CCL2 induces articular chondrocyte MMP expression through ERK and p38 signaling pathways

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ABSTRACT

Objective: In previous studies, we determined an association between increased serum and articular cartilage levels of CCL2 with osteoarthritis (OA) progression, cartilage damage and increased MMP13 in cartilage. Here we analyzed CCL2 downstream signaling mediators that lead to gene expression of cartilage catabolic markers, in healthy and OA human articular chondrocytes.

Design: Human articular chondrocytes obtained from healthy or OA subjects were treated with or without recombinant human CCL2; cell lysates or mRNA were collected for immunoblotting or qRT-PCR. For pathway analysis, chondrocytes were pre-incubated with an inhibitor of CCR2 (the unique CCL2 receptor), ERK inhibitor or p38 inhibitor prior to CCL2 treatment.

Results: CCL2 treatment of both healthy and OA chondrocytes activated ERK and p38 via CCR2. In healthy chondrocytes, short (6h) and prolonged (24–72h) CCL2 treatments led to Ccr2, Mmp-1, Mmp-3, Mmp-13 and Timp1 upregulation. In OA chondrocytes, CCL2 induced expression of Ccr2, Mmp-1 and Mmp-3, but not Mmp1 and Timp1, and only following longer treatments (72h). In both healthy and OA chondrocytes, the CCL2-mediated upregulation of Ccr2 and cartilage catabolic markers was mediated by ERK and p38 signaling.

Conclusions: The triggering of the CCL2/CCR2 axis in articular chondrocytes activates specific MAPK pathways leading to gene expression of cartilage degrading enzymes. However, some differences in the response to CCL2 stimulation are detected in healthy vs OA chondrocytes with respect to the number of activated genes and to the time of exposure to CCL2, suggesting that CCL2 action in articular cartilage may be dependent on OA stage and severity.

1. Introduction

Increased expression of chemokines in cartilage, synovial membrane and subchondral bone are linked to osteoarthritis (OA) progression [1]. Improving our understanding of the individual roles and functions of chemokines in OA is instrumental in dissecting the underlying disease mechanism in order to more effectively interfere with the disregulated chemokine/cytokine network [1]. The chemokine CCL2 (a.k.a. MCP-1) has been recognized as an important potential target in OA. CCL2, its functional mouse homologue mCCL12 [2] and their common unique chemokine receptor 2 (CCR2) are significantly increased in both humans with OA and in rodent models of OA [3,4], and have been shown to mediate OA pain in both humans and rodents [5–8].

In previous clinical studies, we determined that CCL2 serum levels at baseline were significantly associated with radiographic knee OA progression and joint space narrowing at a 5-year follow-up [9]. Our findings in a murine model of OA based on the destabilization of the medial meniscus (DMM), showed that the articular cartilage of OA knees had increased mCCL12 levels at sites of OA lesions, increasing progressively with severity, while it was undetectable in controls [10]. Interestingly, systemic inhibition of CCR2 at early OA stages, led to decreased levels of matrix metalloproteinases (MMPs) in articular cartilage, accompanied by reduced joint damage and pain [10]. Based on these studies, CCL2 and its CCR2 receptor have been recognized as important potential targets in OA.

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was used to identify macroscopic degenerative changes to articular joint [21] and only tissues graded 0–3 were used in this study (Supplementary Table I). For this study, only normal-looking cartilage was used, while areas exhibiting any sign of degenerative change were avoided. Age of donors ranged from 58 to 78 yrs (avg. age, 65.08 ± 6.76) (3 female and 9 male donors) (Supplementary Table I). OA tissue was obtained from patients undergoing total knee arthroplasty at the University of North Carolina; age for OA subjects ranged from 39 to 52 yrs (avg. age, 52.33 ± 10.44) (2 female and 4 male patients) (Supplemental Table II). Given the severity of the lesions in OA donors, it was rare to find enough tissue in less-affected regions, therefore we did not attempt to separate less-affected from more-affected regions and pooled together all the affected cartilage from the same OA joint. Chondrocytes were isolated and cultured as described [22] and used upon 80–90% confluency. Use of human tissue was in agreement with both Rush University Medical Center and the University of North Carolina at Chapel Hill Institutional Review Boards.

2.3. Gene expression analyses by quantitative RT-PCR

Chondrocyte monolayers were left in serum free for 2 h before any further treatment. Serum-free cells were treated with 20ng/ml CCL2 for 6, 24 or 72 h. For experiments where cells were pre-treated with ERK or p38 inhibitors, serum-free chondrocytes were exposed to U0126 or SB203580 (10 μM), for 1 h prior to CCL2 treatment. mRNA was isolated from chondrocytes using Purelink RNA mini kit from Thermo-Fisher (Waltham, MA; #12182018A). cDNA was synthesized using Super-Script IV Vilo Master Mix Kit with ezDNAse from Thermo-Fisher (#11766050). Quantitative RT-PCR was performed using TaqMan® Gene Expression Assays from Thermo-Fischer. A custom array plate was designed with multiple primers (TaqMan 96-well array custom plate; #44132660). Target primer/probes included were MMP1 (Hs00899658_m1); MMP3 (Hs00968305_m1); MMP13 (Hs00942584_m1); CCR2 (Hs00704702_s1); TIMP1 (Hs01092512_g1); COMP (Hs00164359_m1); COL2A1 (Hs00264051_m1); ADAMTS4 (Hs01120103_g1); ADAMTS5L (Hs01538655_g1). Housekeeping genes were GAPDH (Hs00265174_m1) and TBP (Hs00427620_m1). Assays were combined with TaqMan Fast Advance Master Mix (Thermo-Fischer, #4444557) and run for Real-Time PCR on the QuantStudio™ 6 Flex Real-Time PCR System (Thermo-Fisher, #4485689). Data was analyzed using QuantStudio Real Time Software v1.3.

2.4. Analysis of chondrocyte intracellular signaling by immunoblotting

For dose-response experiments, serum-free chondrocytes were treated with increasing dosage of CCL2 (2, 20, 40 and 80ng/ml) for 24h (for secreted MMP13) or 10 min (for ERK and p38 phosphorylation). For all further experiments, serum-free chondrocytes were then treated with or without CCL2 (20ng/ml) for 5, 10 or 15 min; or with FN-f (1 μM). For experiments with the CCR2 inhibitor, serum-free chondrocytes were exposed to RS504393 (20 μM) for 1 h prior CCL2 treatment. After experimental incubations, chondrocytes were rinsed in ice cold PBS and lysed in standard lysis buffer under gentle agitation for 30 min (4°C) prior to centrifugation at 13,000 rpm (10 min) to remove insoluble protein fractions. Quantification of total protein was determined using the Pierce Micro BCA kit (Thermo-Fischer, #23225). Cell lysates (10 μg protein/sample) were then combined with 10% β-mercaptoethanol and 5X loading buffer (reducing conditions) and immunoblotted as previously described [23]. Analyses of phosphoproteins were identified using phosho-specific antibodies for ERK, p38 or JNK. The total protein for ERK, p38 or JNK was used as loading control for each of the respective phosphoproteins. Therefore, blots were stripped and probed with antibodies to the total protein for loading controls. For secreted MMP13 protein detection, equal volumes of conditioned media were run on SDS-PAGE and blotted as above. As previously shown for FN-f [24], MMP2 production was not affected by CCL2 treatment (Supplementary
Fig. 1) and was used as loading control. Secondary antibodies conjugated to HRP were used to visualize the blot with chemiluminescent HRP substrate and imaged. Densitometric analysis of immunoblotting was performed using ImageJ software (NIH, Bethesda, MD). The area of pixilation of band intensity was measured from corresponding histograms. The relative intensity of blot bands were expressed as intensity of the phosphorylated bands normalized to their respective loading controls as previously reported [25].

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. Data are expressed as mean ± SD. In all cases, independent experiments were performed using chondrocytes from different tissue donors. Statistical analyses were performed using ordinary one-way ANOVA followed by Tukey’s post hoc test for multiple comparisons or paired t-tests, when comparing two groups; Statistical significance was set at p ≤ 0.05. Biological replicates are indicated in the figure legends.

Fig. 1. Effect of CCL2 treatment on the expression of Ccr2 receptor, Mmp1, Mmp3, Mmp13 and Timp1 in normal chondrocytes. RNA was isolated from confluent normal human articular chondrocytes treated with recombinant CCL2 (20ng/ml) for 6, 24 or 72 h. Quantitative RT-PCR analyses was performed using the following probes: Ccr2 (n=6), Mmp1 (n = 6), Mmp3 (6h, n = 9; 24–72h, n = 6), Mmp13 (6h, n = 10; 24h, n = 7; 72h, n = 6), and Timp1 (n=6). Data are presented as mean values ± SD. Statistical significance was set at p ≤ 0.05 (Unpaired t-test).
3. Results

3.1. CCL2 treatment of primary culture of chondrocytes leads to an increased production of MMP13 protein

MMP13 is the major collagenase found in cartilage and its upregulation has been implicated in osteoarthritis [26,27]. In addition, our previous mouse studies and findings from other investigators, have reported that CCL2 induces MMP-13 production in articular chondrocytes [10,14]. In dose-response experiments, we confirmed that CCL2 stimulation of human articular chondrocyte cultures leads to increased levels of MMP-13 in the culture media, with optimal levels at 20ng/ml (Supplementary Fig. 1). This dosage matches the dose reported by previous investigators and was therefore used in following experiments [14].

3.2. CCL2 treatment of primary culture of chondrocytes leads to an increased gene expression of its Ccr2 receptor and markers of cartilage breakdown

In light of our previous mouse in vivo finding supporting a role for CCL2/CCR2 axis in cartilage damage during the early stages of injury-induced OA [10], we investigated the effect of CCL2 treatment on the gene expression of its own receptor Ccr2, as well as on cartilage degrading enzymes in primary cultures of articular chondrocytes by qRT-PCR. Stimulation of human primary chondrocytes with CCL2 for different time periods (6, 24 and 72 h), led to an upregulation of Ccr2 expression compared to untreated controls (Fig. 1). Similarly, CCL2 treatments for the same time period led to an upregulation of metalloproteinase’s gene expression (Mmp-1, Mmp-3, and Mmp-13), as well as an increased expression of the tissue inhibitor of metalloproteinases-1 (Timp-1) (Fig. 1). In a few donors CCL2 treatment led to increased gene expression of Comp and Collagen 2; however, the overall analysis did not reach statistical significance due to high variability among donors (Supplementary Fig. 2). We did not find any differences in Adams-4 and Adams-5 gene expression following any length of CCL2 treatments (Supplementary Fig. 2).

3.3. CCL2 treatment of primary culture of chondrocytes leads to an increased phosphorylation of ERK and p38 but not JNK

In order to understand signaling pathways activated by CCL2, we exposed human articular chondrocytes from healthy donors to CCL2

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![Fig. 2. Effect of CCL2 treatment on ERK, p38 and JNK phosphorylation in normal chondrocytes. Confluent normal human articular chondrocytes were treated with recombinant CCL2 (20ng/ml) for 5, 10 or 15 min. FN-f (1 μM) treatment for 30 min was used as positive control. (A) Cell lysates were subjected to immunoblotting to detect phospho-ERK, phospho-p38 and phospho-JNK. Immunoblots are representative of n=4 independent donors. (B) Results of the densitometric analysis showing the effect of CCL2 treatment on the phosphorylation of ERK, p38 and JNK normalized to their respective loading controls. Data are presented as mean values ± SD. Statistical significance was set at p ≤ 0.05 (Asterisks represent 1way ANOVA summary; p values obtained with Tukey’s multiple comparisons are indicated on plots; ns = not significant).](image)
treatment and investigated the effect on the phosphorylation of MAP kinases, including ERK, p38 and JNK, that have been previously demonstrated to be involved in Fibronectin-fragment (FN-f) induced MMP expression, which was used as a positive control in the present experiments [28]. Similar to FN-f, CCL2 stimulation led to a significant increase in ERK and p38 phosphorylation within 5, 10 and 15 min after treatment, with a peak at 10 min. However, unlike FN-f, no significant effect of CCL2 was noted on JNK phosphorylation (Fig. 2A–B). As determined for MMP13 protein levels, 20 ng/ml was confirmed to be the optimal dosage by dose-response experiments using P-ERK and P-p38 as outcomes (Supplementary Fig. 3A and B).

3.4. Inhibition of CCR2 signaling decreases ERK and p38 phosphorylation induced by CCL2

To determine if the CCL2-induced stimulation of ERK and p38 phosphorylation was specific to the CCR2 receptor, we used a small CCR2 antagonist, RS-504393, to block its signaling. RS504393 specifically inhibits the CCR2 receptor (but not the CCR1, CCR3 or any of CXCRs) by occupying the ligand binding site [29]. Our results in Fig. 3A–B, show that pre-treatment with RS504393 modestly decreased CCL2-induced ERK and p38 phosphorylation.

3.5. Inhibition of Erk and p38 signaling decreases CCL-2-induced expression of Mmp3, Mmp13, Mmp1 and Timp1

The results obtained above suggest that CCL2 may exert its action on cartilage catabolic marker expression by activating ERK and p38 MAP kinases. To test this hypothesis, we exposed human articular chondrocytes to ERK or p38 specific inhibitors prior to CCL2 treatment and determined the effect on Mmp3, Mmp13, Mmp1 and Timp1 gene expression. As expected, pre-treatment with U0126 (Erk inhibitor) or SB203580 (p38 inhibitor) abolished the CCL2 action on these signaling pathways (Fig. 4A–B). Most importantly, blockade of either ERK or p38 signaling, significantly decreased the expression of Mmp3, Mmp13, Mmp1 and Timp1, although some differences were detected with respect of length of treatment. Specifically, following a short CCL2 treatment (6 h), both ERK and p38 were shown to mediate the Mmp13 increase, while only p38...
appeared to mediate the \( \text{Mmp1} \) upregulation; although both \( \text{Mmp3} \) and \( \text{Timp1} \) showed a decrease in their expression following ERK and p38 inhibition, none of the results reached statistical significance (Fig. 4C).

With respect to longer treatment (24h), we found that both ERK and p38 were mediating \( \text{Mmp3} \) and \( \text{Timp1} \) upregulation, while \( \text{Mmp13} \) and \( \text{Timp1} \) increased expressions were selectively mediated by p38 and ERK, respectively. Interestingly, following prolonged treatment of 72 h, blockade of either ERK or p38 signaling was able to abolish the CCL2-induced expression of all the genes tested, such as \( \text{Mmp3}, \text{Mmp13}, \text{Timp1} \) (Fig. 4C).

### 3.6. Prolonged exposure of chondrocytes from OA subjects to CCL2 stimulates expression of \( \text{Ccr2}, \text{Mmp3} \) and \( \text{Mmp13} \), but not \( \text{Mmp1} \) and \( \text{Timp1} \)

Following our results on human articular chondrocytes from normal donors, we next investigated whether CCL2 exerts a similar action on human chondrocytes obtained from OA subjects. Stimulation of OA chondrocyte with CCL2 for different time periods (6, 24 and 72 h), led to a significant upregulation of \( \text{Ccr2} \) expression only when the cells were exposed to prolonged treatments of 72 h, compared to untreated controls (Fig. 5). Similarly, CCL2 treatment for 72h, but not 6 or 24h, induced an upregulation of \( \text{Mmp3} \) and \( \text{Mmp13} \). In contrast with the results obtained from normal donor chondrocytes, CCL2 stimulation had no significant effect on \( \text{Mmp1} \) and \( \text{Timp1} \), at any of the time point tested (Fig. 5).

Similarly to normal donor chondrocytes, expression of \( \text{Comp}, \text{Collagen 2}, \text{Adams-4} \) and \( \text{Adams-5} \) did not change following CCL2 stimulation in OA chondrocytes (Supplementary Fig. 4).

### 3.7. Blockade of Erk and p38 signaling in OA chondrocytes decreases CCL-2-induced expression of \( \text{Mmp3} \) and \( \text{Mmp13} \)

To determine whether the CCL2-induced activation of \( \text{Mmp3} \) and \( \text{Mmp13} \) in OA chondrocytes was mediated by ERK and/or p38 signaling, we exposed OA articular chondrocytes to ERK and p38 specific inhibitors prior to CCL2 treatment for 72h. As expected, pre-treatment with U0126 or SB203580 blocked the CCL2 action on ERK and p38, respectively (Fig. 6A–B). Most importantly, as obtained for normal chondrocytes, blockade of ERK or p38 signaling, significantly decreased the expression of \( \text{Mmp3} \) and \( \text{Mmp13} \) following the 72 h treatment (Fig. 6C), indicating a critical role of these MAPKs in CCL2 signaling in OA chondrocytes.

### 4. Discussion

The articular chondrocytes express on their surface several mecha-noreceptors, cytokine and chemokine receptors. Several in vivo and \textit{in vitro} studies have supported a critical role for \( \text{Ccr2} \) signaling in cartilage metabolism that may be relevant to OA [30–32]. \( \text{Ccr2} \) activation can affect the anabolic activities of chondrocytes shifting them to a degra-dative phenotype accompanied by the secretion of many degradative enzymes, including the MMPs, leading to articular cartilage breakdown [30–35]. Although the \( \text{Ccr2} \) receptor binds several ligands (such as \( \text{CCL7}, \text{CCL8}, \text{CCL12} \) and \( \text{CCL13} \)) [2], our previous studies in human OA patients, and in accordance with others, have shown that increasing CCL2 serum levels correlated with radiographic knee OA progression and joint space narrowing at 5-year follow up [9]; therefore, in the present study, we focused our attention on the CCL2 ligand.

Although many studies have shown a critical role for the CCL2/\( \text{Ccr2} \) axis in mediating chondrocyte metabolism, the specific signaling pathways activated during cartilage breakdown are still unknown. In the present study, we used normal and OA articular chondrocytes to determine whether CCL2 stimulation leads to activation of specific MAP kinases (MAPK) and whether different MAPKs are associated with the expression of specific cartilage degradation markers.

First, we determined that the exposure of primary chondrocytes from healthy donors to CCL2 stimulation for different amount of time leads to a significant increase in the expression of the \( \text{Ccr2} \) receptor, suggesting a positive feedback on its activation. Furthermore, we found that CCL2 stimulation triggers the expression of \( \text{Mmp1}, \text{Mmp3}, \text{Mmp13} \) and \( \text{Timp1} \). Interestingly, in OA chondrocytes, CCL2 stimulation showed slightly different results, where only the upregulation of \( \text{Mmp3} \) and \( \text{Mmp13} \) was noticed, while \( \text{Mmp1} \) and \( \text{Timp1} \) expression was unchanged. In addition, \( \text{Mmp3} \) and \( \text{Mmp13} \) upregulation in OA chondrocytes appeared significant only when treatments were carried out for 72 h, in contrast with healthy chondrocytes that showed an increase in \( \text{Mmp3} \) and \( \text{Mmp13} \) expression at both short (6 h) and prolonged (24h and 72h) treatments.

In previous in vivo studies in a murine model of post-injury OA, we observed that the OA articular cartilage showed increased protein levels of different CCR2 ligands (CCL2, CCL7, CCL8, CCL12, CCL13) when compared to non-OA cartilage samples; therefore the endogenous high concentration of different CCR2 ligands in OA cartilage might cause a saturation of the CCR2 receptor, and further increase of ligand becomes less effective. In support to this hypothesis, we found that, in contrast with healthy chondrocytes, in OA chondrocytes the CCL2 feedback on the activation of its own \( \text{Ccr2} \) receptor reached significance only after prolonged exposure to the ligand (72 h). One explanation for such differences between normal and OA chondrocytes in response to CCL2 treatment, may be found in the known chemokine heterodimerization property leading to chemokine cooperativity, which is the ability of different chemokines to enhance each other’s function when a high gradient of concentration is reached [6,36–41]. Therefore, in OA chondrocytes, only longer exposure of the CCL2 stimulation is able to enhance the \( \text{Ccr2} \) receptor leading to increased expression of cartilage catabolic genes. However, when compared to healthy chondrocytes, only the CCL2-induced \( \text{Mmp3} \) and \( \text{Mmp13} \) expression reached significance, while \( \text{Mmp1} \) and \( \text{Timp1} \) did not change, even after prolonged treatment. These results suggest that the cartilage susceptibility to the CCL2 action may vary, and different genes can be activated depending on OA stages. We also analyzed the action of CCL2 stimulation on \( \text{Collagen 2} \) expression, as well as on \( \text{Comp} \), which has been demonstrated to be a biomarker for cartilage breakdown, and on the \( \text{ADAMTS-4} \) and \( \text{-5} \). Our data show that expression of \( \text{Adams-4} \) and \( \text{-5} \) are not directly affected by CCL2 stimulation, in both normal and OA chondrocytes. With respect to expression of \( \text{Comp} \) and \( \text{Collagen 2} \), our data show a higher variability in different donors, across normal and OA chondrocytes, at all treatment time points (Supplementary Figs. 2 and 4), suggesting that donor age may represent an additional variable for the response of articular chondrocytes to CCL2.

We next examined the possible downstream signaling cascade activated by the CCL2/\( \text{Ccr2} \) axis. Different studies have highlighted the critical role of MAP kinases in the pathogenesis of OA [25,28], with a particular relevance for p38, extracellular-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK), which are upregulated in OA tissues and are associated with the production of cartilage degradation enzymes [42,43]. In addition, ERK and p38 were shown to mediate CCL2-induced
chemotaxis and cell adhesion in a monocytic cell line [12], therefore we focused on these signaling pathways. Our data showed that chondrocyte stimulation with CCL2 leads to increased phosphorylation of ERK and p38, but does not seem to increase JNK activation when compared to untreated chondrocytes, suggesting that ERK and p38 might mediate the CCL2 action. This was supported by the finding that inhibition of either ERK or p38 could affect the CCL2 mediated MMP expression in normal and OA chondrocytes, although results were different with respect to the length of the CCL2 exposure.

Although CCR2 is the unique receptor for CCL2, it is not excluded that in the experimental in vitro conditions, where CCL2 concentrations are higher than physiological so that the pathological state can be studied, heterodimers may exert their signaling through other receptors. We used a CCR2 inhibitor, RS504393, to determine the specificity of CCL2 mediated action on ERK and p38 in chondrocytes. RS504393 specifically inhibits the CCR2 receptor (but not the CCR1, CCR3 or any of the CXCRs) by occupation of the ligand binding site [29]. As expected, the blockade of CCR2 signaling decreased ERK and p38 phosphorylation, although some activity was still present compared to the untreated control. Further studies are required to determine whether other chemokine receptors may be partially involved in mediating the CCL2 action on ERK and p38 MAPK activation in chondrocytes, when high levels of CCL2 are present.

Our findings demonstrate the importance of CCL2/CCR2 signaling in cartilage degradation during OA and define ERK and p38 as critical signaling molecules that mediate the CCL2 action, opening new strategies to restore proper cartilage homeostasis during disease progression. However, our results show that both MAPKs have a different time of action that might vary for different cartilage catabolic genes, especially when referring to short and medium-length treatment; in some cases, ERK and p38 were only partially mediating the short response to CCL2 (such as in Mmp3 upregulation), suggesting that other pathways might contribute to enhance the CCL2 action on chondrocyte degradation, at least in some condition. Interestingly, following prolonged treatment of 72 h, both MAPK pathways were shown to mediate the CCL2 action on all...
Fig. 6. Effect of CCL2 treatment on expression of MMPs in OA chondrocytes in presence or absence of ERK and p38 inhibition. Human OA chondrocytes were treated with recombinant CCL2 (20ng/ml) for 10 min. Where indicated, chondrocytes were pre-incubated with ERK inhibitor (U0126, 10 μM) or p38 inhibitor (SB203580, 10 μM), for 1h prior CCL2 treatment. (A) Cell lysates were subjected to immunoblotting to detect phospho-ERK and phospho-p38. Immunoblots are representative of n=4 independent donors. (B) Results of the densitometric analysis showing the effect of CCL2 treatment on the phosphorylation of ERK and p38 with or without ERK or p38 inhibition normalized to their respective loading controls. (C) Quantitative RT-PCR analyses were performed using the following probes: Mmp3 (n=6) and Mmp13 (n=6). Data are presented as mean values ± SD. Statistical significance was set at p ≤ 0.05 (Asterisks represent 1way ANOVA summary; p values obtained with Tukey’s multiple comparisons are indicated on plots; ####p ≤ 0.0001).
the cartilage degradation markers tested, mirroring the results obtained in OA chondrocytes and supporting the hypothesis that excessive exposure to CCL2 might affect pathway specificity through the binding with other chemokines and, consequently, possible activation of other receptors. Taken together, these findings, highlight the susceptibility of chondrocytes to CCL2 concentration and emphasize that a certain chemokine balance might be critical for the activation of selective degradation markers, suggesting the need of addressing OA potential treatment as stage specific. This is also supported by our previous studies in a mouse model of post-traumatic OA, showing that systemic blockade of CCR2 during OA led to decreased MMP13 protein levels and reduced OA pathological changes in articular cartilage only when treatment was delivered at early or moderate stages of the disease.

Although CCL2 stimulation leads to increased expression of its own receptor (Ccr2) in certain experimental conditions, we did not show a direct increase in CCR2 downstream signaling pathway. Future studies are needed to investigate how CCL2 stimulation affects levels of phosphorylated NF-κB-p65 in both normal and OA articular chondrocytes; however, our results in OA chondrocytes highlight that changes in cartilage catabolic genes mediated by CCL2 were only effective at higher Ccr2 gene expression levels, suggesting that the increased transcription might translate into increased cell signaling.

OA is a disease of multiple tissues and in the effort to identify potential treatments we need to analyze other tissues involved in the joint degeneration (bone, synovium, meniscus etc.). With the respect to CCL2/CCR2 signaling, we know that bone and inflammatory cells are also susceptible to CCL2 stimulation and the CCL2/CCR2 axis plays a role in OA disease progression also by increasing bone damage and monocyte infiltration. Therefore, future studies are indicated to analyze signaling pathways that mediate the CCL2 response in bone and inflammatory cells.

Declaration of competing interest

Authors do not have conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jocarto.2020.100136.

Contributions

1) Study conception and design: H.W., R.F.L., LL. Acquisition of data: H.W., H.O., S.C., LL. Data analysis and interpretation: S.C., R.F.L., LL. Obtaining of funding: LL., R.F.L. and S.C.

2) Drafting and revision of manuscript: all authors have contributed to the draft and revision of the manuscript.

3) Final Approval of the Manuscript: all authors have reviewed the final version of the manuscript and approved the version to be published.

Lara Longobardi takes full responsibility for data integrity and analysis, from inception to finished article (lara_longobardi@med.unc.edu).

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Sponsors had no involvement in the study design, collection, analysis, interpretation of data and in the manuscript writing.

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