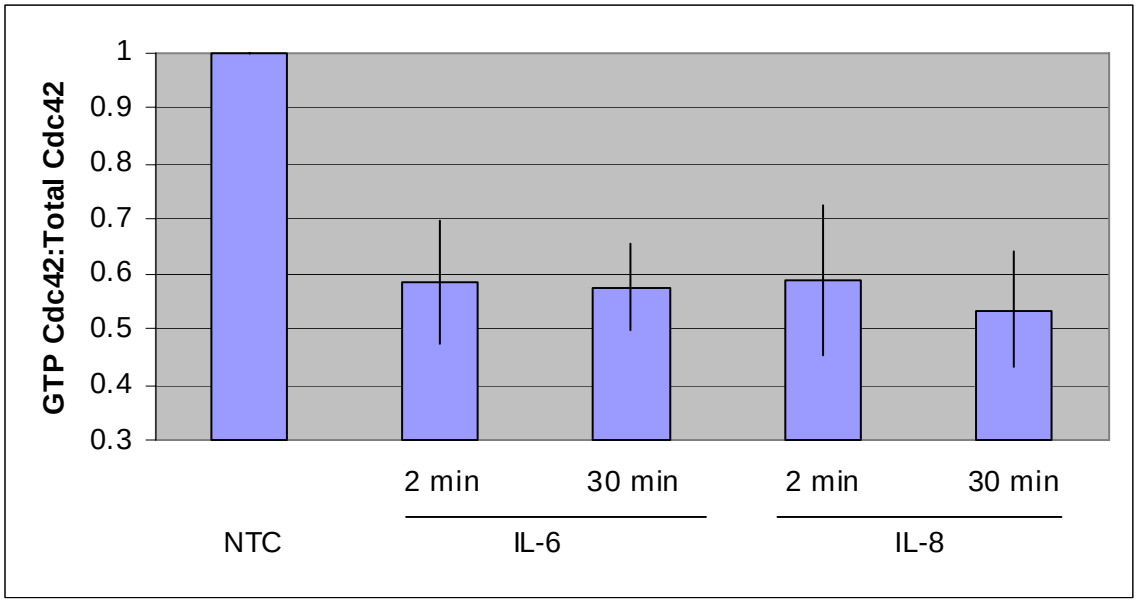

GTP Cdc42:Total Cdc42
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2 min
30 min
2 min
30 min
NTC
IL-6
IL-8

Figure 8

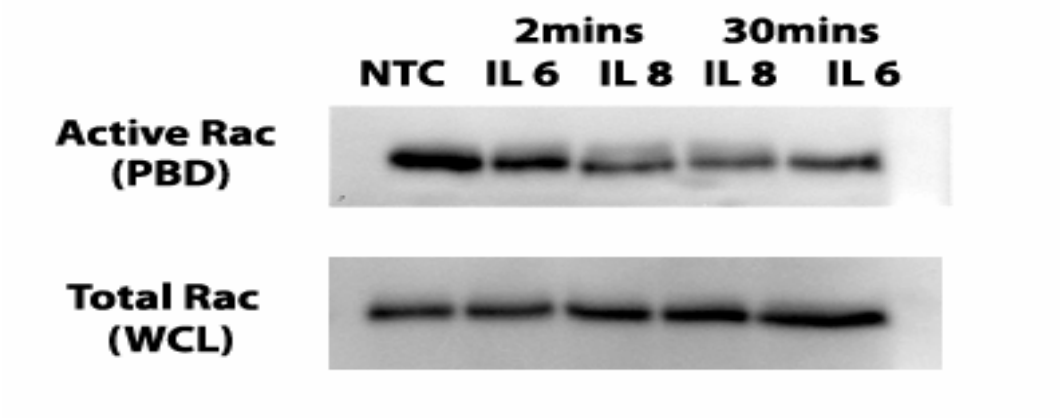
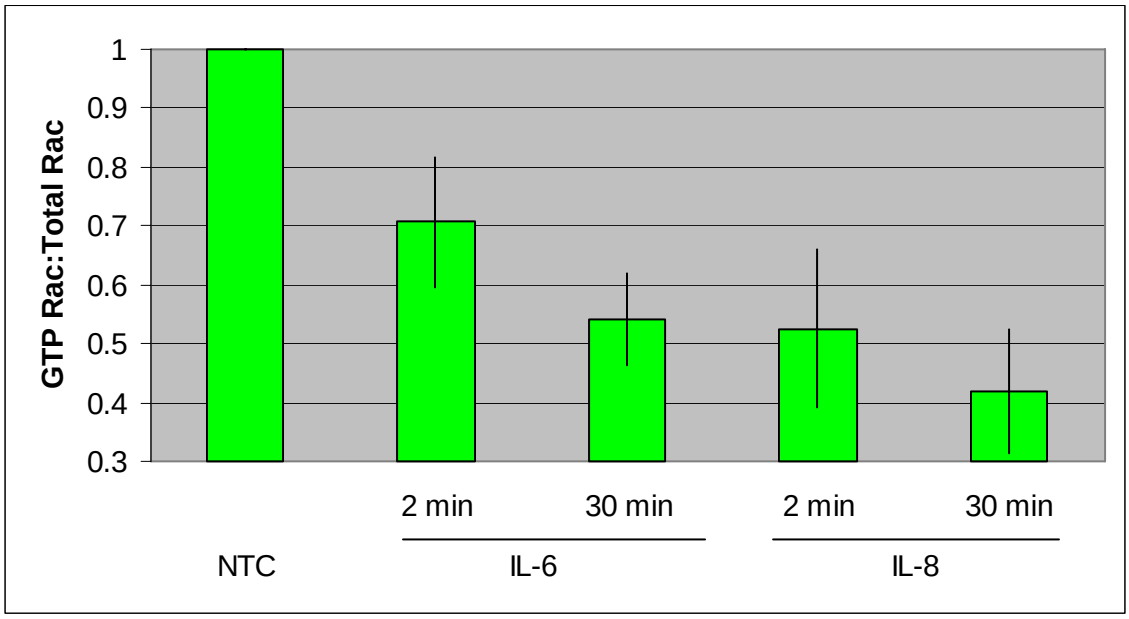


Figure 9


GTP Rac:Total Rac
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2 min
30 min
2 min
30 min
NTC
IL-6
IL-8