

Title

Cartilage inflammation and degeneration is enhanced by pro-inflammatory (M1) macrophages *in vitro*, but not inhibited directly by anti-inflammatory (M2) macrophages

Running title

The direct effect of macrophage phenotypes on cartilage

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Introduction

Knee osteoarthritis (OA) is a degenerative joint disease characterized by erosion of cartilage coupled with inflammation of the joint, eventually leading to pain and loss of function. If conservative treatment fails, total joint replacement may be the only option left open. This indicates that new insights into possibilities for delaying OA progression are necessary. Macrophages, together with fibroblasts, are present in the synovial lining of joints. They are involved in synovial inflammation, and have been shown to play a prominent role in the progression of OA [1]. Macrophages can become activated and may acquire a phenotype, ranging from pro-inflammatory (M1) to anti-inflammatory (M2) [2]. *In vitro*, pro-inflammatory macrophages develop when monocytes are subjected to interferon- γ (IFN- γ) and lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF α) [3]. They have high microbicidal activity and secrete large amounts of pro-inflammatory cytokines. Anti-inflammatory macrophages can be further divided into subtypes. One of these subtypes, develops when monocytes are exposed to IL-4 or IL-13, sometimes referred to as M2a, and these cells are considered anti-inflammatory due to their ability to down regulate pro-inflammatory stimuli [4]. Another subtype develops when monocytes are stimulated with IL-10, sometimes referred to as M2c, and downregulates pro-inflammatory cytokines and plays a role in tissue remodeling [5]. In earlier studies, it has been shown *in vitro* that bone-marrow derived macrophages [6] and peritoneal macrophages [7] secrete enzymes that may be responsible for cartilage degeneration. However, the direct effect of different macrophage phenotypes on cartilage is not clear. We hypothesized that pro-inflammatory macrophages exacerbate the progression of cartilage degeneration and anti-inflammatory macrophages can inhibit the progression of cartilage degeneration. Therefore, the direct effect of macrophage phenotypes on cartilage was assessed by culturing human articular cartilage with phenotype specific macrophage conditioned medium (MCM).

Methods

Preparation of subtype specific MCM

Monocytes were isolated from a total of six buffy coats (males, 61 \pm 11 years; Sanquin blood bank, Amsterdam, the Netherlands) using Ficoll (GE Healthcare, Little Chalfont, UK) density gradient separation and CD14 magnetic-activated cell sorting microbeads (MACS; Miltenyi, Bergisch Gladbach, Germany) as

previously described [8]. To prepare MCM, monocytes of three buffy coats were pooled, seeded in culture flasks at 500,000 monocytes/cm² and cultured in X-VIVO-15 (Lonza, Verviers, Belgium) containing 20% heat-inactivated fetal calf serum (FCS; Lonza), 50 µg/mL gentamicin (Gibco) and 1.5 µg/mL amphotericin B (Gibco) at 37°C and 5% CO₂. Monocytes were stimulated with 10 ng/mL Interferon-γ (IFNγ; PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL Tumor Necrosis Factor-α (TNFα, PeproTech) to obtain pro-inflammatory M(IFNγ+TNFα) macrophages. The anti-inflammatory M(IL-4) subtype was obtained after stimulation with 10 ng/mL Interleukin-4 (IL-4; PeproTech). M(IL-10) was acquired by stimulation with 10 ng/mL IL-10 (PeproTech). After 48h, the medium used to differentiate the macrophages containing serum and stimuli was removed and the macrophages were washed twice with 0.9% NaCl (Sigma-Aldrich) before the addition of serum-free Dulbecco's Modified Eagle Medium, low glucose (DMEM; Gibco) supplemented with 1% Insulin-Transferrin-Selenium (ITS+ premix, Biosciences, New Jersey, USA), 50 µg/mL gentamicin, 1.5 µg/mL amphotericin B and 25 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich) to obtain MCM. After 24h, the MCM was harvested, centrifuged at 200g and stored at -80°C until use. Non-conditioned DMEM supplemented with 1% Insulin-Transferrin-Selenium (ITS+ premix, Biosciences, New Jersey, USA), 50 µg/mL gentamicin, 1.5 µg/mL amphotericin B and 25 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich) was also incubated, centrifuged and frozen to serve as control medium. Cells were harvested for DNA quantification using a modified CyQUANT assay (Invitrogen, Carlsbad, USA) as described previously [9].

Cartilage explant culture in MCM

Human articular cartilage was obtained with implicit consent as waste material from patients undergoing total knee replacement surgery for which the patients had the rights to refuse. This protocol was approved by the medical ethical committee of the Erasmus Medical Center, protocol number MEC-2004-322. Full thickness cartilage explants (ø=5mm) were harvested from macroscopically intact areas of the femoral condyles and tibia plateau and washed twice with 0.9% NaCl. Samples were pre-cultured for 24h in DMEM, followed by culture with 50% MCM (*n*=6 cartilage donors, 68±7Y), or a combination of 50% M(IFNγ+TNFα) plus 50% M(IL-4) or M(IL-10) MCM (*n*=3 cartilage donors, 68±6 years). To simulate acute inflammation, cartilage explants (*n*=3 cartilage donors, 61±4 years) were pre-stimulated for 24h with 10 ng/mL IFNγ and 10 ng/mL TNFα, followed by culture with 50% MCM. The MCM was completed with 50% fresh medium to replenish potential nutrient depletion. Explants cultured in DMEM instead of MCM were included as controls.

All MCM used for culture and analysis were frozen and thawed once. To account for the cell numbers by which the MCM was produced, the average DNA contents of all macrophage phenotypes were defined as 50% and the percentage MCM used in culture was adjusted for the DNA content per MCM phenotype as described previously [10]. The first MCM batch was used on explants of donor 1-3 and the second batch was used on explants of donor 4-10. Donor demographics and culture conditions are presented in Table 1.

Characterization of MCM

To confirm macrophage polarization, IL-6, CCL18 and sCD163 were measured in the second MCM batch. In our previous work, we have characterized M(IFN γ +TNF α), M(IL-4) and M(IL-10) primary human monocyte-derived macrophages based on gene expression and protein production [3, 8, 10, 11]. IL-6 was found to be a good marker for M(IFN γ +TNF α), CCL18 for M(IL-4) and soluble CD163 (sCD163) for M(IL-10) which was also supported by others [12, 13]. IL-6 (PeproTech), CCL18 (R&D Systems) and sCD163 (PeproTech) protein concentrations were quantified in the MCM using enzyme-linked immunosorbent assays (ELISAs) according to manufacturer's instructions. To check for possible nutrient depletion of the conditioned medium, glucose was measured. Glucose concentration was 0.83 g/L for M(IFN γ +TNF α) MCM, 0.93 g/L for M(IL-4) MCM, 0.92 g/L for M(IL-10) MCM and 1.0 g/L in non-conditioned medium. Since MCM was mixed 1:1 with fresh medium, the difference in glucose between the conditions was maximally 5.5% and considered negligible.

Gene expression analysis of cartilage explants

mRNA isolation and cDNA synthesis of the cartilage explants were executed as described previously [9]. qPCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to assess gene expression, matrix metalloproteinase-1 (*MMP1*; Fw: CTCAATTTAC*TTCTGTTTCTG; Rev: CATCTCTGTCGGCAAATTCGT; Probe: CACAACTGCCAAATGGGCTTGAAGC), *MMP13* (Fw: AAGGAGCATGGCGACTTCT; Rev: TGGCCCAGGAGGAAAAGC; Probe: CCCTCTGGCCTGCGGCTCA), A disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS4*; Fw: CAAGGTCCCATGTGCAACGT; Rev: CATCTGCCACCACCAGTGTCT; Probe: CATCTGCCACCACCAGTGTCT), *ADAMTS5* (Fw: TGCCTGCCAGCGGATGT; Rev: ACGGAATTACTGTACG*GCCTACA; Probe:

ACGGAATTACTGTACGGCCTACA), aggrecan (*ACAN*), Collagen type II (*COL2A1*) [9], Interleukin-1 β (*IL1B*; Fw: CCCTAAACAGATGAAGTGCTCCTT; Rev: GTAGCTGGATGCCGCCAT), *IL6*, Tumor Necrosis Factor- α (*TNFA*) [8], suppressor of cytokine signaling 1 (*SOCS1*; Fw: CCCTGGTTGTTGTAGCAGCTT; Rev: TTGTGCAAAGATACTGGGTATATGT) and *SOCS3* (Fw: TCGGACCAGCGCCACTT; Rev: CACTGGATGCGCAGGTTCT) were measured. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [8] was found stable and therefore further used as housekeeper gene. The nucleotide marked with an asterisk denotes a mismatch in the primer sequence. Gene expression levels of *ADAMTS5* and *MMP1* in OA cartilage were compared with those found in literature [14] and this was similar. Furthermore, since all primer sequences were developed using NCBI BLAST according to a protocol to be specific for the gene of interest, we are confident that these primer-probe combinations are specific despite a single nucleotide mismatch. Amplification efficiency was confirmed using a dilution range of universal human cDNA and the efficiency was calculated using the following formula: $10^{(-1/\text{slope})-1}$. The primer was only approved if the slope was between -3.60 and -3.20, and if the efficiency was between 0.9 and 1.1. In case the primers were used for a SYBR GR assay, gel electrophoresis was conducted on the PCR products to ensure that no primer-dimers were formed and to confirm the product size. In case of a Taqman assay, a specific probe was designed to be used in combination with the primers to increase specificity. Finally, the following amplification protocol was used: 10 min at 95°C, then 39 cycles of 95°C for 15s, 60°C for 60s, finishing with a melt curve from 65°C to 95°C with 0.5°C increments of 5s. The relative expression of the genes of interest was determined by the $2^{-\Delta CT}$ formula.

Nitric oxide (NO) and glycosaminoglycan (GAG) quantification

NO production was measured in the MCM and in the medium of the cartilage explants using Griess reagent (Sigma-Aldrich) as an indication of the degree of inflammation. The reaction was monitored at 540 nm using a spectrophotometer (VersaMax; Molecular Devices, Sunnyvale, USA). Sodium nitrite (NO₂; Chem-lab, Zedelgem, Belgium) was used as standard.

GAG release in the MCM and in the medium of the explants was measured with a 1,9-Dimethylmethylene Blue assay (Sigma-Aldrich) [15] as indication for cartilage degeneration. The reaction was monitored using a VersaMax at 530 and 590 nm. Shark chondroitin sulphate C (Sigma-Aldrich) was used as standard.

Statistics

IBM SPSS 22.0 (IBM, New York, USA) and Microsoft Excel 2010 were used for statistical evaluation. Per donor, a minimum of three cartilage samples were used that were randomly divided per experimental condition. For PCR analysis, all samples were processed and analyzed individually. These values were used for statistical analysis. For the biochemical assays, the mean of a duplicate measurement per sample was used for statistical analysis. To take donor variability into account, a mixed linear model after log transformation was used. In the model, single or combined phenotype specific MCM (e.g., M(IFN γ +TNF α), M(IL-4) and M(IL-10) MCM) and cartilage state (e.g., end-stage OA or pre-stimulated with IFN γ +TNF α) were defined as fixed factors, while the cartilage donor was considered as random factor. The tests were adjusted for multiple comparisons by a Bonferroni's *post hoc* comparisons test. Differences were considered statistically significant for $P < 0.05$.

Results

MCM

To confirm that the MCM was produced by different macrophage subtypes, IL-6, CCL18 and sCD163 were measured. IL-6 protein concentration was high in M(IFN γ +TNF α) MCM, while undetectable (<62.5 pg/mL) in M(IL-4) and M(IL-10) MCM. CCL18 protein concentration was highest in M(IL-4) and lower in M(IFN γ +TNF α) and M(IL-10). sCD163 was high in M(IL-10) and undetectable (<156 pg/mL) in M(IFN γ +TNF α) and M(IL-4) (Figure 1). NO₂ concentrations were undetectable (<1.25 μ M) in MCM of all phenotypes, indicating that no NO was released into the MCM. Since NO was no longer produced by the macrophages, this could be used as a parameter to assess the induction of inflammation in cartilage. Average GAG concentrations in the MCM were 0.30 μ g/mL for all phenotypes. These GAG concentrations were further reduced, since 50% MCM was used in the cultures. The final GAG concentrations in the MCM constituted for less than 0.04% of the average GAG release of cartilage explants, indicating that GAG production by macrophages can be considered negligible.

Pro-inflammatory macrophages affect cartilage matrix genes and induce inflammation in osteoarthritic cartilage

To assess the direct effect of macrophages with different phenotypes on cartilage, OA cartilage was cultured with M(IFN γ +TNF α), M(IL-4) and M(IL-10) MCM and assessed by gene expression analysis, NO production and GAG release. Expression of *IL1B*, *IL6*, *TNFA*, *SOCS1*, *MMP13* and *ADAMTS5* was higher when cartilage was cultured with M(IFN γ +TNF α) MCM than when the cartilage was cultured with non-conditioned DMEM, while *COL2A1* and *ACAN* were decreased. M(IL-4) MCM did not significantly affect any of the genes of interest, while *IL1B* and *SOCS1* expression was higher in cartilage cultured with M(IL-10) than when cultured with DMEM (Figure 2A). NO production by the cartilage was statistically significantly increased when cultured with M(IFN γ +TNF α) and M(IL-10) MCM than when cultured with DMEM, while GAG release was significantly increased by the explants cultured with M(IFN γ +TNF α) (Figure 2B).

Anti-inflammatory macrophages do not counteract pro-inflammatory processes in OA cartilage

To further investigate possible effects of anti-inflammatory macrophages directly on cartilage, OA cartilage was cultured with a combination of M(IFN γ +TNF α) MCM and M(IL-4) MCM or M(IL-10) MCM. No inhibiting effects were seen on gene expression levels of the genes of interest when the cartilage was cultured with the combinations of conditioned media of M(IFN γ +TNF α) and M(IL-4) or M(IFN γ +TNF α) and M(IL-10) compared to M(IFN γ +TNF α) only (Figure 3A). NO production by the cartilage when cultured with M(IFN γ +TNF α) was not counteracted by the addition of M(IL-4) or M(IL-10) MCM, as NO levels remained elevated (Figure 3B). Culture with single phenotype MCM or in combinations did not significantly affect GAG release of the cartilage explants (Figure 3C).

M(IL-4) and M(IL-10) do not inhibit acute inflammation or cartilage degeneration in stimulated cartilage

Since we could not detect a clear effect of M(IL-4) and/or M(IL-10) MCM directly on OA cartilage, we further investigated the potential effects of the anti-inflammatory macrophages. For this reason, acute inflammation was simulated in OA cartilage by 24h pre-stimulation with IFN γ +TNF α . After this pre-stimulation, expression of *IL6*, *TNFA*, *SOCS1*, *MMP1* and *ADAMTS4* was higher in stimulated cartilage

than in unstimulated OA cartilage, while *COL2A1* was lower (Figure 4A). NO production was higher in pre-stimulated cartilage than in unstimulated cartilage and GAG release was not significantly affected (Figure 4B). These data combined confirm that inflammation was induced, coupled with upregulation of genes that are associated with inflammation and matrix degradation, as well as suppression of collagen production. After the 24h pre-stimulation, the cartilage was cultured for another 48h in the presence of M(IFN γ +TNF α), M(IL-4) or M(IL-10) MCM. As expected, culturing the pre-stimulated cartilage with M(IFN γ +TNF α) MCM did not affect the genes of interest, indicating that pro-inflammatory macrophages did not further enhance the inflammation that was induced during the pre-stimulation period. Unexpectedly, after culturing the pre-stimulated cartilage explants with M(IL-4) MCM, only *ACAN* expression was affected and its expression was lower than when cultured in DMEM. Culturing cartilage with M(IL-10) did not affect any of the genes of interest (Figure 5A). NO production and GAG release were unaffected by the conditioned medium of either macrophage phenotype (Figure 5B).

Discussion

We have shown in this study that pro-inflammatory macrophages exacerbate processes involved in degeneration of OA cartilage and induce inflammation, while anti-inflammatory macrophages do not directly affect OA cartilage, or inhibit effects of pro-inflammatory macrophages on cartilage. This suggests that inhibition of pro-inflammatory macrophages or enhancing the performance of anti-inflammatory macrophages may be relevant targets to consider when developing therapies that are aiming at inhibiting cartilage degeneration.

Since inflammation plays an important role in the development and progression of OA, development of therapies focusing on synovial inflammation is increasing. However, *in vitro* studies that acknowledge macrophage-mediated inflammation in their culture models did not consider the spectrum of phenotypes that may be present in *in vivo* situations [6, 7, 16]. To our knowledge, we are the first to describe the direct effects of specific macrophage phenotypes on human articular cartilage. Although the M1/M2-classification of macrophages is mainly applicable to *in vitro* models, by generating subtypes of M2 macrophages, we aimed to include a range of macrophages phenotypes that mimic *in vivo* situations. The macrophage phenotypes were confirmed by the protein secretion profiles of IL-6, CCL18 and sCD163. Since these three proteins are also

found in the synovial fluid of OA patients [17-21], this suggests that macrophages of various phenotypes may be linked to OA pathogenesis and progression.

In this study, M(IFN γ +TNF α) MCM affected OA cartilage by inhibiting genes associated with matrix production, upregulation of matrix degenerating genes and induction of inflammation. This was expected and in line with previous work, where we have shown that synovial macrophages and monocyte-derived pro-inflammatory macrophages negatively affected chondrogenesis of mesenchymal stem cells [10, 22]. In contrast, M(IL-4) did not affect OA cartilage, while M(IL-10) seemed to induce inflammation to some extent, although less intense than M(IFN γ +TNF α). Both M(IL-4) and M(IL-10) were unable to counteract the pro-inflammatory effects of M(IFN γ +TNF α) MCM, since NO levels were not reduced when M(IFN γ +TNF α) MCM was combined with M(IL-4) or M(IL-10) and expression levels of the majority of the genes of interest, were not rescued by M(IL-4) and M(IL-10) MCM. This was somewhat unexpected since it was demonstrated earlier that monocytes stimulated with macrophage colony-stimulating factor (M-CSF), enhanced chondrogenesis *in vitro* [23]. This discrepancy could be explained by the fact that processes during chondrogenesis are different than processes occurring in mature, degenerating cartilage. Furthermore, the anti-inflammatory macrophages in our study were not polarized by M-CSF, but with IL-4 and IL-10. Even when extra inflammation was induced in OA cartilage, M(IL-10) MCM in our study did not have an effect on any of the genes of interest. M(IL-4) MCM on the other hand, reduced *ACAN* expression of the stimulated cartilage to some extent. When M1 and M2 MCM were combined, anti-inflammatory macrophages were also unable to inhibit the inflammatory effects of M(IFN γ +TNF α) MCM. In the current study, a concentration of 50% MCM was used. Although with this concentration M(IFN γ +TNF α) MCM affected OA cartilage, while M2 MCM did not have a clear effect, it cannot be ruled out that higher concentrations of M2 MCM would have affected cartilage. Our results suggest that the products of anti-inflammatory macrophages only may not be sufficient or potent enough for directly suppressing inflammation in cartilage in this system, but interaction between macrophages of different phenotypes may be required to initiate biological actions. One should therefore realize that other *in vitro* or *in vivo* models may be needed to further investigate the mechanisms of the role of macrophage subtypes on either protecting cartilage or inducing degeneration and inflammation. A second limitation of our study is that the macrophage phenotypes were characterized based on three main factors, which were shown in our previous work [11] to be good markers for our generated phenotypes. More extensive characterization or profiling of soluble macrophage factors may help to pinpoint which active factor in

M(IFN γ +TNF α) MCM actually affected the cartilage. That knowledge may then be used to develop targeted therapies.

This *in vitro* model enabled us to study the direct effect of factors secreted by macrophages of different phenotypes on cartilage inflammation and degeneration. The use of human cartilage explants and primary human macrophages makes translation of the results to the clinical situation more realistic. The variability of the cartilage between donors and within samples of the same donors is, however, an inevitable disadvantage of the use of human cells and tissues. Although macroscopically preserved cartilage was used in the experiments, differences in local degeneration of the used tissue resulted in variable baseline levels of GAG, varying chondrocyte viability and inflammatory state, as seen by the high variability within donors in our experiments. In this model, MCM had clear effects on production of NO. In the majority of our experiments, however, GAG release was unaffected by MCM. The release of GAG is influenced by multiple processes such as production and breakdown. Longer culture periods may be necessary to be able to observe effects on GAG release. Though an effect on GAG release was not detected, processes associated with cartilage degeneration were initiated by the macrophages, as indicated by decreased levels of *ACAN* and increased gene expression of cartilage degrading enzymes.

Based on the data of this study, management of the behavior of synovial macrophages may seem a suitable approach to prevent cartilage degeneration. Additionally, the importance of the role of macrophages during wound healing and tissue regeneration *in vivo* models has been reported by others [24, 25]. Though we were unable to show a direct protective effect of anti-inflammatory macrophages on cartilage, we believe that this phenotype should not be completely disregarded, since an obvious interplay and balance between macrophage phenotypes and other cell types may contribute to their regenerative or protective capacity. More studies will be needed to fully understand this mechanism prior to clinical application.

Conclusion

M(IFN γ +TNF α) pro-inflammatory macrophages have a prominent direct effect on OA cartilage while M(IL-4) and M(IL-10) do not inhibit the inflammatory and degenerative effects of M(IFN γ +TNF α). This

knowledge may be taken into consideration when developing therapies aiming at inhibition of cartilage degeneration, by inhibiting pro-inflammatory macrophages or stimulating anti-inflammatory macrophages.

Author contributions

LU designed the study, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. YMBJ interpreted the data and edited the manuscript. JANV edited the manuscript. GJVMvO designed the study, interpreted the data and edited the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors have nothing to disclose

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Ethical approval

The experiments involving human cartilage have been conducted with the approval of the medical ethical committee of the Erasmus Medical Center, registration number MEC2004-322. The cartilage was obtained with implicit consent as waste material from patients who underwent total knee replacement surgery. The patients had the right to refuse as stated by the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org).

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Table 1: Cartilage donor demographics and culture conditions

Donor	Gender	Age	24h pre-stimulation	48h treatment	No. of explants/condition
1	Male	65	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	6
2	Male	65	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
3	Male	70	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
4	Female	59	Non-stimulated IFN γ +TNF α stimulation	None (analyzed after 24h) DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
5	Female	78	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
6	Male	66	Non-stimulated IFN γ +TNF α stimulation	None (analyzed after 24h) DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
7	Female	63	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM M(IFN γ +TNF α)+M(IL-4) MCM M(IFN γ +TNF α)+M(IL-10) MCM	3
8	Female	66	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM M(IFN γ +TNF α)+M(IL-4) MCM M(IFN γ +TNF α)+M(IL-10) MCM	3
9	Male	58	Non-stimulated IFN γ +TNF α stimulation	None (analyzed after 24h) DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM	3
10	Female	75	Non-stimulated	DMEM M(IFN γ +TNF α) MCM M(IL-4) MCM M(IL-10) MCM M(IFN γ +TNF α)+M(IL-4) MCM M(IFN γ +TNF α)+M(IL-10) MCM	3

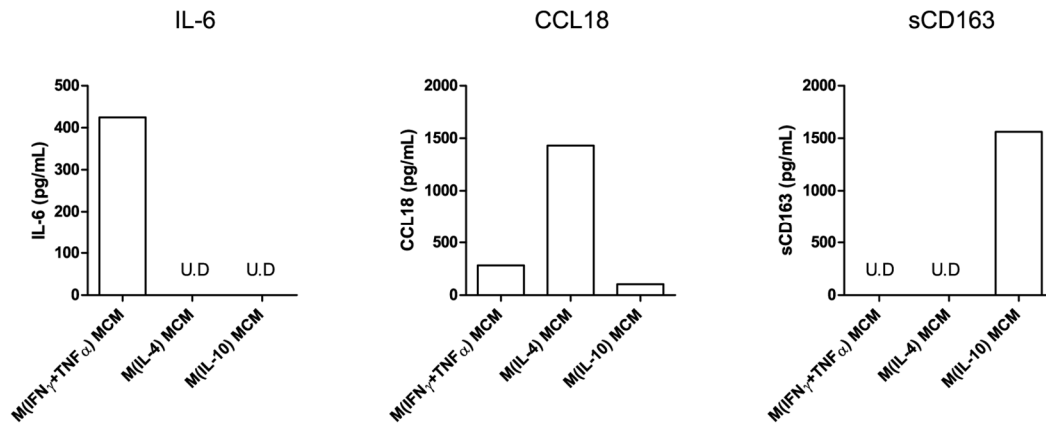


Figure 1: Protein concentrations in MCM. IL-6, CCL18 and sCD163 protein concentrations of M(IFN γ +TNF α), M(IL-4) and M(IL-4) MCM. Bars represent the mean of a duplicate measurement. U.D: undetectable.

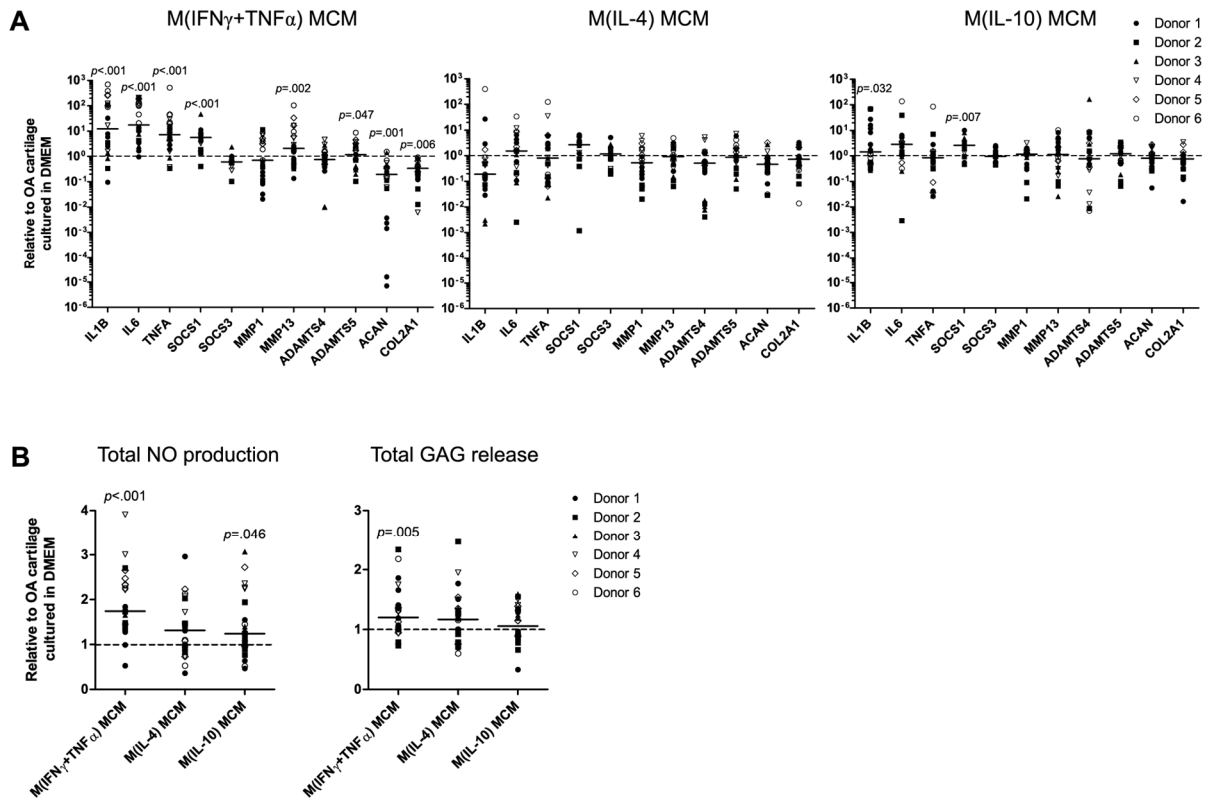
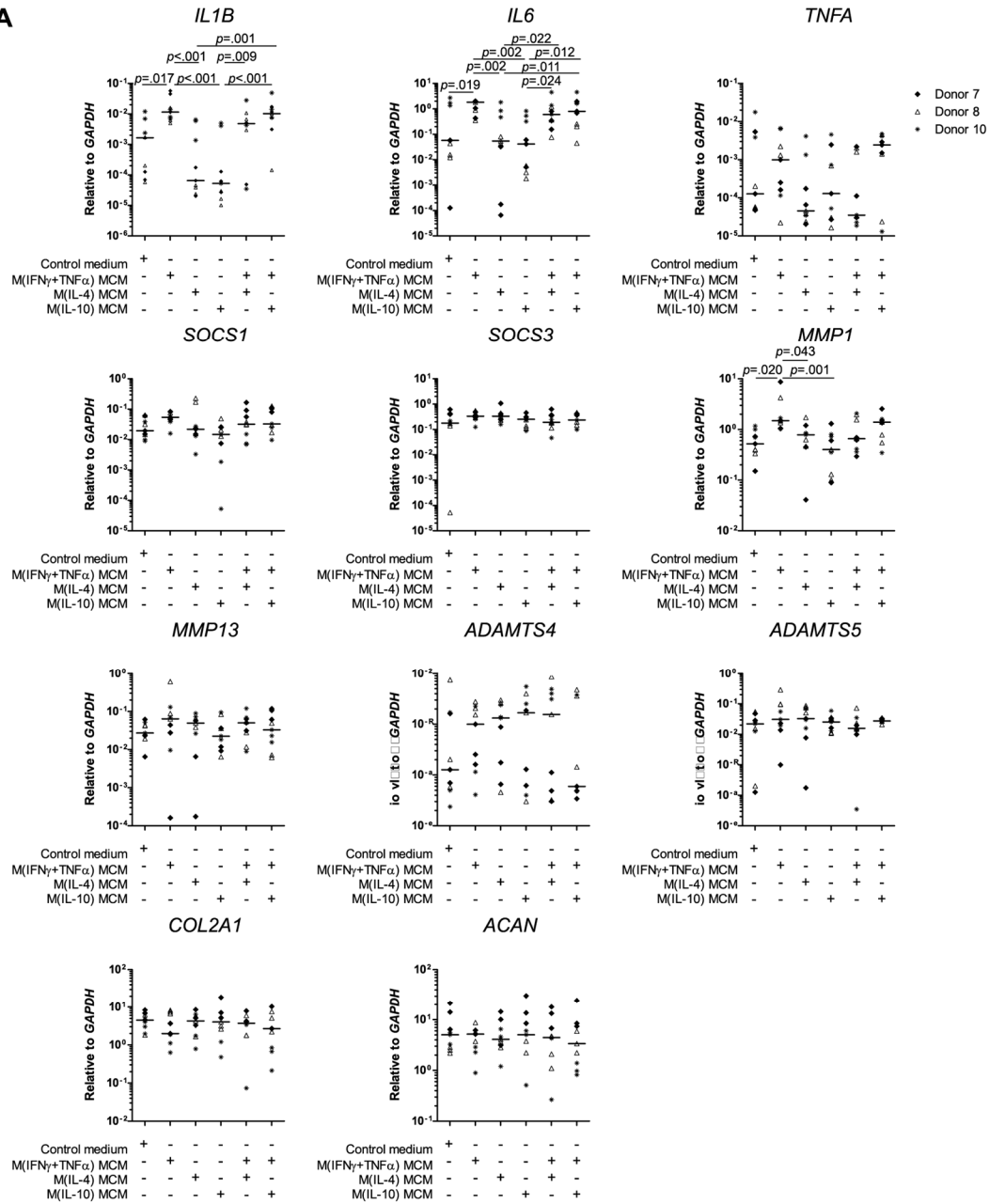
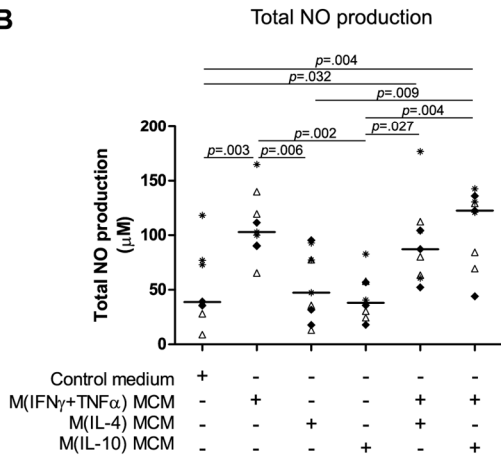


Figure 2: OA cartilage is significantly affected by pro-inflammatory macrophages. (A) Expression of genes encoding for inflammatory proteins: *IL1B*, *IL6*, *TNFA*, *SOCS1* and *SOCS3*, genes associated with matrix degeneration: *MMP1*, *MMP13*, *ADAMTS4* and *ADAMTS5*, and genes associated with matrix components: *ACAN* and *COL2A1* of OA cartilage cultured with MCM. The expression is shown relative to the expression of cartilage cultured with non-conditioned DMEM control medium represented by the dotted line. Data of all samples presented as dot plots including the grand median for $n=6$ cartilage donors with three samples per donor (six samples for donor 1). **(B)** NO production by cartilage after culture with MCM as an indication of the inflammatory state and GAG release as an indication of cartilage degeneration after culture with MCM. Data is presented relative to the NO production and GAG release of cartilage cultured in non-conditioned DMEM control medium represented by the dotted line for $n=6$ cartilage donors with three samples per donor (six samples for donor 1 and 2). Statistical evaluation was conducted with a linear mixed model after log transformation followed by a Bonferroni's *post hoc* test. Abbreviations: *IL1B*: Interleukin-1 β ; *IL6*: Interleukin-6; *TNFA*: Tumor Necrosis Factor- α ; *SOCS1*, -3: Suppressor of cytokine signaling 1, -3; *MMP1*, -13: matrix metalloproteinase-1, -13; *ADAMTS4*, -5: A disintegrin and metalloproteinase with thrombospondin motifs-4, -5; *ACAN*: aggrecan; *COL2A1*: collagen type II

A



B



C

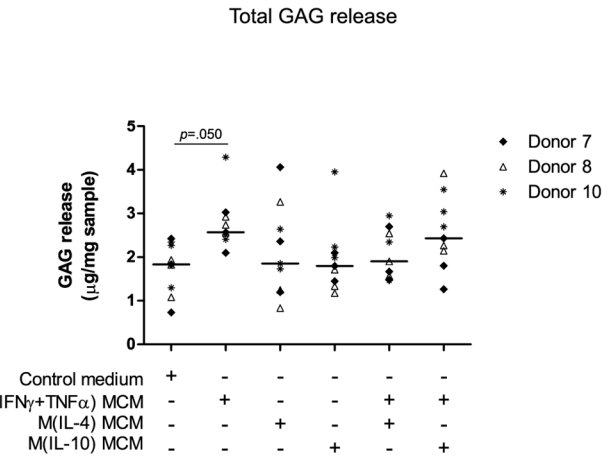


Figure 3: M(IL-4) and M(IL-10) macrophages do not counteract the inflammatory effects of M(IFN γ +TNF α) on OA cartilage. (A) Gene expression levels, (B) NO production and (C) GAG release of OA cartilage after culture with single phenotype MCM or a combination of phenotypes. For the cultures with single phenotype MCM, the MCM was completed 1:1 with fresh DMEM to reach 50% MCM. For the cultures with combined phenotype MCM, 50% of each phenotype was used in the cultures. For the control medium, previously frozen non-conditioned DMEM was combined 1:1 with fresh DMEM. Data of all samples presented in dot plots including the grand median for $n=3$ cartilage donors with three samples per donor. Statistical evaluation was conducted with a linear mixed model after log transformation followed by a Bonferroni's *post hoc* test.

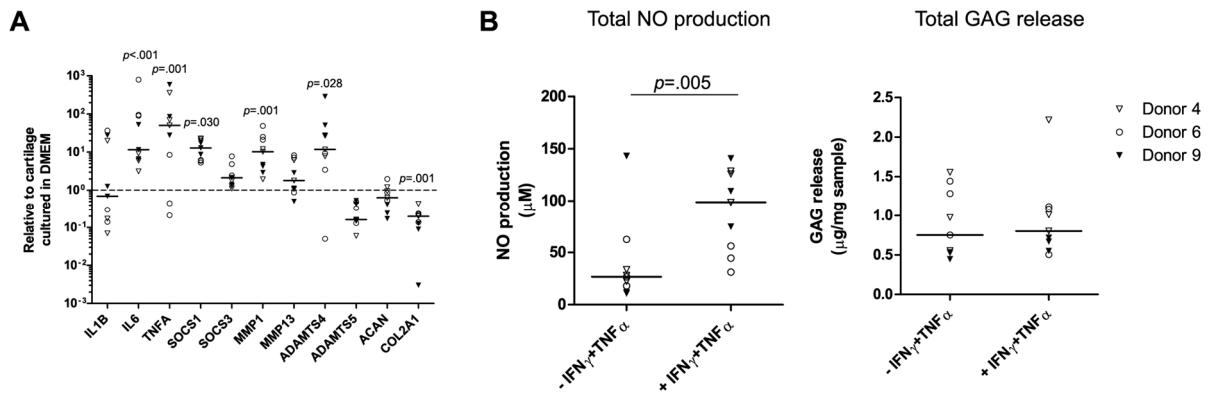


Figure 4: Stimulation of OA cartilage with IFN γ +TNF α as a cartilage inflammatory model. To simulate acute inflammation, OA cartilage explants were pre-stimulated for 24h with IFN γ +TNF α . **(A)** The effect of IFN γ +TNF α pre-stimulation on gene expression levels, **(B)** NO production and release of GAG. Gene expression levels are shown relative to the expression of OA cartilage explants cultured in DMEM as represented by the dotted line. Data of all samples is presented in dot plots including the grand median for $n=3$ cartilage donors with three samples for each donor. For statistical evaluation, a mixed linear model after log transformation was used followed by a Bonferroni's *post hoc* test.

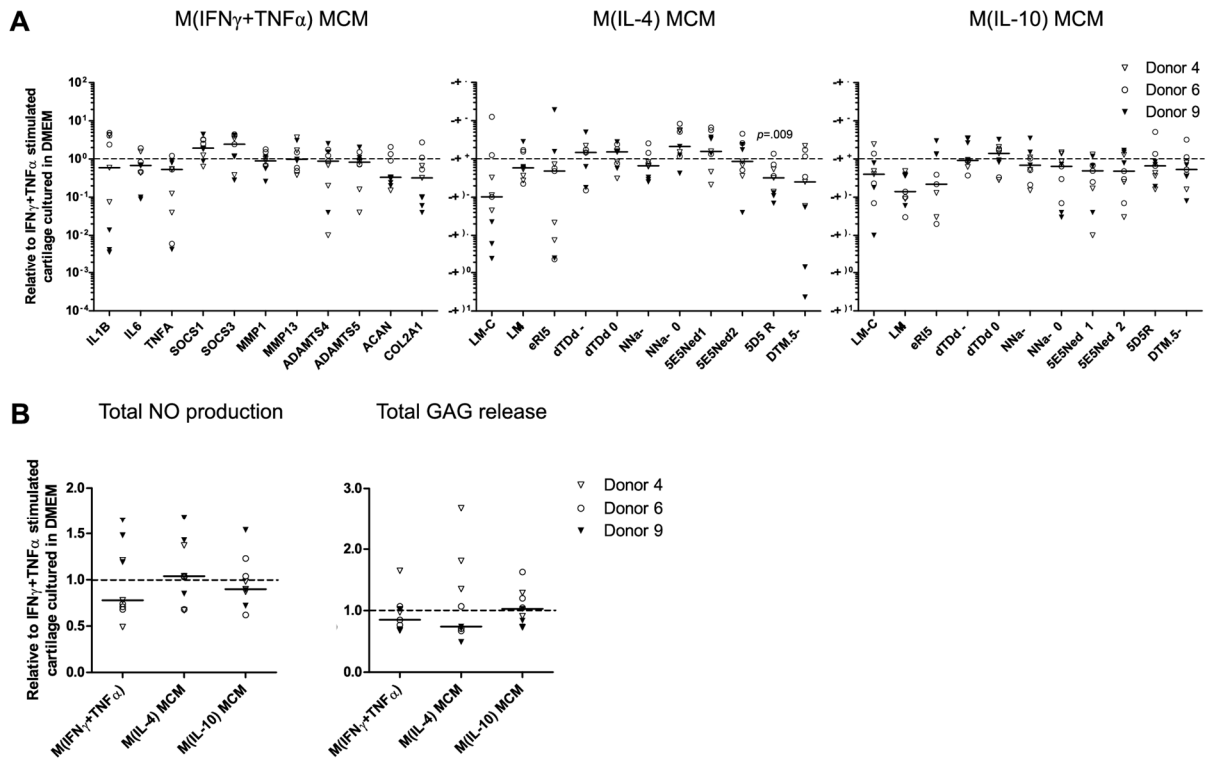


Figure 5: The effect of anti-inflammatory macrophages on acute inflamed cartilage. To assess potential effects of anti-inflammatory macrophages, acute inflamed cartilage (i.e. pre-stimulated with IFN γ +TNF α) was cultured with MCM. **(A)** Expression levels of IFN γ +TNF α pre-stimulated cartilage cultured with MCM. Expression is shown relative to the expression of pre-stimulated cartilage explants cultured in non-conditioned DMEM without IFN γ +TNF α as represented by the dotted line. **(B)** NO production and GAG release by IFN γ +TNF α pre-stimulated cartilage after culture with MCM relative to the controls cultured in DMEM as represented by the dotted line. Data of all samples is presented in dot plots including the grand median for $n=3$ cartilage donors with three samples for each donor. For statistical evaluation, a mixed linear model after log transformation was used followed by a Bonferroni's *post hoc* test.