# Guiding synovial inflammation by macrophage phenotype modulation: an *in vitro* study towards a therapy for osteoarthritis

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## ABSTRACT

*Objective:* The aims of this study were to modulate inflammation in synovial explants with the compounds: dexamethasone, rapamycin, bone morphogenetic protein 7 (BMP-7) and pravastatin, and to investigate the modulatory capacity of the compounds on specific macrophage phenotypes.

*Design:* Synovial explants from osteoarthritis (OA) patients were treated with $10^{-6}$M dexamethasone, 100 ng/mL rapamycin, 500 ng/mL BMP-7 or 50 μM pravastatin. Half of the explants were pre-stimulated with IFNγ+TNFα to simulate acute inflammation. Inflammatory state of the synovium was assessed with gene expression analysis. Primary human monocytes were isolated and stimulated towards macrophage phenotypes M(IFNγ+TNFα), M(IL-4) and M(IL-10) with the respective cytokines, followed by treatment with the compounds.

*Results:* Dexamethasone had an anti-inflammatory effect on IFNγ+TNFα stimulated and osteoarthritic synovium, likely due to suppression of pro-inflammatory M(IFNγ+TNFα) macrophages while enhancing anti-inflammatory M(IL4) and M(IL10) macrophages. Rapamycin and BMP-7 further enhanced inflammation in stimulated synovium, but rapamycin did not have a clear effect on non-stimulated synovium. Rapamycin suppressed M(IL-4) and M(IL-10) macrophages without affecting M(IFNγ+TNFα). BMP-7 suppressed M(IFNγ+TNFα) and enhanced M(IL-10) in the macrophage cultures. Pravastatin did not affect synovium, but enhanced M(IL-10).

*Conclusions:* These data indicate that macrophage phenotype modulation can be used to guide joint inflammation and thereby contribute to the development of new therapies to delay the progression of OA. The varying effects of the compounds on synovium of different degrees of inflammation, indicate that the modulatory capacity of the compounds depends on OA stage and underlines the importance of identifying this stadium for adequate treatment.

## INTRODUCTION

Osteoarthritis (OA), a chronic degenerative disease affecting the whole joint, is characterized by erosion of articular cartilage and synovial inflammation. Macrophages, along with fibroblasts, reside in the synovial lining of joints. These cells can become activated by stimuli from their microenvironment, a process that results in a spectrum of phenotypes[47]. This spectrum ranges from pro-inflammatory (M1) macrophages, induced by stimuli such as interferon (IFN), tumor necrosis factors (TNFs)[125] or lipopolysaccharides (LPS)[45], to anti-inflammatory macrophages (M2), which can be further divided into specific subtypes depending on their stimuli such as IL-4 or IL-10[45]. Because of their importance in diseases and homeostasis, macrophages are frequently the focus of development of new interventions and treatment strategies[126]. Early *in vivo* studies have shown that depletion of phagocytic synovial lining cells, resulted in less influx of polymorphonuclear neutrophils, reduced proteoglycan degradation and reduced chondrocyte death of cartilage after induction of collagen induced arthritis (CIA)[127] . It was suggested that monocytes/macrophages are mainly responsible for this cartilage damage[128]. Moreover, osteophyte formation was also reduced after depletion of synovial macrophages in an OA mouse model[51]. The fact that high levels of pro-inflammatory cytokines were found in synovial fluid of OA joints and after trauma[93,94], together with the role macrophages seem to play in the development of OA and other inflammatory joint diseases, led to the hypothesis that a potentially specific strategy to control inflammation would be modulating the polarization state of macrophages. Therefore, the aim of this study was to guide synovial inflammation with four compounds representing commonly used classes of drugs in the clinics, that may have the potential to modulate macrophage phenotype: dexamethasone, rapamycin, bone morphogenetic protein 7 (BMP-7) and pravastatin. Dexamethasone is a corticosteroid with well-known anti-inflammatory effects[129] and is used to treat numerous inflammatory-based diseases. Rapamycin, known by its non-propriety name sirolimus, is an immunosuppressive commonly used in transplantation medicine[130]. It inhibits T-cell activation by inhibiting signal transduction via mammalian target of rapamycin (mTor)[131]. BMP-7 is a member of the transforming growth factor beta (TGF-β) superfamily and is clinically used in orthopedic studies and applications, such as long bone non-union fractures[132,133]. Additionally, it has been shown that BMP-7 directed the polarization of THP-1 monocytes into an anti-inflammatory state[134]. Pravastatin is a member of the statins, a group of cholesterol synthesis inhibitors with a broad spectrum of effects, including anti-inflammatory effects[135]. Since all compounds are known to affect inflammatory processes, we investigated their effects on synovium of different degrees of inflammation. For this reason, OA synovium was additionally stimulated with IFNγ+TNFα to simulate acute inflammation. Furthermore, to examine the

modulatory effects of the compounds on macrophage phenotypes, human monocyte-derived macrophages were polarized to specific phenotypes and treated with the same compounds.

## MATERIALS AND METHODS

### Modulation of synovium

To examine the effect of stimulation with IFNγ+TNFα, synovial tissue was obtained from OA patients (*n*=4, 60±13Y) undergoing total knee replacement surgery. The synovium was washed twice with 0.9% NaCl (Sigma-Aldrich, St. Louis, USA) and cut into pieces of 30-35 mg wet weight. To simulate acute inflammation[93,94], the explants were cultured for 24h with 10 ng/mL Interferon-γ (IFNγ; PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL Tumor Necrosis Factor-α (TNFα, PeproTech) in medium (Dulbecco's Modified Eagle Medium, low glucose (DMEM; Gibco, Carlsbad, USA) supplemented with 1% Insulin-Transferrin-Selenium (ITS+ Premix, Corning, New York, USA), 50 µg/mL gentamicin (Gibco), 1.5 µg/mL amphotericin B (Fungizone; Gibco) and 25 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich)). After 24h of stimulation, the synovial explants were harvested and stored at -80°C until assessment for their inflammatory state after stimulation using gene expression analysis. To examine the effect of the compounds, synovial explants of other patients (*n*=4; 63±3Y) were pre-cultured for 24h with or without IFNγ+TNFα as described above to obtain acute inflamed synovium. Then, $10^{-6}$ M dexamethasone (Sigma-Aldrich), 100 ng/mL rapamycin (R&D Systems, Minneapolis, USA), 500 ng/mL BMP-7 (PeproTech), or 50 µM pravastatin (Sigma-Aldrich) were added to the medium and the explants were cultured for an additional 3 days. Doses were chosen based on literature[134,136-141]. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as vehicle for dexamethasone and rapamycin and the final DMSO concentration in the cultures was 0.01%. Donor demographics and culture conditions are shown in Table 5.1. The medium including compounds and stimuli was refreshed 24h prior to harvest and the explants were stored at -80°C until further processing for gene expression analysis.

### Monocyte isolation, stimulation and macrophage modulation

Primary human monocytes were polarized to specific macrophage phenotypes and cultured with the compounds. Monocytes were isolated by Ficoll density gradient separation and $CD14^+$ selection as described previously[43] from human buffy coats of male donors, 52±14Y (Sanquin Blood bank, Amsterdam, the Netherlands). For every experiment, monocytes were pooled from at least two donors and plated in monolayers in 48-well plates (Corning Costar, NY, USA) at a density of 500,000 monocytes/$cm^2$ in

**Table 5.1: Donor demographics and culture conditions of synovial explants**

| Donor | Gender | Age | Non-stimulated/IFNγ+TNFα stimulated | Compound treatment |
|---|---|---|---|---|
| 1 | female | 42 | Non-stimulated<br>IFNγ+TNFα stimulation | None (analyzed after 24h) |
| 2 | female | 74 | Non-stimulated<br>IFNγ+TNFα stimulation | None (analyzed after 24h) |
| 3 | male | 65 | Non-stimulated<br>IFNγ+TNFα stimulation | None (analyzed after 24h) |
| 4 | male | 65 | Non-stimulated<br>IFNγ+TNFα stimulation | None (analyzed after 24h)<br>DMEM (vehicle control)<br>DMSO (vehicle control)<br>Dexamethasone<br>Rapamycin<br>BMP-7<br>Pravastatin |
| 5 | male | 65 | Non-stimulated<br>IFNγ+TNFα stimulation | DMEM (vehicle control)<br>DMSO (vehicle control)<br>Dexamethasone<br>Rapamycin<br>BMP-7<br>Pravastatin |
| 6 | male | 65 | Non-stimulated<br>IFNγ+TNFα stimulation | DMEM (vehicle control)<br>DMSO (vehicle control)<br>Dexamethasone<br>Rapamycin<br>BMP-7<br>Pravastatin |
| 7 | male | 64 | Non-stimulated<br>IFNγ+TNFα stimulation | DMEM (vehicle control)<br>DMSO (vehicle control)<br>Dexamethasone<br>Rapamycin<br>BMP-7<br>Pravastatin |

X-VIVO™ 15 medium (Lonza, Verviers, Belgium) supplemented with 20% heat-inactivated fetal calf serum (FCS; Lonza), 50 µg/mL gentamicin and 1.5 µg/mL amphotericin B. To model a range of phenotypes of activated macrophages, the monocytes were stimulated 1 h after plating with 10 ng/mL IFNγ and 10 ng/mL TNFα (from now on referred to as M(IFNγ+TNFα)), 10 ng/mL Interleukin-4 (IL-4; PeproTech), to obtain M(IL-4) or 10 ng/mL IL-10 (PeproTech) to obtain M(IL-10)[45,125]. The macrophages were cultured and stimulated for 3 days at 37°C and 5% $CO_2$. After the stimulation period, the polarized macrophages were treated either with $10^{-6}$ M dexamethasone, 100 ng/mL rapamycin, 500 ng/mL BMP-7 or 50 µM pravastatin and cultured for an additional 3 days. The final DMSO concentration used in the cultures was 0.01%. The medium including compounds and

stimuli was refreshed 24h prior to harvest. After culture, the medium was collected, centrifuged at 200 x *g* to remove detached cells, and the supernatants were stored at -80ºC until cytokine measurements. The cells were harvested in PBS/0.1% Triton X-100 (Sigma-Aldrich) for DNA quantification or in RLT lysis buffer/1% β-mercaptoethanol (Qiagen, Hilden, Germany/Sigma-Aldrich) for mRNA isolation.

## Gene expression analysis

mRNA isolation, cDNA synthesis and PCR analysis were performed as described previously[80]. Gene expression of Interleukin-6 (*IL6*), Interleukin-1β *(IL1B*), Tumor Necrosis Factor-α (*TNFA*), chemokine (C-C motif) ligand 18 (*CCL18*), Interleukin-1 Receptor Antagonist (*IL1RA*), Mannose receptor, C type 1/CD206 (*MRC1/CD206*), cluster of differentiation 163 (*CD163*), Toll-Like-Receptor 4 (*TLR4*), and Transforming Growth Factor β1 (*TGFB1*) was evaluated. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and ubiquitin C (*UBC*) were all tested as housekeepers, where *GAPDH* was found the most stable (data not shown) and was therefore further used as normalization for the genes of interest. The amplification efficiency of all primers (Supplementary Table S5.1) was between 0.90 and 1.05 and the relative expression was determined by the $2^{-\Delta CT}$ formula.

## Quantification of cytokine production

Production of IL-6, CCL18 and soluble CD163 (sCD163) by the macrophages was quantified by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions of the human IL-6 ELISA Development Kit (PeproTech), human CCL18 DuoSet Development Kit (R&D Systems) and human soluble CD163 DuoSet Development Kit (R&D Systems). As an indication of the number of cells in the monolayers and to normalize cytokine production, the DNA content of the monolayers was measured using a modified CyQUANT assay (Invitrogen, Carlsbad, USA) as described previously[80].

## M1/M2-index calculation

An M1/M2-index was calculated based on the expression of the pro-inflammatory (M1) genes (*IL6*, *IL1B* and *TNFA*) and the anti-inflammatory (M2) genes (*CCL18*, *IL1RA*, *CD206* and *CD163*). The mean of the relative expression of M1 genes per sample to the overall M1 gene expression of all samples, was divided over the mean of the relative expression of M2 genes per sample to the overall M2 gene expression of all samples[43].

## Statistics

MS Excel 2010 and PASW Statistics 21.0 (SPSS Inc., Chicago, USA) were used for statistical evaluation. To take into account the variability between donors, a mixed

linear model after log transformation was used, followed by a Bonferroni's *post hoc* comparisons test. For the synovial explant experiments, inflammation state of the synovium (i.e., stimulation with IFNγ+TNFα) and donor were defined as random factors, while compound treatment was defined as a fixed factor. For the macrophages cultured in monolayers, polarization together with compound treatment were defined as fixed factors, while individual experiments were considered as random factors in the model. Differences were considered to be statistically significant for $P<0.05$.

## RESULTS

### Stimulation of osteoarthritic synovium leads to a difference in gene expression profiles

To investigate the modulatory capacity of the compounds on synovium of different degrees of inflammation, a culture model with or without IFNγ+TNFα stimulation was set up. Gene expression of *IL6*, *IL1B*, *TNFA, CCL18* and *IL1RA* was higher in OA synovium stimulated with IFNγ+TNFα than of synovium without stimulation when cultured in DMEM only (Figure 5.1A). The expression profile of IFNγ+TNFα stimulated synovium resulted in a higher M1/M2-index (Figure 5.1B), indicating that acute inflammation was indeed induced in the culture model.

### Dexamethasone, rapamycin, BMP-7 and pravastatin can modulate the gene expression profile of synovium

In IFNγ+TNFα stimulated OA synovium treated with dexamethasone, *IL1B*, *IL6*, *TNFA*, *IL1RA* and *CCL18* were lower than in the stimulated synovium cultured in DMSO control medium, while *CD206* and *CD163* were higher. This expression profile resulted in a lower M1/M2-index than in the controls. Treatment with rapamycin increased *IL6,* while lowering *CCL18* in stimulated synovium. This resulted in a higher M1/M2-index than in the DMSO controls. BMP-7 lowered expression of *IL1RA* compared to the DMEM controls, while pravastatin did not affect any of the genes of interest. For both BMP-7 and pravastatin, this expression profile resulted in a higher M1/M2-index than in the controls (Figure 5.2A).

In non-stimulated OA synovium treated with dexamethasone, *IL1B* was lower than in non-stimulated synovium cultured in DMSO control medium, while *CD163* was higher, eventually resulting in a lower M1/M2-index. Rapamycin did not affect the genes of interest, but the overall inflammation was reduced as indicated by a lower M1/M2-index. BMP-7 lowered *IL1B* and increased *CCL18*, resulting in a lower M1/M2-index than in the DMEM controls. Pravastatin did not affect the genes of interest and did not alter the overall inflammatory state of non-stimulated synovium (Figure 5.2B).

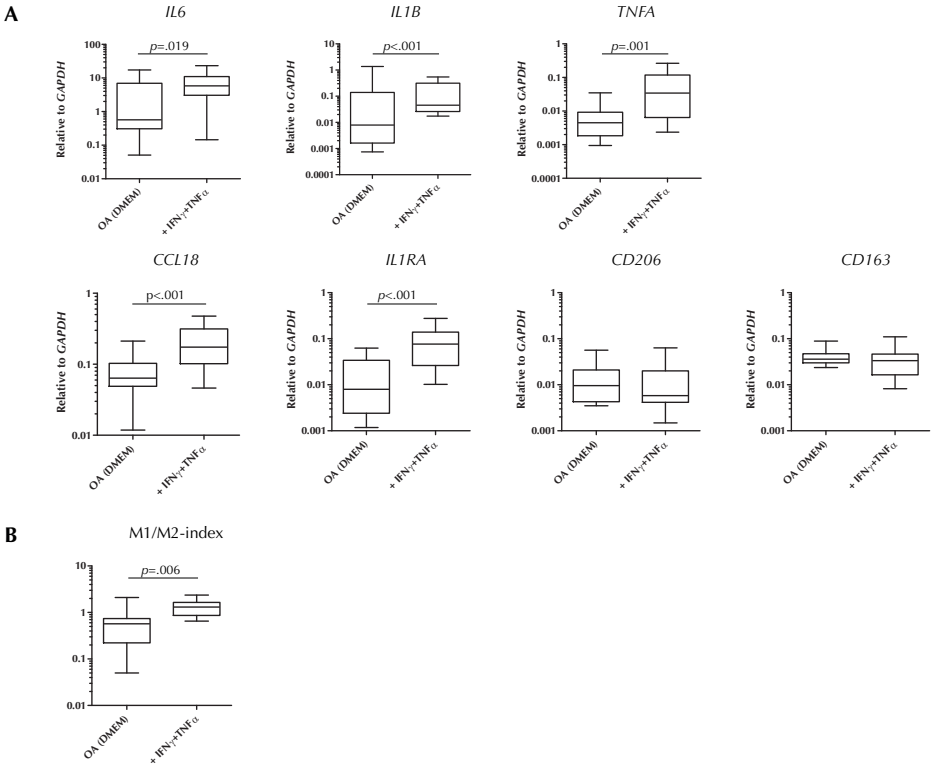


**Figure 5.1: Gene expression profile of osteoarthritic synovium with and without IFNγ+TNFα stimulation.** (**A**) Expression of genes encoding for pro-inflammatory proteins (*IL1B*, *IL6* and *TNFA)* and genes encoding for anti-inflammatory proteins (*IL1RA*, *CCL18*, *CD206* and *CD163)* relative to the expression of Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). (**B**) An M1/M2-index based on expression of all measured pro-inflammatory genes and anti-inflammatory genes to provide an overall assessment of the inflammatory state of the synovium with or without IFNγ+TNFα stimulation. Data is presented as boxplots with whiskers from minimum to maximum for *n*=4 donors in triplicate.

## Characterization and modulation of primary polarized macrophages

To assess the modulatory capacity of the compounds on specific macrophage phenotypes, primary monocyte-derived macrophages were polarized towards specific macrophage phenotypes and treated with the compounds. Without treatment, M(IFNγ+TNFα) had high expression levels of *IL6*, *IL1B, TNFA, IL10* and *TFGB1* and high protein production of IL-6. M(IL-4) expressed high levels of *CC18*, *IL1RA* and *CD206* and had high CCL18 protein production. M(IL-10) had higher expression of *CD206* than M(IFNγ+TNFα), but was lower than in M(IL-4). *CD163* was mainly expressed in anti-inflammatory macrophages and expression levels were higher in M(IL-10) than in M(IL-4). sCD163 protein production was high in M(IL-10) (Figure 5.3A and B). This confirmed that

stimulation with IFNγ+TNFα resulted in pro-inflammatory macrophages and IL-4 or IL-10 stimulation resulted in the development of anti-inflammatory macrophages of distinct phenotypes. As the IL-6, CCL18 and sCD163 protein production patterns were also specific for our generated phenotypes, we decided to use these three proteins as read-out parameters for detailed examination of the effects of the compounds on macrophages in monolayer.

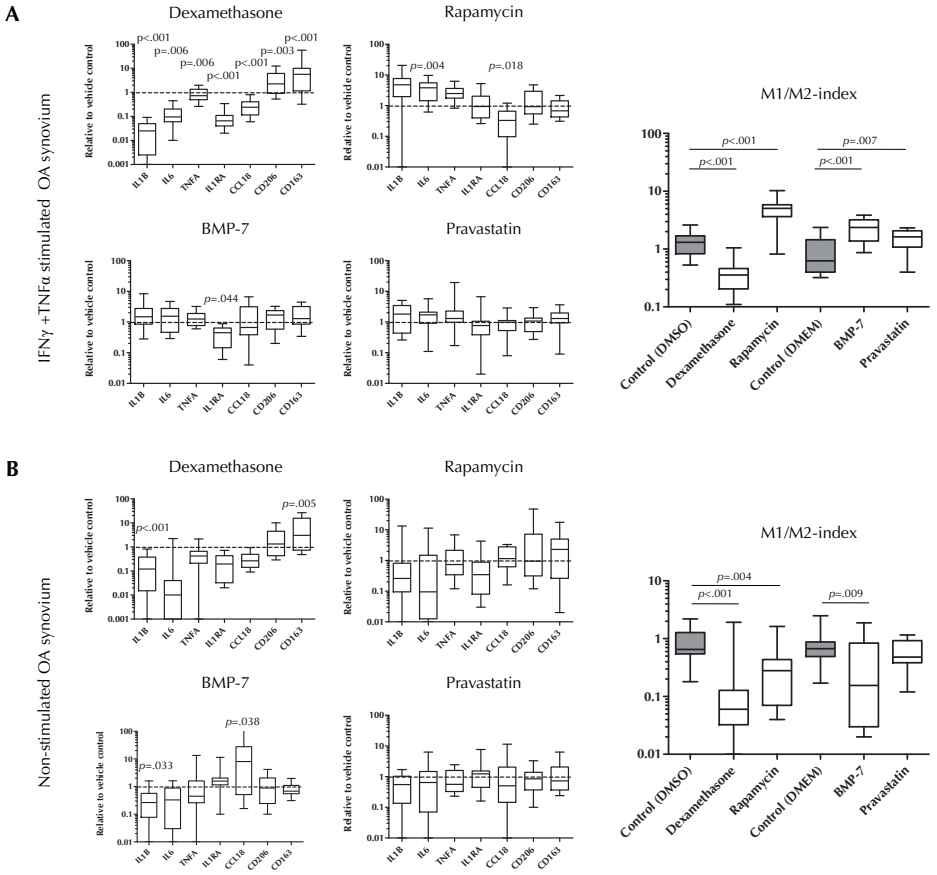


**Figure 5.2: Modulation of stimulated and non-stimulated osteoarthritic (OA) synovial tissue.** (**A**) Gene expression profiles of IFNγ+TNFα stimulated OA synovium, and (**B**) non-stimulated OA synovium after treatment with dexamethasone, rapamycin, BMP-7 or pravastatin relative to the expression levels of the vehicle controls as represented by the dotted line. 0.01% DMSO was used as vehicle control for dexamethasone and rapamycin and X-VIVO medium was used as vehicle control for BMP-7 and pravastatin. The M1/M2-index provides an overview of the overall inflammatory state of the synovial tissue after treatment with the compounds based on expression of all the measured pro-inflammatory genes and anti-inflammatory genes. Data is presented in boxplots with whiskers from minimum to maximum for *n*=4 donors in triplicate.

Interestingly, after treatment with dexamethasone, IL-6 production by the M(IFNγ+TNFα) was decreased, while production levels were maintained in M(IL-4) and M(IL-10). Production of CCL18 by M(IFNγ+TNFα) was maintained as well, whereas CCL18 production was increased in M(IL-4) and M(IL-10). sCD163 production was highly increased in all three macrophage phenotypes after treatment with dexamethasone (Figure 5.4A). Treatment with rapamycin lowered the IL-6 and CCL18 production of M(IL-4), CCL18 production of M(IL-10) and sCD163 production of M(IL-4) (Figure 5.4B). BMP-7 treatment did not have an effect on the IL-6, CCL18 and sCD163 protein production of M(IFNγ+TNFα) and M(IL-4), while CCL18 and sCD163 production was increased in M(IL-10) (Figure 5.4C). Pravastatin did not have an effect on the IL-6 and CCL18 production of either macrophage phenotypes, yet sCD163 was higher in M(IL-10) than in the untreated controls (Figure 5.4D).

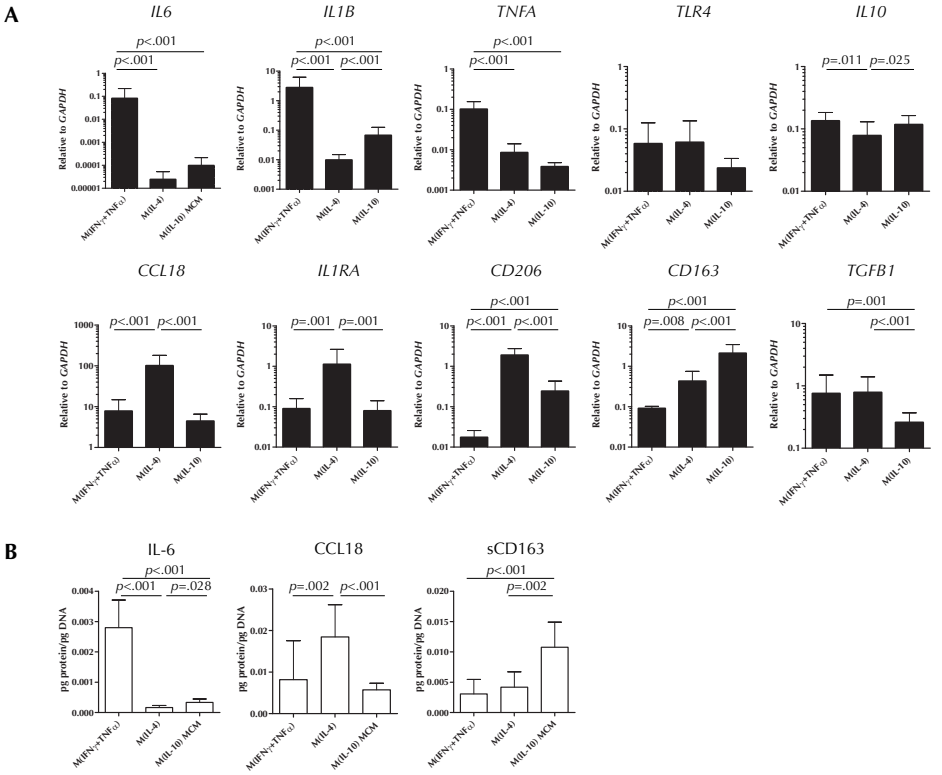


**Figure 5.3: Characterization of primary human macrophages stimulated with IFNγ and TNFα (M(IFNγ+TNFα)), IL-4 (M(IL-4)) and IL-10 (M(IL-10))**. (**A**) Gene expression profile relative to *GAPDH* expression and (**B**) protein production of IL-6, CCL18 and sCD163 corrected for amount of DNA. Data is shown as mean ± SD for *n*=3 donors in 5-fold. The error bars represent the variation between donors.

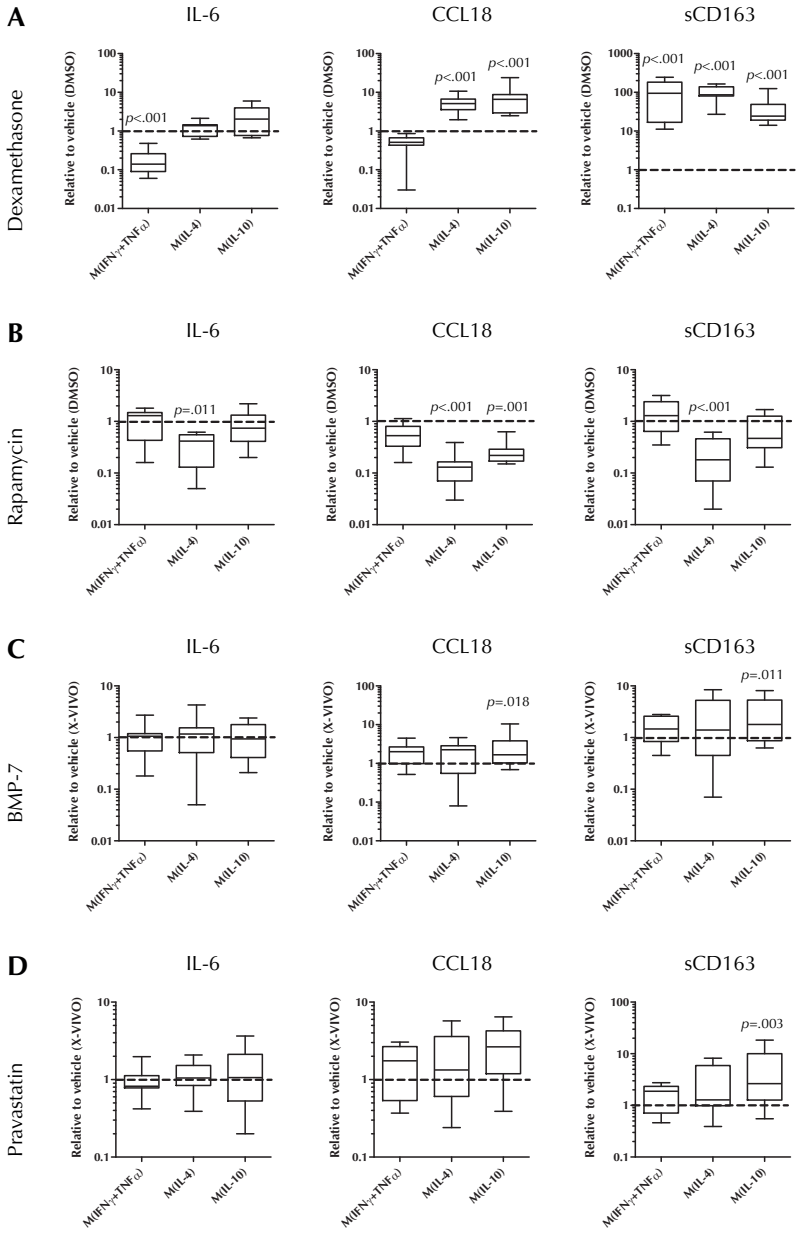


**Figure 5.4: Modulation of primary polarized macrophages.** IL-6, CCL18 and sCD163 protein production of M(IFNγ+TNFα), M(IL-4) and M(IL-10) macrophages after modulation with **A**) dexamethasone, **B**) rapamycin, **C**) BMP-7, and **D**) pravastatin. Data is presented for *n*=3 independent experiments in triplicate as boxplots with whiskers from minimum to maximum and relative to the compound vehicle controls represented as a dotted line. 0.01% DMSO was used as vehicle control for dexamethasone and rapamycin and X-VIVO medium was used as vehicle control for BMP-7 and pravastatin.

## DISCUSSION

In this study, we have shown that the overall inflammatory state of synovial explants can be modulated by dexamethasone, rapamycin, and BMP-7. Because macrophages play a crucial role in inflammatory processes that contribute to inflammation, phenotype modulation can be used to control synovial inflammation, and subsequently may also inhibit the progression of post-traumatic OA.

Nowadays, many compounds are being tested for their anti-inflammatory effects *in vitro* as potential new interventions for OA. However, completely suppressing inflammation, either via macrophages or by directly inhibiting cytokines, may not be the most successful method to treat OA, as inflammation to a certain extend is required for proper wound healing[142]. Since the composition of synovial tissue varies at different stages of OA[2] and even between patients, we can assume that the composition of macrophage phenotypes in the synovium also differs, which was indicated in our previous work[5]. To our knowledge, we are the first to show modulation of synovium of different degrees of inflammation using compounds that are already clinically applied. In addition, we showed that these compounds target specific macrophage phenotypes by either enhancing or suppressing their function. Our culture model using human synovium allowed us to study *in vitro* the effects of the compounds on synovial macrophages in their natural microenvironment. We are aware that we cannot rule out the possibility that by treating the explants with the compounds, fibroblasts or other cells present in the synovium were also affected by the compounds. Therefore, we compared the modulatory effects of the compounds on the entire synovial explants in terms of change in overall synovial inflammation, with their effect on different phenotypes of macrophages cultured in monolayers. Macrophages, once migrated into tissue, are in an activated state. With the polarization of M(IFNγ+TNFα), M(IL-4) and M(IL-10), we aim to model this range of phenotypes. Our aim was to modulate already polarized macrophages in synovial tissue and not to intervene with the process of polarization from monocyte to macrophage. Therefore, assessment of the effects of the compounds on unstimulated macrophages is beyond the scope of this study. Additionally, we have shown that compounds that were initially known for their anti-inflammatory effect, can either behave in a pro-inflammatory or anti-inflammatory manner and that the behavior of the compounds differs and depends on the macrophage phenotypes present in the tissue that is treated. The specific modulatory effects of the compounds on synovium and macrophages could provide valuable insights for the development of specialized therapies aiming at modulation of specific macrophage phenotypes.

In our study, dexamethasone had an anti-inflammatory effect on synovium and suppressed the pro-inflammatory macrophage phenotype, while enhancing the anti-inflammatory macrophages. Since *CD163* expression can be assumed as a marker

for M(IL-10), these data combined suggests that dexamethasone has the capacity to modulate synovial tissue and to specifically enhance the macrophage phenotypes resembling M(IL-10). The effect of dexamethasone on macrophages was shown earlier to vary depending on macrophage origin, as phagocytic activity in alveolar macrophages was induced more abundantly than in peritoneal macrophages[143]. The difference in anti-inflammatory capacity per macrophage source suggests that treatment with dexamethasone may result in responses that differ according to macrophage type or origin. Intra-articular delivery of dexamethasone was shown to have chondroprotective effects and reduced inflammation in a post-traumatic OA animal model[144]. This could be explained by the fact that dexamethasone enhanced M(IL-10), which was seen in our present study, as well as of others[81], on gene expression level and by increased levels of sCD163 in the macrophage cultures. Although this subtype expressing CD163 has been described as the tissue repair phenotype[38], is has also been associated with (chronic) inflammatory diseases[145,146]. Additionally, CD163 expressing macrophages induced by intra-articular injection of triamcinolone acetonide, also a corticosteroid, have been linked as well with the prevention of osteophyte formation in an OA rat model[147].

Rapamycin exerted an overall pro-inflammatory effect on IFNγ+TNFα stimulated synovium in this study, while the effect on non-stimulated OA synovium was less clear as no differences in expression levels were seen of any of the genes of interest. Our data indicated that the increase of inflammation of stimulated synovium in response to rapamycin was likely due to suppression of anti-inflammatory macrophages, without affecting the pro-inflammatory macrophages, as indicated by reduced IL-6, CCL18 and sCD163 protein production in the anti-inflammatory macrophages, while these levels were maintained in M(IFNγ+TNFα) after treatment with rapamycin. This was in line with an earlier study, where inhibition of the mTOR signaling pathway was shown to regulate macrophage polarization. Murine macrophages in which *Tsc1* was specifically deleted, and therefore mTOR complex 1 constitutively activated, were unable to polarize towards M2, while the pro-inflammatory response to LPS was enhanced. Treatment of these macrophages with rapamycin rescued this M2 polarization deficiency[136]. Furthermore, rapamycin seemed to shift the polarization of human macrophages towards a pro-inflammatory phenotype *in vitro*[148]. BMP-7 had an overall pro-inflammatory effect on IFNγ+TNFα stimulated synovium, while an anti-inflammatory effect on non-stimulated OA synovium. The effect on non-stimulated OA synovium can partially be explained by the effects seen on polarized macrophages, where BMP-7 increased the CCL18 and sCD163 production by M(IL-10), suggesting an anti-inflammatory effect. These data were in line with another study[134], where it was shown that BMP-7 directed the polarization of THP-1 monocytes into an anti-inflammatory state. The anti-inflammatory effect of BMP-7 indicate that the modulatory capacity of BMP-7 may depend on the phenotype

of the cells that are present in the tissue, assuming that the majority of the macrophages in OA synovium have a similar phenotype to IL-10 stimulated primary macrophages. On the other hand, the reason for the overall pro-inflammatory effect of BMP-7 seen in INFγ+TNFα stimulated synovial tissue remains unclear, although is likely due to a decrease of *IL1RA* expression which led to a reduced M1/M2-index. However no effect was seen on pro-inflammatory macrophages in the monolayer cultures. These data combined suggest that BMP-7 may also have an effect on other cells than macrophages that are present in synovium that are involved in inflammatory processes as well. Other studies have shown that intra-articular administration of BMP-7[137,149-151] and rapamycin[152-154] protected OA progression after induction of experimental OA. These studies focused mainly on articular cartilage quality in end-stage OA and have not taken into account the direct effect of BMP-7 and rapamycin on the synovial membrane. The varying effects of these two compounds on IFNγ+TNFα stimulated and non-stimulated synovium, underlines the importance of identifying OA stadium in order to clinically use BMP-7 or rapamycin as a therapy for OA.

Although statins are known for their pleiotropic effects, pravastatin did not have a clear modulatory effect on OA synovial tissue in our study. On polarized macrophages however, pravastatin increased sCD163 production in both anti-inflammatory phenotypes, which is in line with other studies that have shown anti-inflammatory effects of statins on macrophage polarization[155,156], chondrocytes[157,158], and progression of OA and arthritis *in vivo*[159,160]. Additionally, systemic statin use has also been shown to be associated with reduced progression of knee OA[161] and seemed to reduce the activity of rheumatoid arthritis in humans[162]. We chose to assess the effects of pravastatin over other statins such as simvastatin, since the latter needs to be bio-activated by the liver, or manually if it is not administered orally[163]. The fact that pravastatin was unable to modulate synovial tissue, suggests that it may not be suitable to be used intra-articular for guiding synovial inflammation by macrophage phenotype modulation, unless specifically targeting M(IL-10) like macrophages.

To conclude, dexamethasone, rapamycin and BMP-7 can modulate the overall inflammatory state of OA synovium by altering their expression profile. Future research could include large population patient studies to specifically correlate initial synovitis to the response after treatment. Directing synovial inflammation to delay the progression of OA may therefore be a suitable personalized medicine approach for which the optimal compound can be selected when the inflammatory state of the synovium has been determined.

# APPENDIX CHAPTER 5: INFLUENCING SYNOVIAL INFLAMMATION VIA MACROPHAGE PHENOTYPE MODULATION TO SUPPRESS CARTILAGE DEGENERATION: PROOF OF CONCEPT

## INTRODUCTION

An approach to impede cartilage degradation could be improving the inflammatory state of the synovial tissue via modulation of the residing macrophages. In previous work[68], we have shown that the overall inflammatory state of synovium can be modulated by corticosteroids and the modulatory capacity seemed to be associated with to alteration of the phenotype of macrophages. Additionally, we have shown that during OA induced in mice knees by intra-articular injection of collagenase or destabilization of the medial meniscus, the presence of anti-inflammatory CD163$^{+}$ macrophages inversely correlated with the presence of osteophytes[61]. Moreover, triamcinolone acetonide (TA) injections in rat knees increased the number of cells expressing receptor for folic acid (FRβ) and prevented the formation of osteophytes after induction of experimental OA[147]. FRβ is co-expressed on CD206+ macrophages[164], suggesting that TA enhances M2 macrophages. We therefore hypothesized that alteration of the inflammatory state of synovial tissue, due to modulation of the phenotype of synovial macrophages, could also influence cartilage degradation. Therefore, the aim of this study was to provide proof of concept whether modulation of the synovial macrophages *in situ*, would affect human OA cartilage. To explore this, the inflammation in synovial tissue was modulated using rapamycin or TA. Rapamycin exerted pro-inflammatory effects on macrophages cultured in monolayer, while TA, a corticosteroid has well known anti-inflammatory effects by specifically enhancing anti-inflammatory macrophages[68]. Based on these previous findings, these two compounds were selected to modulate the synovium, either to a more pro-inflammatory or anti-inflammatory state.

## MATERIALS AND METHODS

### Preparation of conditioned medium of modulated synovium (SCM)

Synovial tissue was obtained from osteoarthritis patients ($n$=4, 68±9Y) as waste material after total knee replacement surgery (approval number MEC2004-322). The synovium was washed twice with saline (0.9% NaCl; Sigma-Aldrich, St. Louis, USA) and cut into pieces of 40-60 mg wet weight. Simulation of acute inflammation was simulated by 24h pre-stimulation of half of the explants with 10 ng/mL Interferon-γ (IFNγ; PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL Tumor Necrosis Factor-α (TNFα, PeproTech) in

blanc medium (Dulbecco's Modified Eagle Medium, low glucose (DMEM-LG; Gibco, Carlsbad, USA) supplemented with 1% Insulin-Transferrin-Selenium (ITS+ Premix, Corning, New York, USA), 50 µg/mL gentamicin (Gibco), 1.5 µg/mL amphotericin B (Fungizone; Gibco), and 25 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich)). This stimulation has previously shown to increase the overall inflammatory state of OA synovium by enhancing expression levels of Interleukin (*IL*)6, *IL1B*, *TNFA*, chemokine (C-C motif) ligand 18 (*CCL18*), and *IL1RA*[68]. After stimulation, the synovial explants were treated with 0.1µM (100 ng/mL) rapamycin (R&D Systems, Minneapolis, USA) or 1 µM (435 ng/mL) triamcinolone acetonide (TA; Sigma-Aldrich) for 3 days. Concentrations of the compounds were based on literature and previous work where clear effects were seen with these concentrations[68]. Dimethyl sulfoxide (DMSO) was used as vehicle with a final concentration of 0.01% in all the cultures. After 3 days of modulation, the explants were washed twice with saline and cultured for an additional 24h in basic medium without any medications or stimuli to prepare synovium conditioned medium (SCM; 200 mg synovium/mL medium). The SCM was harvested, centrifuged at 200 x *g* to remove cells and debris, and stored at -80°C. Non-conditioned medium was also incubated, centrifuged and frozen to serve as control medium. The synovial explants that produced the SCM were stored at -80°C for gene expression analysis.

## The effect of SCM on human OA cartilage

Human articular cartilage was obtained from OA patients (*n*=3, 66±3Y) undergoing total knee replacement surgery as waste material (approval number MEC2004-322). Full thickness cartilage explants (ø=5mm) were harvested from the bone and washed twice with saline. The cartilage explants were cultured with 75% SCM that was pooled from all synovium donors and completed with 25% fresh medium to prevent depletion of the medium. Explants cultured in 75% SCM of non-modulated synovium were included as controls. The complete experimental setup is depicted in Figure 5.5. All the frozen medium was thawed only once. After culture, the medium was collected and stored at -20°C for biochemical analysis, and the cartilage explants were stored at -80°C for gene expression analysis.

## Gene expression analysis of synovium and cartilage explants

Synovium and cartilage explants were processed and analyzed as described previously[60,80]. For the synovial explants, gene expression of interleukin *IL6*, *IL1B, CCL18,* and *CD163* were measured. The cartilage explants that were cultured in SCM were assessed for expression levels of Matrix metalloproteinase (*MMP*)*1*, *MMP13,* A disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*)*4, ADAMTS5*, aggrecan (*ACAN),* collagen type II (*COL2A1*), and cartilage oligomeric matrix protein (*COMP*), collagen type X (*COL10A1*), alkaline phosphatase (*ALPL*), Runt-related

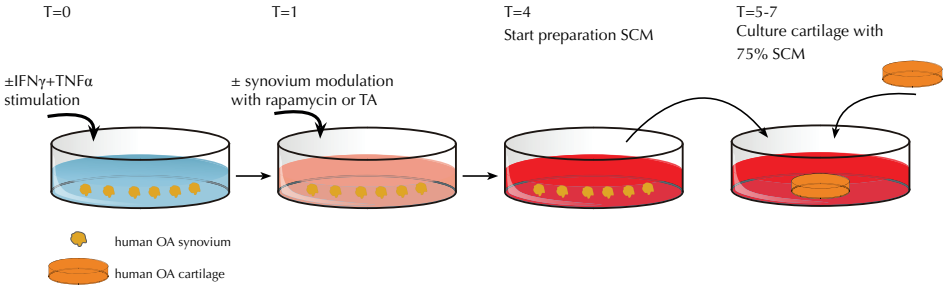


**Figure 5.5: Experimental setup.** Human OA synovium was cultured with or without 10 ng/mL IFNγ+TNFα to simulate acute inflammation, followed by modulation with either 0.1µM (100 ng/mL) rapamycin or 1 µM (435 ng/mL) triamcinolone acetonide (TA), or 0.01% as vehicle control. The medium containing rapamycin and TA was then replaced with fresh medium (200 mg synovium/mL) and collected to be cultured with human OA cartilage explants.

transcription factor 2 (*RUNX2*), *RUNX3,* and indian hedgehog (*IHH)* were measured. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as housekeeper and the relative expression was determined by the $2^{-\Delta CT}$ formula. All used primer sequences are listed in Supplementary Table S5.2.

## Biochemical analysis

As an indication of inflammation, NO production was measured in the SCM and in the medium of the cartilage explants using Griess reagent (Sigma-Aldrich) according to manufacturer's instructions with sodium nitrite ($NO_2$; Chem-lab, Zedelgem, Belgium) as standard. As an indication of cartilage degradation, release of GAGs in the SCM and in the medium of the explants was measured with a 1,9-Dimethylmethylene Blue assay (Sigma-Aldrich)[83] with shark chondroitin sulphate C (Sigma-Aldrich) was used as standard.

## Statistics

MS Excel 2010 and PASW Statistics 21.0 (SPSS Inc. Chicago, USA) were used for calculation and statistical evaluation. To take into account the variability between donors, a mixed linear model after log transformation was used, followed by a Bonferroni's *post hoc* comparisons test. To assess the effects of modulation on the synovium, the compound treatment was considered as fixed factor, while the synovium donor was defined as random factor. To evaluate the response of the SCM on the cartilage, the conditions of the modulated synovium were considered as fixed factors, while the cartilage donors were defined as random factors in the model. The adjusted *p*-values are presented in the figures and differences were considered to be statistically significant for $p<0.05$.

## RESULTS

### Characterization of modulated synovium and synovial conditioned medium

As indication of the inflammatory state of the synovium, expression of *IL1B*, *IL6*, and *CCL18* were measured in the synovial explants that produced the SCM. Expression of *IL1B* was lower in the unstimulated and IFNγ+TNFα stimulated explants that were treated with TA than in the stimulated controls. *IL6* was only reduced in the stimulated explants after TA treatment and *CCL18* expression was unaffected by either medication. Expression levels of CD163 were higher in the stimulated synovium treated with TA than in the stimulated control samples that were not treated with TA. Treatment with rapamycin did not statistically significantly affect the levels of the genes of interest, irrespective of IFNγ+TNFα stimulation (Figure 5.6).

### Modulation of acute inflamed synovium suppresses cartilage degeneration

SCM of both unstimulated and IFNγ+TNFα stimulated synovial explants that were modulated with rapamycin, did not statistically significantly affect the gene expression levels of the genes of interest of the cartilage. Culturing the cartilage in SCM of unstimulated synovium that was modulated with TA, resulted in lower levels of *MMP1* and *COL10A1* and higher levels of *ACAN* than in cartilage that was cultured in SCM of unmodulated synovium. Culturing the cartilage explants in SCM of IFNγ+TNFα stimulated synovium that was modulated with TA, had lowered levels of *MMP1* and *MMP13* and higher *ACAN* levels than the cartilage that was cultured in SCM of non-modulated IFNγ+TNFα stimulated synovium (Figure 5.7A). *ALPL*, *IHH*, *RUNX2*, and *RUNX3* were not detected in the cartilage samples in any of the conditions (data not shown).

Release of GAGs from the cartilage explants was inhibited when cultured with the SCM of IFNγ+TNFα stimulated synovium that was treated with TA than when the cartilage was cultured in the SCM of the non-treated synovium (Figure 5.7B).

## CONCLUSION

*In situ* modulation of macrophages in acute inflamed synovial tissue with TA may inhibit degradation processes in cartilage as indicated by lower expression levels of *MMP1* and *MMP13*, and higher levels of *ACAN*. On a functional level, the release of GAGs is also inhibited after modulation. In addition, modulation of end-stage OA synovium may inhibit chondrocyte hypertrophy by reducing expression of *COL10A1*.

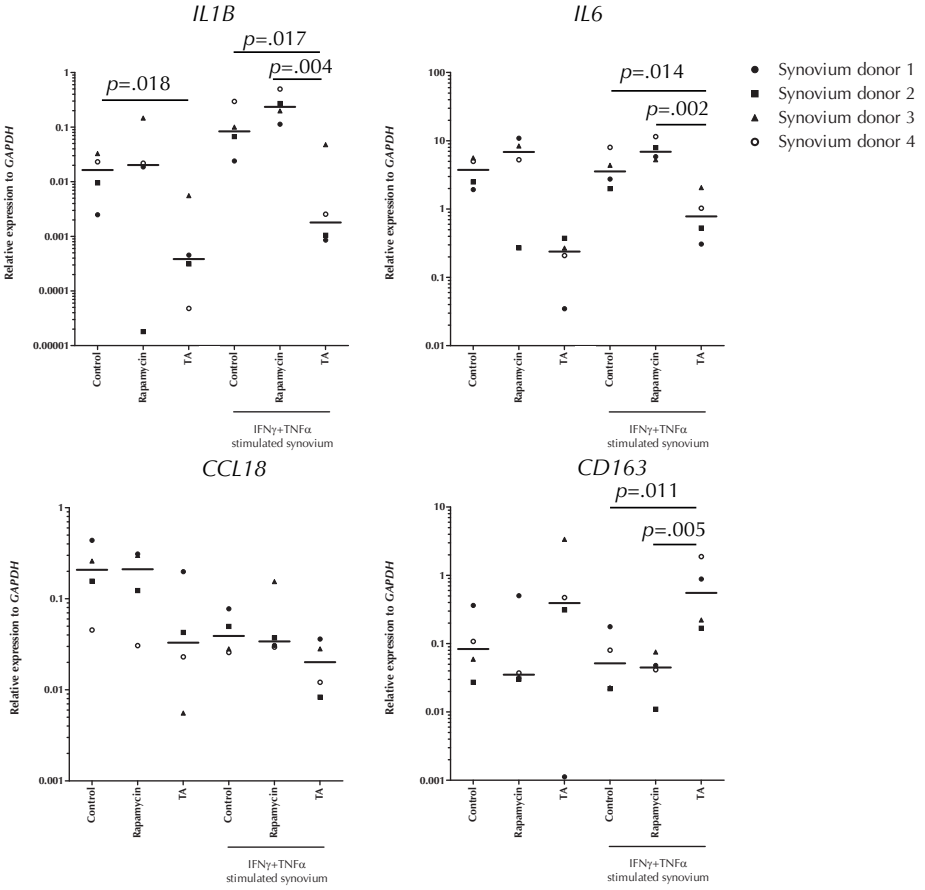


**Figure 5.6: Modulated synovium.** Gene expression levels of *IL1B*, *IL6*, *CCL18,* and *CD163* of the modulated synovial explants that produced the SCM. 0.01% DMSO was used as vehicle control. Data is depicted relative to expression levels of *GAPDH* of pooled synovium samples of *n*=4 synovium donors.

These findings suggest that *in situ* modulation of macrophages in either early or late stage OA may be a suitable method to inhibit cartilage degradation and methods to specifically deliver drugs to synovial macrophages may increase the beneficial effects of the macrophages.

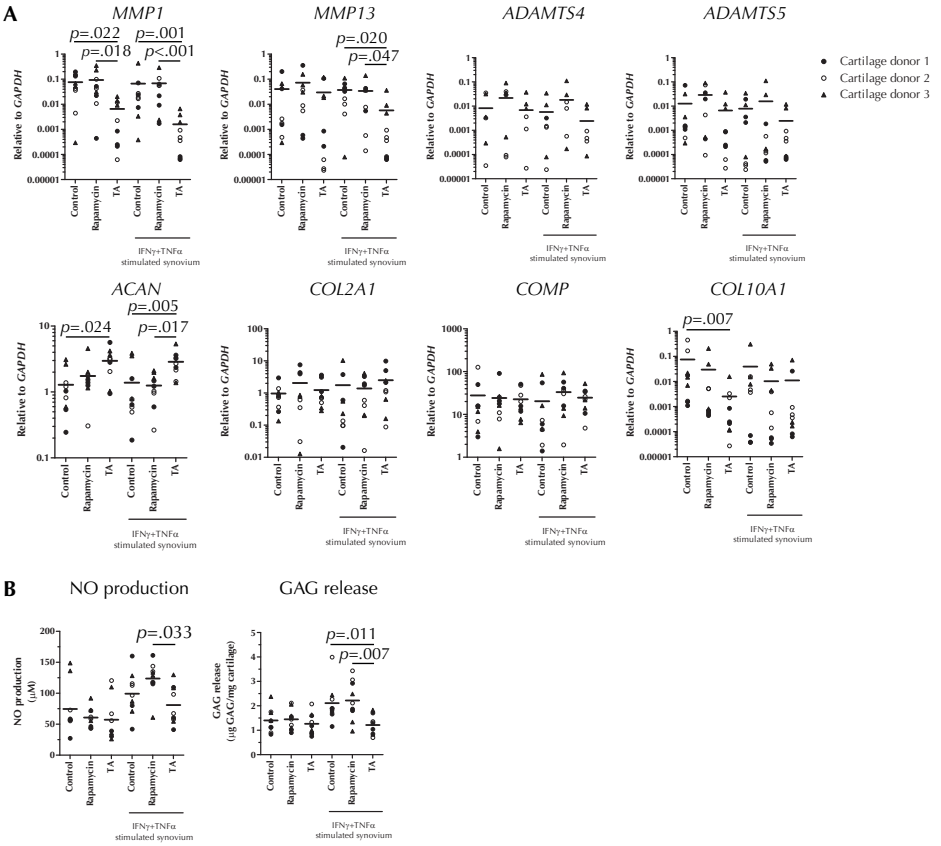


**Figure 5.7: The direct effect of modulated synovium on human articular cartilage. (A)** Gene expression levels, and **(B)** nitric oxide production and GAG release of cartilage after culture with conditioned medium of unstimulated and stimulated synovium modulated with rapamycin or triamcinolone. Gene expression levels are shown relative to the gene expression of cartilage cultured in conditioned medium of synovium cultured with the DMSO vehicle (dotted line). *ALPL*, *IHH*, *RUNX2*, and *RUNX3* were undetectable. NO was not detected (< 1.25 μM) in the SCM of unstimulated synovium, while found in a low concentration (9 μM) when the synovium was

## SUPPLEMENTARY DATA

**Supplementary Table S5.1: Primers and probes used for qRT-PCR analysis**

| Gene | Primer sequences |
|---|---|
| *IL6* | Fw: TCGAGCCCACCGGGAACGAA<br>Rev: GCAGGGAAGGCAGCAGGCAA |
| *IL1B* | Fw: CCCTAAACAGATGAAGTGCTCCTT<br>Rev: GTAGCTGGATGCCGCCAT |
| *TNFA* | Fw: GCCGCATCGCCGTCTCCTAC<br>Rev: AGCGCTGAGTCGGTCACCCT |
| *CCL18* | Fw: GCACCATGGCCCTCTGCTCC<br>Rev: GGGCACTGGGGGCTGGTTTC |
| *IL1RA* | Fw: AACAGAAAGCAGGACAAGCG<br>Rev: CCTTCGTCAGGCATATTGGT |
| *CD206* | Fw: TGGCCGTATGCCGGTCACTGTTA<br>Rev: ACTTGTGAGGTCACCGCCTTCCT |
| *CD163* | Fw: GCGGGAGAGTGGAAGTGAAAG<br>Rev: GTTACAAATCACAGAGACCGCT |
| *TLR4* | Fw: GGCATGCCTGTGCTGAGTT<br>Rev: CTGCTACAACAGATACTACAAGCACACT |
| *IL10* | Fw: CCTGGAGGAGGTGATGCCCCA<br>Rev: GACAGCGCCGTAGCCTCAGC |
| *TGFB1* | Fw: GTGACAGCAGGGATAACACACTG<br>Rev: CATGAATGGTGGCCAGGTC<br>Probe: ACATCAACGGGTTCACTACCGGC |
| *GAPDH* | Fw: CAACGGATTTGGTCGTATTGGG<br>Rev: TGCCATGGGTGGAATCATATTGG<br>Probe: GGCGCCCCAACCAGCC |

**Supplementary Table S5.2: Sequences of used primers**

| Gene | Sequence |
|---|---|
| *IL6* | Fw: TCGAGCCCACCGGGAACGAA<br>Rev: GCAGGGAAGGCAGCAGGCAA |
| *IL1B* | Fw: CCCTAAACAGATGAAGTGCTCCTT<br>Rev: GTAGCTGGATGCCGCCAT |
| *CCL18* | Fw: GCACCATGGCCCTCTGCTCC<br>Rev: GGGCACTGGGGGCTGGTTTC |
| *CD163* | Fw: GTTGGCCATTTTCGTCGCATT<br>Rev: CTCTCCTCTTGAGGAAACTGCAA |
| *MMP1* | Fw: CTCAATTTCACTTCTGTTTTCTG<br>Rev: CATCTCTGTCGGCAAATTCGT<br>Probe: CACAACTGCCAAATGGGCTTGAAGC |
| *MMP13* | Fw: AAGGAGCATGGCGACTTCT<br>Rev: TGGCCCAGGAGGAAAAGC<br>Probe: CCCTCTGGCCTGCGGCTCA |
| *ADAMTS4* | Fw: CAAGGTCCCATGTGCAACGT<br>Rev: CATCTGCCACCACCAGTGTCT<br>Probe: CATCTGCCACCACCAGTGTCT |
| *ADAMTS5* | Fw: TGTCCTGCCAGCGGATGT<br>Rev: ACGGAATTACTGTACGGCCTACA<br>Probe: ACGGAATTACTGTACGGCCTACA |
| *ACAN* | Fw: TCGAGGACAGCGAGGCC<br>Rev: TCGAGGGTGTAGCGTGTAGAGA<br>Probe: ATGGAACACGATGCCTTTCACCACGA |
| *COMP* | Fw: CCCCAATGAAAAGGACAACTGC<br>Rev: GTCCTTTTGGTCGTCGTTCTTC |
| *COL10A1* | Fw: CAAGGCACCATCTCCAGGAA<br>Rev: AAAGGGTATTTGTGGCAGCATATT<br>Probe: TCCAGCACGCAGAATCCATCTGA |
| *ALPL* | Fw: GACCCTTGACCCCCACAAT<br>Rev: GCTCGTACTGCATGTCCCCT<br>Probe: TGGACTACCTATTGGGTCTCTTCGAGCCA |
| *RUNX2* | Fw: ACGTCCCCGTCCATCCA<br>Rev: TGGCAGTGTCATCATCTGAAATG<br>Probe: ACTGGGCTTCTTGCCATCACCGA |
| *RUNX3* | Hs01566408_m1 (Thermo Scientific) |
| *IHH* | Hs01081800_m1 (Thermo Scientific) |
| *GAPDH* | Fw: ATGGGGAAGGTGAAGGTCG<br>Rev: TAAAAGCAGCCCTGGTGACC<br>Probe: CGCCCAATACGACCAAATCCGTTGAC |