Expression of *CCL2* in synovium is inversely correlated with articular cartilage degeneration in hip osteoarthritis patients

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Lizette Utomo
Yvonne M. Bastiaansen-Jenniskens
Giuseppe Talò
Jan A.N. Verhaar
Luigi Zagra
Gerjo J.V.M. van Osch
Matteo Moretti
Silvia Lopa

ABSTRACT

Objective: Synovial inflammation is an important feature of osteoarthritis (OA). Chemokines facilitate and regulate the migration of monocytes, which polarize into macrophages and contribute to the overall inflammatory state of the joint. The aims of this study were to determine chemokine gene expression profiles in the synovial tissue of hip OA patients, and to correlate this with the presence of different macrophage phenotypes in the synovium and with the degenerative state of the articular cartilage.

Design: Synovial tissue and cartilage from the same patient were obtained from end-stage hip OA patients (n=15). Gene expression levels of C-C motif chemokine ligands (*CCL*)*2*, *CCL3*, *CCL4*, *CCL5*, and *CX3CL1* and immunohistochemical staining for CD11c, CD163, and CD206 as markers for macrophage phenotypes were evaluated in the synovium samples. Cartilage was histologically evaluated using the Mankin score and for gene expression of matrix metalloproteinase (*MMP*)*1*, *MMP13*, and cartilage oligomeric matrix protein (*COMP*).

Results: All measured chemokines were expressed in the synovium and the expression of *CCL2* inversely correlated with articular cartilage degeneration. Immunohistochemical stainings revealed that the synovium samples contained predominantly $CD206^{+}$ tissue repair macrophages and $CD163^{+}$ anti-inflammatory macrophages.

Conclusions: Chemokines, in particular CCL2, may play a role in end-stage hip OA. The recruitment of additional monocytes into the synovial membrane by chemokines appears to result into the polarization of anti-inflammatory or tissue repair phenotype. Methods to regulate chemokines could be favorable for preventing further cartilage degeneration in end-stage hip OA.

INTRODUCTION

Osteoarthritis (OA) is an inflammatory, degenerative joint disease characterized by the degeneration of cartilage in which macrophages are pivotal[4,50-52]. Macrophages are plastic cells derived from blood monocytes and when activated, they can acquire a phenotype that may range from pro-inflammatory (M1-like) to anti-inflammatory and tissue repair (M2-like)[47,59]. Depending on this phenotype, macrophages express surface markers and secrete cytokines[47] that may influence and contribute to inflammation and joint tissue degradation[5,55]. We have shown previously *in vitro* that pro-inflammatory macrophages contribute to catabolic processes in OA cartilage from the knee and enhance cartilage degeneration[60]. In previous *in vivo* work, it was shown that the presence of different macrophage phenotypes varies during the course of knee OA and that the presence is associated with the development and progression of OA features such as cartilage damage, presence of osteophytes, and synovial thickness (unpublished results)[61]. An association was found between the inflammatory biomarker C-reactive protein (CRP) in the serum of hip OA patients and cam deformity of the hip as well as the development of future hip OA[62]. It was also reported that levels of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNFα) in the synovial fluid of patients with terminal hip OA were higher than that of patients with early hip OA[63]. Moreover, gene expression levels of *IL1B*, *IL8*, and matrix metalloproteinase (*MMP)3* in the synovium were reported to be higher in hip OA patients than patients presenting with femoroacetabular impingement[64]. These reports combined imply that like in knee OA[4], inflammation does play a certain role during hip OA. Studies have demonstrated prominent influx of macrophages in inflamed synovium during OA[2,6,65], which can make up for 30-40% of the cell content in inflamed synovium[66,67]. Since monocytes are precursors of macrophages, understanding mechanisms that are responsible for monocyte migration into synovial tissue may be an approach to develop new strategies to delay OA progression. For instance, reducing the numbers of migrated blood monocytes into the tissue might lead to reduced synovial inflammation, and consequently impede OA progression. Chemokines at the local site of inflammation facilitate and regulate monocyte migration into the tissue, yet expression profiles of chemokines in synovial tissue of hip OA patients have not been reported previously. Therefore, the aims of this study were 1) to explore gene expression of chemokines associated with monocyte extravasation in the synovium of hip OA patients, and 2) to correlate the expression of these chemokines with the presence of different macrophage phenotypes in the synovium and with the degenerative state of the articular cartilage.

MATERIALS AND METHODS

Collection of synovial tissue and cartilage from hip OA patients

Synovial tissue and cartilage from the same hip OA patient were obtained as waste material during total hip replacement surgery (*n*=15, 52±11Y, 2 female, 13 male; BMI: 28±4.8 kg/m^2) in accordance with local ethics guidelines and with the approval of the Scientific Directorate of the IRCCS Galeazzi Orthopaedic Institute, Milan, Italy. The synovium was washed twice with phosphate buffered saline (PBS; Gibco, Carlsbad, USA) and macroscopically dissected from the adipose tissue. The synovium was then divided over three tubes and stored at -80ºC until processing for gene expression analysis. Full thickness cartilage chips were harvested from the femoral head, washed twice with PBS, divided and stored over three tubes at -80ºC until processing for gene expression analysis. The cartilage was harvested from both macroscopically intact areas and areas with clear lesions and fissures. Synovium and cartilage explants were also harvested and fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, Dallas, USA) until processing for histological analysis.

Polarization of monocyte-derived macrophages

Monocytes were isolated from buffy coats that were obtained from the local blood bank (ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy) from healthy donors by Ficoll gradient density separation, followed by positive magnetic selection using human cluster of differentiation (CD)14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Monocytes were differentiated and polarized towards activated macrophages based on a protocol by Spiller, *et al*[45]. Briefly, the monocytes were plated in culture plates (Cellstar, Kremsmünster, Austria) at a density of 150,000/cm^2 in macrophage culture medium (RPMI-1640 (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Hyclone, GE Healthcare, Little Chalfont, UK) and 20 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; PeproTech, New Jersey, USA) and cultured for 7 days to allow differentiation into macrophages. To obtain a spectrum of phenotypes, the macrophages were stimulated for 3 additional days with 100 ng/mL recombinant human interferon-γ (IFNγ; PeproTech) and 100 ng/mL recombinant human TNFα (PeproTech) to obtain pro-inflammatory M1-like macrophages, from now on referred to as M(IFNγ+TNFα). To obtain M2a-like tissue repair macrophages, stimulation was carried out with 20 ng/mL recombinant human IL-4 and 20 ng/mL recombinant human IL-13 (both from PeproTech) for now on referred to as M(IL4+IL13). For M2c-like anti-inflammatory macrophages, 40 ng/mL IL-10 (PeproTech) was added to the culture medium to obtain M(IL10) macrophages.

Gene expression analysis

The synovium and cartilage explants were disrupted and homogenized using a TissueRuptor (Qiagen, Hilden, Germany) and TRIzol reagent (Thermo Fisher Scientific, Waltham, USA). The macrophages were directly collected in TRIZol. Messenger RNA (mRNA) was isolated from the aqueous phase using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA) including on-column DNAse treatment (Purelink DNAse kit; Invitrogen) according to the manufacturer's instructions. Quantification of total extracted mRNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) at 260/280 nm. Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. qPCR analysis was accomplished with a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems) with an input of 20 ng cDNA. For the synovial explants, gene expression of the following C-C motif chemokine ligands (CCL) was measured: *CCL2*/monocyte chemoattractant protein (*MCP1;* Hs00234140_m1), *CCL3*/macrophage inflammatory protein 1α (*MIP1A;* Hs00234142_m1), *CCL4*/macrophage inflammatory protein 1β (*MIP1B;* Hs99999148_m1), *CCL5/RANTES* (Hs00982282_m1), and C-X3-C motif chemokine ligand 1 *CX3CL1/FRACTALKINE* (Hs00171086_m1). As an indication of cartilage degeneration, the expression of matrix metalloproteinase (*MMP*)*1* (Hs00899658_m1), *MMP13* (Hs00233992_m1), and cartilage oligomeric matrix protein (*COMP;* Hs00164359_m1) was measured in the cartilage. To confirm the polarization of the macrophages into a pro-inflammatory or anti-inflammatory phenotype, expression of the following genes were measured as they were previously shown to be good markers for polarized macrophages *in vitro*[43,68,69]: *IL1B* (Hs01555410_m1) and *TNFA* (Hs00174128_m1) as pro-inflammatory macrophage markers, *CCL18* (Hs00268113_m1) and cluster of differentiation (*CD*)206 (Hs00267207_m1) as tissue repair markers, and *CD163* (Hs00174705_m1) as anti-inflammatory macrophage marker. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH;* Hs99999905_m1*)* was used as housekeeper as it was previously found to be stable for macrophages[68] and cartilage[60]. The relative expression was determined by the $2^{-\Delta CT}$ formula. All primers were obtained from Thermo Fisher Scientific.

Histological assessment of synovium and cartilage explants

Paraffin embedded synovium and cartilage tissues were sectioned at 6 µm. The cartilage was stained with safranin-O (Sigma-Aldrich) to stain glycosaminoglycans and for the evaluation of cartilage damage using a modified Mankin score[70,71]. Cartilage damage of the explants was scored in a blinded manner in two sections of two random explants per donor. In each section, three subdomains were scored: tissue structure (0-6), cellularity (0-3) and matrix staining (0-4), and combined resulting in a maximum possible score of 13 (Supplementary Figure S2.1). The median scores of four sections were used to determine the degenerative state of the cartilage.

The synovium sections were immunohistochemically stained for CD11c, CD206, and CD163 to assess the inflammation status. These CD-markers have been associated with different macrophage phenotypes[42,61,68,72,73]. Sections were subjected to 30 min heat-induced epitope retrieval and pre-incubated with 10% normal goat serum (Southern Biotech, Birmingham, AL, USA) in phosphate-buffered saline PBS/1% bovine serum albumin (BSA; Sigma-Aldrich)/1% Elk milk powder (Campina, Amersfoort, the Netherlands). The sections were then incubated for 60 minutes with the primary antibodies against CD11c (Genetex, Irvine, USA), CD206 (Abcam, Cambridge, UK), and CD163 (Abcam), or rabbit IgG as negative isotype control. An enzyme-streptavidin conjugate (HK-326-UR/HK-321-UK; Biogenex, Fremont, USA) in PBS/1% BSA was used as link and label. Finally, the sections were counterstained with Gill's haematoxylin (Sigma-Aldrich) and mounted with VectaMount (Vector Laboratories, Burlingame, USA). To quantify the percentage of staining over the entire tissue, a region of interest was selected on sections which was equal for all three stainings. The area fraction of the staining was then quantified using ImageJ (NIH, Bethesda, Maryland, USA). After scale calibration using the scale bar of the image, the background was removed with the intensity auto threshold function of ImageJ, and the area occupied by the tissue was measured. To measure the positively stained areas in the images, a manual color threshold was applied. Microsoft Excel 2016 (Redmond, Washington, USA) was used to calculate the positively stained surface area as a percentage of the total sample area.

Statistics

Microsoft Excel 2016 and IBM SPSS 23.0 (IBM, New York, USA) were used for calculations and statistical evaluation. Per donor, three random samples were harvested of the cartilage and synovial tissue. For PCR analysis, all samples were processed and analyzed individually. The mean values of these samples were used in the graphical representations and for the statistical analysis. To evaluate associations between parameters of interests, non-parametric Spearman correlation tests were conducted including bootstrap-based calculations to determine the 95% confidence interval (95%CI). Strengths of the correlations were based on the correlation coefficient (ρ_s) and were defined as follows: 0.0-0.19: very weak; 0.20-0.39: weak; 0.40-0.59: moderate; 0.60-0.79: strong; 0.80-1.00: very strong. Wilcoxon signed-rank tests after log-transformation followed by Bonferroni *post-hoc* correction were conducted to determine differences between expressions of the genes of interest in the synovium and polarized macrophages. To confirm macrophage polarization, Mann-Whitney U tests were conducted after log-transformation to compare expression levels of macrophage markers and chemokines between unstimulated (M0) macrophages and a macrophage phenotype (i.e., M(IFNγ+TNFα, M(IL4+IL13), or M(IL10)). To assess the polarization of primary macrophages, expression levels of macrophage markers were evaluated within the macrophage phenotypes. To take into account variation in donors,

linear mixed models after log-transformation were conducted. In the model, phenotypes were considered as fixed factors and donors as random factors. The corrected *p*-values are depicted in the figures. For all statistical tests, differences were considered statistically significant for $p<0.05$. In case a sample was lost during processing, or expression of the housekeeper gene was undetectable, the sample was excluded from the study. The actual number of donors and samples used for statistical analysis is therefore stated in each figure or figure legend.

RESULTS

Correlations between gene expression of chemokines in synovium and articular cartilage degeneration

CCL2, *CCL3*, *CCL4*, *CCL5*, and *CX3CL1* were all expressed in the synovium of hip OA patients, with *CCL2* being the chemokine that was expressed the highest, and *CX3CL1* the lowest. In the majority of the cartilage samples, *MMP1* and *MMP13* were undetectable whereas *COMP*, a cartilage turnover marker, was expressed (data not shown). *CCL2* expression in the synovium had a strong inverse correlation with the degenerative state of the cartilage as assessed by the Mankin score for OA (Figure 2.1). No correlations were found between patient age or BMI and expression of chemokines in the synovium or the degenerative state of the cartilage (data not shown).

A

Chemokines in synovium		Cartilage damage
CCL2	Correlation coefficient	**-0.69**
	95%CI [lower-upper]	**-0.932 - -0.269**
CCL3	Correlation coefficient	-0.38
	95%CI [lower-upper]	-0.779 - 0.143
CCL4	Correlation coefficient	-0.27
	95% CI [lower-upper]	-0.842 - 0.381
CCL5	Correlation coefficient	-0.23
	95% CI [lower-upper]	0.735 - 0.505
CX3CL1	Correlation coefficient	-0.17
	95% CI [lower-upper]	-0.727 - 0.512

B

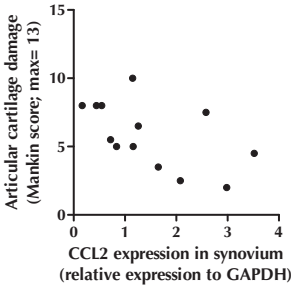


Figure 2.1: *CCL2* expression in synovium correlates with structural cartilage degeneration in hip OA patients. (A) Non-parametric Spearman correlations between chemokine expressions in the synovium and structural cartilage damage as evaluated by the Mankin score. The median Mankin score for all patients was 5.5 on a scale of 0 to 13. Values in bold denote statistically significant associations. **(B)** Scatterplot of the correlation between *CCL2* expression ($2^{-\Delta CT}$) and cartilage degeneration of *n*=13 patients for which both gene expression data of synovium and histological data of the cartilage was available.

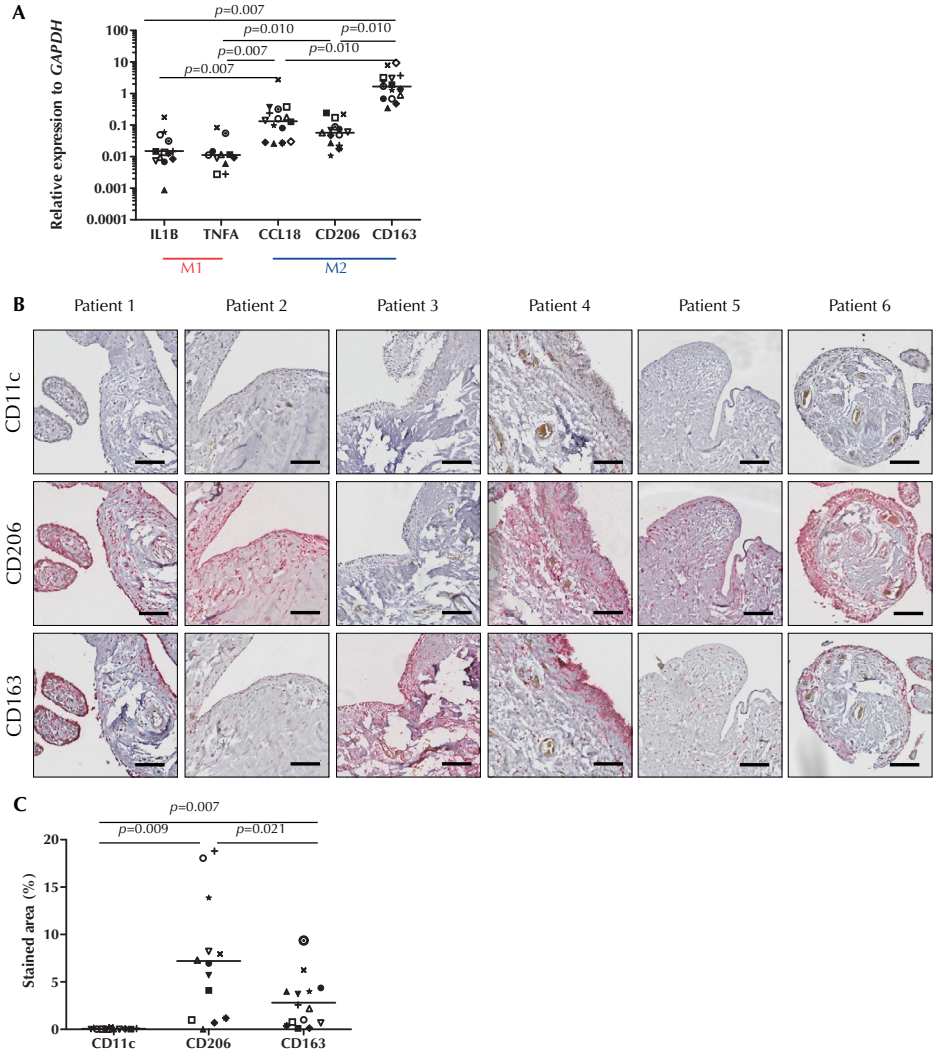


Figure 2.2: Macrophages in synovium of hip OA patients. (A) Gene expression levels in synovium (*n*=15) of pro-inflammatory macrophage markers *IL1B* and *TNFA* and anti-inflammatory markers *CD206*, *CCL18*, and *CD163*. Each symbol represents a patient. (**B**) Examples of immunohistochemical stainings for CD11c (M1 marker) and CD206 and CD16 (M2 markers) of synovium samples of patients 1 to 8. Scale bar: 100 µm. **(C)** Quantification of the immunohistochemical stainings for all patients (each symbol represents a patient).

Macrophages in the synovium of hip OA patients have a predominant anti-inflammatory and tissue repair phenotype

Expression levels of the anti-inflammatory macrophage markers *CCL18*, *CD206*, and *CD163* in the synovium were higher than the expression levels of the pro-inflammatory

macrophage markers *IL1B* and *TNFA* (Figure 2.2A). On tissue level, only a few cells were positively stained for CD11c in the synovial lining, indicating a low level of pro-inflammatory macrophages. The percentages of stained tissue area for CD206 and CD163 were statistically significantly higher than of the CD11c staining (Figure 2.2B and 2.2C), indicating that tissue repair and anti-inflammatory macrophages were abundantly present.

Chemokines are expressed by different macrophage phenotypes

To gain insights into the potential role of certain macrophage phenotypes in producing chemokines, the expression levels of the chemokines were measured in primary macrophages that were polarized by different cytokines. We confirmed the polarization protocol applied to induce the different phenotypes. Pro-inflammatory M(IFNγ+TNFα) macrophages were characterized by high expression of *TNFA* and low expression of *CD206*. Tissue repair M(IL4+IL13) macrophages were characterized by high expression of *CD206* and *CCL18,* and downregulation of *IL1B*. Anti-inflammatory M(IL10) macrophages were characterized by high expression of *CCL18* and additionally high expression levels of *CD163* (Supplementary Figure S2.2).

Regarding the expression of chemokines in polarized macrophages, all the phenotypes expressed the analyzed chemokine genes, except for *CX3CL1*. In particular, *CCL5* expression was significantly higher in M(IFNγ+TNFα) and lower in M(IL10) than in unstimulated M0 macrophages (Figure 2.3).

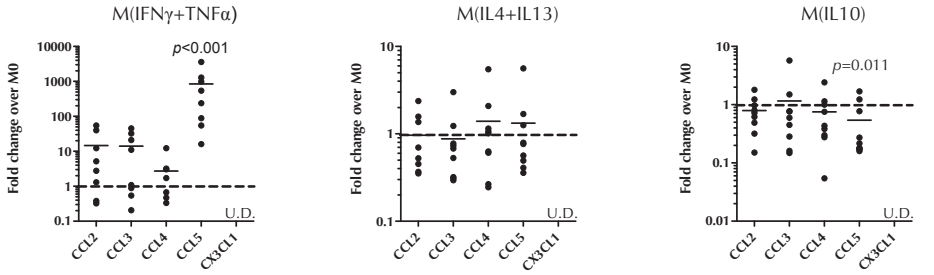


Figure 2.3: Chemokine expression in primary polarized macrophages. Chemokine expression in primary polarized macrophages. Expression levels are shown as $2^{-\Delta CT}$ fold change over unstimulated macrophages (M0; dotted line). U.D.: undetectable.

DISCUSSION

Macrophages are important in synovial inflammation and OA[4,50-52]. However, the inflammatory component of hip OA is not extensively explored and only little is reported in literature. In this study, we aimed to investigate the link between macrophages and

chemokines in synovium of hip OA patients and the degenerative state of the articular cartilage. We have shown expression of chemokines by hip OA synovium and that the expression of *CCL2* had a strong inverse association with cartilage degeneration. Moreover, we showed that the macrophages in OA hip synovium mainly had an anti-inflammatory phenotype and that macrophages with different phenotypes were able to express *CCL2*, *CCL3*, *CCL4*, and *CCL5*.

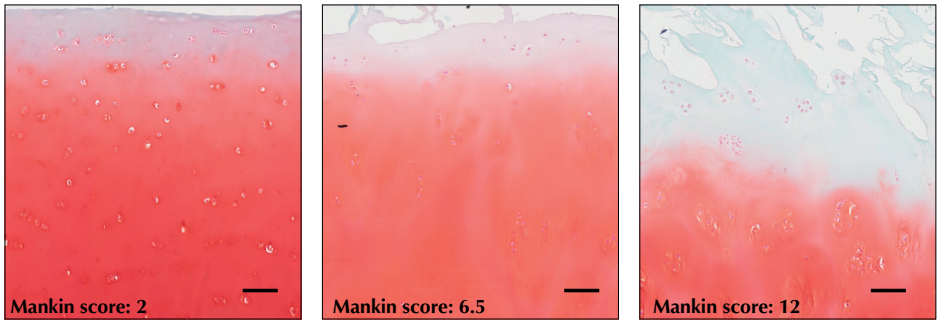
As infiltration of macrophages is seen during hip OA[74], we sought to identify the phenotype of the macrophages in the synovial membrane of the end-stage hip OA patients. The macrophage population that was predominantly present in the synovium were mainly anti-inflammatory and tissue repair macrophages, as indicated by high transcriptional levels of *CD163*, *CCL18*, and *CD206*, markers that identify anti-inflammatory and tissue repair macrophages[60,68]. These findings were also supported by the immunohistochemical stainings of the synovium, where the staining intensity for CD163 and CD206 was higher than the staining for CD11c. To gain more knowledge regarding which chemokines can be produced by which macrophage subset, the expression levels of the chemokines of interests were measured in primary polarized macrophages. *CX3CL1* was not expressed by any of the macrophage phenotypes, indicating that it is mainly expressed by other cells in the synovium such as fibroblasts, since it could be detected on a transcriptional level in the synovial tissue. *CCL2*, *CCL3*, *CCL4*, and *CCL5* were expressed by macrophages of all three phenotypes, including anti-inflammatory and tissue repair macrophages, the predominant phenotype present in the synovium of these hip OA patients. We may assume that the inverse correlation found between *CCL2* expression in the synovium and the degenerative state of the cartilage, is due to degrading cartilage that causes upregulation of *CCL2* expression in the synovium. This assumption is somewhat supported by a study by Raghu *et al*.[75], where it was reported that OA synovial fibroblasts secreted *in vitro* more CCL2 after stimulation with cartilage debris than when the fibroblasts remained unstimulated. Also, chemokines in the synovium, in particular CCL2, could potentially induce the recruitment of additional monocytes into the synovium. These monocytes then subsequently polarize towards M2 macrophages to dampen the initial inflammation phase, indicated by the high numbers of $CD206^+$ and $CD163^+$ cells in the synovium.

We initially hypothesized that chemokines are secreted by the synovium, facilitate the recruitment of monocyte-derived macrophages, and subsequently exacerbate the degeneration of the articular cartilage. Somewhat unexpected, we found that the expression of *CCL2* in the synovium had an inverse correlation with structural cartilage degeneration. Although our study is not a longitudinal study and we have no baseline measurement of the *CCL2* levels in the synovium prior to OA onset, our findings suggests that during end-stage hip OA in humans, severe cartilage damage is associated with low expression of *CCL2* in synovium. This partially reflects the findings of other studies

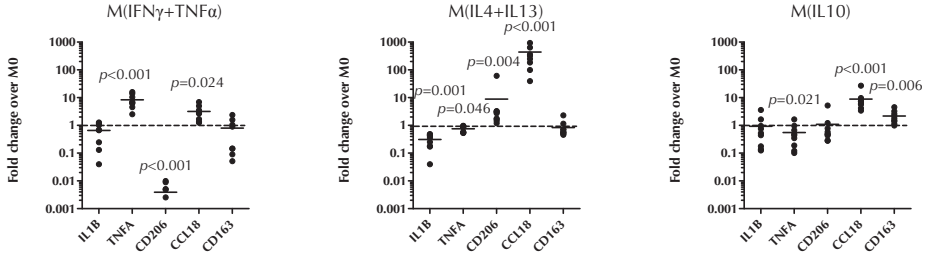
that focus on knee OA. Li *et al.* reported that CCL2 levels in the synovial fluid of OA knees significantly correlated with pain scores in OA patients, yet not with radiographic OA[76]. On the other hand, studies focusing on the role of CCL2 during experimental knee OA are slightly conflicting. Raghu *et al.* and Miotla Zarebska and colleagues both reported that monocyte recruitment was reduced in *CCL2* and *CCR2* deficient mice[75,77]. Raghu *et al.* reported that these mice were also protected from developing knee OA[75]. However, the study by Miotla Zarebska *et al.* showed that the CCL2/CCR2 axis contributes to pain but not to chondropathy[77]. Taken together, these studies indicate that the involvement of CCL2/CCR2 in OA progression in humans needs to be further explored on a molecular level. Future research could focus on exploring differences in monocyte subsets in hip OA patients, as it has been shown that the receptor for CCL2 (i.e. CCR2) is only found on the $CD14^{++}CD16^{-}$ classical monocyte subset and not on non-classical $CD14^{+}CD16^{++}$ monocytes[78], meaning that in particular classical monocytes will be attracted by CCL2. It would be interesting to know whether specific monocyte subsets have a preference in acquiring a certain macrophage phenotype, as it is currently only known that all monocyte subsets can polarize into macrophages upon stimulation[79]. By exploring such mechanisms, the role of monocytes/macrophages in the inflammatory component of hip OA can be further elucidated.

The findings of our study suggest that chemokines, in particular CCL2, may play a role in end-stage hip OA. We can speculate that during end-stage hip OA, the recruitment of additional monocytes results into polarization into anti-inflammatory macrophages. Caution is needed however, since the macrophages can also polarize towards the so-called tissue repair phenotype and contribute to synovial fibrosis. The findings of our study provide novel insights in the potential dynamics between chemokines, the phenotype of synovial macrophages, and the degenerative state of cartilage in end-stage hip OA.

SUPPLEMENTARY DATA



Supplementary Figure S2.1: Mankin score for cartilage degeneration. Examples of three degrees of articular cartilage degeneration as evaluated by the Mankin score. Stain: safranin-O; scale bar: 100 µm.



Supplementary Figure S2.2: Confirmation of macrophage polarization into pro-inflammatory M(IFNγ+TNFα), tissue repair M(IL4+IL10), and anti-inflammatory M(IL10) macrophages. Expression levels are shown as $2^{-\Delta CT}$ fold change over unstimulated macrophages (M0; dotted line).