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# Chemokine (C-C Motif) Ligand 2/Chemokine Receptor 2 (CCR2) Axis Blockade to Delay Chondrocyte Hypertrophy as a Therapeutic Strategy for Osteoarthritis

Authors' Contribution:

Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

ACE 1 **Zidong Wang**  
CF 2 **Bei Wang**  
DF 1 **Jian Zhang**  
BD 3 **Zhensong Wu**  
BF 1 **Liankui Yu**  
ADG 1 **Zhongye Sun**

1 Department of Orthopedic Surgery, Liaocheng People's Hospital, Liaocheng, Shandong, PR China  
2 Department of Imaging, Liaocheng Infectious Disease Hospital, Liaocheng, Shandong, PR China  
3 Department of Joint Sports Medicine, Zaozhuang Municipal Hospital, Zaozhuang, Shandong, PR China

**Corresponding Author:** Zhongye Sun, e-mail: 593694199@qq.com  
**Financial support:** Departmental sources

**Background:** Chondrocytes play a vital role in the later stages of osteoarthritis (OA). The roles of chemokine (C-C motif) ligand 2 (CCL2) and its receptor, chemokine receptor 2 (CCR2), are as yet poorly elucidated in chondrocyte hypertrophy (CH). Here, we aimed to regulate the CCL2/CCR2 axis and explore its effect on progression of CH.

**Material/Methods:** Chondrocytes isolated from patients with OA were used in the present study. In vitro experiments were conducted to test hypertrophic gene and CCL2/CCR2 expression in chondrocyte degeneration caused by interleukin (IL)-17A or CCL2 protein stimulation. In addition, inhibition of CCL2 and CCR2 was used to assess the role of CCL2 and CCR2 blockade in CH. Relative gene expression was determined with real-time polymerase chain reaction, western blot, or immunofluorescence. Hypertrophic changes were assessed with cell area measurement. Moreover, the viability of chondrocytes was analyzed using an MTT assay and flow cytometry was used to assess cell apoptosis.

**Results:** CCL2 and CCR2 were upregulated in IL-17A-treated chondrocytes. The exogenic CCL2 stimulation also promoted CH and increased the expression of Type 10 collagen, *RUNX2*, and *IHH*, which could be reversed via suppression of CCR2. Inhibition of CCL2 and CCR2 expression was sufficient to: 1) protect Type 2 collagen synthesis; 2) alleviate IL-17A-induced overexpression of Type 10 collagen, *RUNX2*, and *IHH*; and 3) improve chondrocyte proliferation and apoptosis.

**Conclusions:** Blockading the CCL2/CCR2 axis plays a role in delaying the development of CH.

**Keywords:** **Chondrocytes • Hypertrophy • Osteoarthritis**

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/930053>

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## Background

Osteoarthritis (OA) is characterized by loss of cartilage, changes in subchondral bone, synovitis, and other joint tissue destruction. It is a chronic, degenerative disease that mainly affects weight-bearing joints; the typical clinical manifestations are joint pain, swelling, and limited activity [1,2]. Treatment of advanced OA is still mainly based on joint replacement, and there are currently no effective preventive measures. Cartilage hypertrophy can occur in normal embryonic chondrogenesis. However, chondrocytes that are hypertrophic express some typical biological markers, which mediate the continuing progression of OA [3]. As OA becomes more advanced, chondrocytes undergo differentiation, hypertrophic dedifferentiation, terminal mineralization, and, finally, apoptosis, which is similar to the differentiation of chondrocytes that occurs during ossification of cartilage. Disorders of chondrocyte growth regulation and a lack of factors that inhibit differentiation from maturation to hypertrophy accelerate OA development [4].

Chondrocytes in OA can produce a variety of inflammatory factors, such as interleukin (IL)-1 $\beta$  and tumor necrosis factor- $\alpha$ , which further promote the production of other inflammatory factors, thereby changing the composition of collagen and accelerating the decomposition of cartilage extracellular matrix (ECM), which is one of the leading causes of chondrocyte hypertrophy (CH) [5]. Chemokine (C-C motif) ligand 2 (CCL2), also known as MCP1, is an essential proinflammatory factor of the CC subfamily of chemokines [6,7]. It has a specific chemotactic activation effect on monocytes, macrophages, and other inflammatory factors that are commonly found in the synovial fluid of patients with OA [8]. The impact of chemokine-induced inflammation has been demonstrated in in vitro stimulation of hypertrophic chondrocyte differentiation related to *CXCL8* and *CXCL1* [9], both of which are also overexpressed in OA cartilage [10]. CCL2 deficiency is associated with a notable improvement in multiple inflammatory diseases, including OA [11], atherosclerosis [12], and chronic hepatic injury [13]. However, whether CCL2 is involved in the hypertrophic progress of OA is still unclear.

Chondrocytes, the only type of cells in the cartilage, play a vital role in the pathology of OA because of the character of the dedifferentiation and loss of the chondrogenic phenotype. Collagen type X is currently the central specific marker molecule for CH. Type II collagen is mainly present in the ECM of cartilage, which is secreted by normal chondrocytes and is a vital molecule marker of the chondrogenic phenotype. During the process of hypertrophy, production of Type II collagen in chondrocytes is significantly reduced, and the main components of the ECM change to Type X collagen, with increased levels of metalloproteinase-13 and proteoglycan enzymes, which promote cartilage calcification [14]. The cell surface receptors for

CCL2 are C-C chemokine receptor 4 (CCR4) and chemokine receptor 2 (CCR2); CCR4 is a receptor for various chemokine CC subfamily members, but CCR2 mainly recognizes CCL2 [15]. We hypothesized that the CCL2-CCR2 ligand-receptor axis might play a role in the development of CH. The aim of the present study was to clarify the potential role of the CCL2/CCR2 axis in OA by examining Type 10 collagen and other specific molecular markers of hypertrophy [16].

## Material and Methods

### Reagents

The Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and bovine serum albumin (BSA) were from Gibco (Dun Laoghaire, Dublin, Ireland). The human recombinant interleukin (IL)-17A, penicillin-streptomycin mixture, trypsin, Type XI collagenase, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay kits were from Sigma (St. Louis, Missouri, United States). The human recombinant CCL2 protein (Cat: 10134-H08Y) was from Sino Biological (Beijing, China). The CCR2 antagonist (CAS, Cat: 445479-97-0) was from Santa Cruz (Santa Cruz, California, United States). The bindarit (Bin) (Cat: S3032) was from Selleck (Shanghai, China). The anti-Type 10 collagen, anti-CCR2, anti-CCL2, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies were from Abcam (Cambridge, United Kingdom). The Alexa Fluor488 and 568, TRizol reagent, and Annexin V-FITC/PI kit were from Invitrogen (Carlsbad, California, United States). The universal polymerase chain reaction (PCR) Master Mix was from TOYOBO (Osaka, Japan). Unless specifically indicated, all of the other reagents were from Beyotime (Shanghai, China).

### Cartilage Tissue Collection

Human cartilage was collected from patients with OA who were undergoing total knee arthroplasty at Liaocheng People's Hospital. Informed consent was obtained from the patients before surgery. The study protocol was approved by the hospital's Ethics Committee. A total of 23 patients donated their knee joints. Before surgery, cartilage with mild degeneration was obtained from 6 of them: 3 men and 3 women; average age 49 years, range 38 to 73 years. The inclusion criterion was absence of primary diseases other than OA, such as diabetes, hypertension, and cancer. The tissues were conserved in sterile growth medium immediately after they were cut from the patients and transferred to the laboratory for chondrocyte isolation.

### Chondrocyte Isolation and Culture

We divided the superficial part of the cartilage to isolate the chondrocytes (n=6). The samples were washed with sterile

**Table 1.** Primer sequences for RT-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen X	ATGCTGCCACAAATACCCCTTT	GGTAGTGGGCCTTTTATGCCT
Collagen II	TGGACGATCAGCGCAAACC	GCTGCGGATGCTCTCAATCT
Runx-2	TGGTTACTGTATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
Ihh	GGACGCTATGAAGGCAAGATCG	CAGCGAGTTCAGGCGGCTCT
CCL2	AGAATCACCAGCAGCAAGTGCC	TCCTGAACCCACTTCTGCTTGG
CCR2	CAGGTGACAGACTCTTGGGA	GGCAATCCTACAGCCAAGAGCT
GAPDH	ACAACCTTGGTATCGTGAAGG	GCCATCAGCCACAGTTTC

phosphate-buffered saline (PBS) and cut into pieces as small as possible. The diced cartilage was then incubated with 0.15% trypsin and 0.2% Type XI collagenase overnight at 37°C for digestion. The digestion solution was filtered and centrifuged to obtain the chondrocyte pellets. The pellets were resuspended with growth medium (DMEM with 10% FBS and 1% penicillin-streptomycin mixture) and cultured at 37°C. For cell treatment, we used the human recombinant cytokine IL-17A to induce CH. The human active CCL2 protein was used to upregulate cellular CCL2 expression. The CCL2 inhibitor (Bin) and CCR2 antagonist (CAS) were used to block CCL2 and CCR2 expression during IL-17A treatment.

### Immunofluorescence

Protein expression of Type 10 collagen and CCL2 was analyzed with immunofluorescence (IF) methods using the following steps. Chondrocytes were seeded in a 24-well plate with a density of 7500 per well. After the drug treatments, the chondrocytes were washed using PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Then, the non-specific binding of the protein was blocked with 5% BSA for 1 h at room temperature. The chondrocytes then were incubated with the anti-Type 10 collagen and anti-CCL2 primary antibody overnight at 4°C. The next day, the chondrocytes were washed and incubated with the Alexa Fluor488 and 568 secondary antibodies for 1 h in the dark at room temperature. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). The staining intensity was measured using Image-Pro Plus software to determine protein expression.

### Real-Time PCR Analysis

We performed real-time PCR (RT-PCR) to analyze CCL2, CCR2, Type 2 collagen, Type 10 collagen, *RUNX2*, and *IHH* gene expression at the mRNA level. Briefly, total RNA was isolated from the chondrocytes after treatment using TRIzol reagent according to the manufacturer's instructions. Then, the RNA was transcribed into complementary deoxyribose nucleic acid (cDNA) and applied to the RT-PCR analysis using Universal PCR Master Mix. The mixture contained 6 µL of RNase-free H<sub>2</sub>O, 12 µL of

PCR Master Mix, and 2 µL cDNA. The reaction protocol was as follows: 95°C for 8 min, and 42 cycles at 95°C for 15 s, 60°C for 60 s, and 4°C for 5 min. Relative gene expression was normalized by the amount of GAPDH according using the 2<sup>-ΔΔCt</sup> method. The primers are listed in **Table 1**.

### Western Blot

The total protein in the chondrocytes was isolated using the RIPA buffer. An equal amount of protein from each group was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane. The membranes were incubated with anti-Type 10 collagen, anti-CCL2, anti-CCR2, or GAPDH overnight at 4°C. After incubation with the secondary antibody, the membranes were exposed to enhanced chemiluminescence substrate and the band was measured using ImageJ software.

### Cell Area Measurement

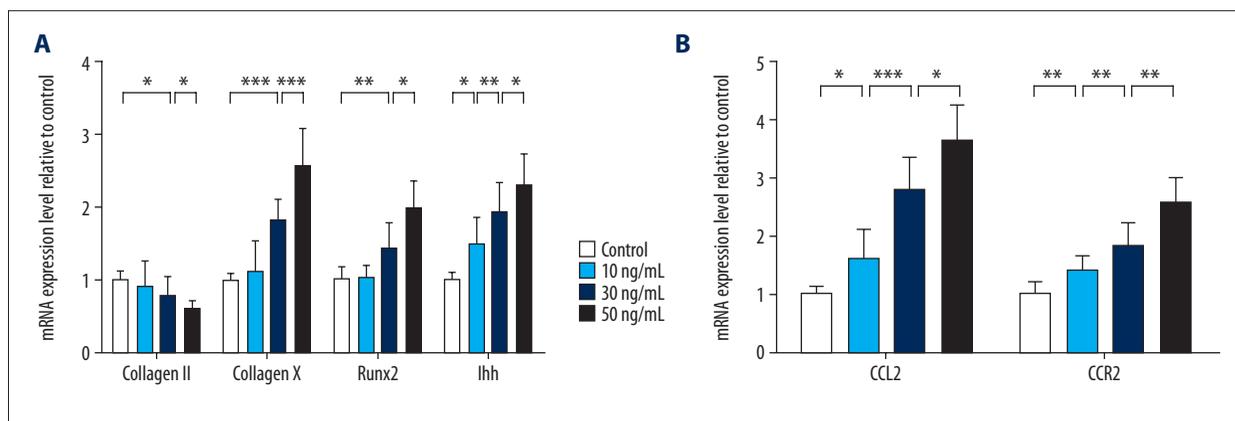
After treatment, the chondrocytes were washed 3 times with PBS and fixed with 4% paraformaldehyde for 15 min. Then, a white field image of the chondrocytes was examined under a microscope and the cell area was determined using ImageJ software.

### Flow Cytometric Analysis

Flow cytometry (FCM) was performed to identify the apoptotic chondrocytes in each group. The chondrocytes were harvested and resuspended in a 100-µL annexin-binding buffer. The Annexin V-FITC/PI kit was added to the cell suspension and incubated at 37°C for 15 min according to the manufacturer's instructions. After incubation, emissions at 530 and 570 nm were used for FCM.

### MTT Assay

To assess the proliferation of chondrocytes after each treatment, we performed the MTT assay. Briefly, 10<sup>4</sup> chondrocytes



**Figure 1.** Chemokine (C-C motif) ligand 2 (CCL2) and CCR2 are increased in interleukin 17A-induced chondrocyte hypertrophy. Real-time polymerase chain reaction was used to assess mRNA expression of (A) Type 2 collagen, Type 10 collagen, *RUNX2*, and *IHH* and (B) CCL2 and CCR2. Data are shown as means±standard deviations vs the control group. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ .

were seeded in 96-well plates following the desired procedures. Viable cells were incubated with MTT assay kits according to the manufacturer's instructions. The wavelength density of the spectrophotometric measurement was 570 nm.

### Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) software, version 22.0 (IBM, Armonk, New York, United States). The data are shown as means±standard deviations. Differences between 2 groups were analyzed by using a *t* test. A comparison among multiple groups was made using a one-way analysis of variance test followed by a post hoc test (least significant difference). For each parameter, \*  $P<0.05$ , \*\*  $P<0.01$ , or \*\*\*  $P<0.01$  between the groups were considered statistically significant.

## Results

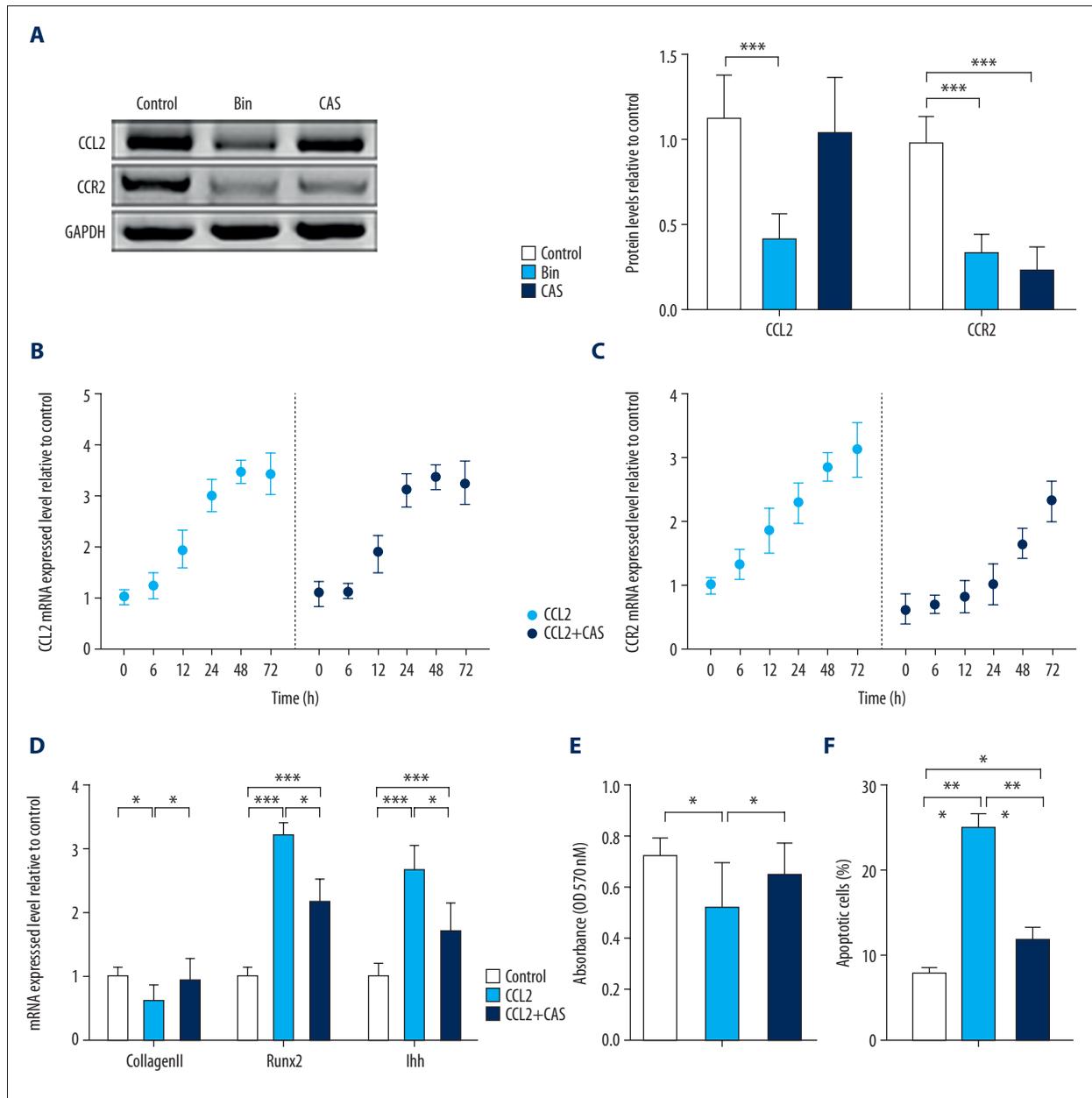
### CCL2 and CCR2 Increased IL-17A-Induced Chondrocyte Hypertrophy

The proinflammatory cytokine IL-17 has been confirmed to enhance chondrocyte dedifferentiation [17]. We used a range of concentrations of IL-17A protein (10 ng/mL, 30 ng/mL, and 50 ng/mL) to incubate chondrocytes for 48 h and analyzed their chondrogenic or hypertrophic gene expression. The higher doses of IL-17A resulted in higher hypertrophic gene expression. After treatment with 30 ng/mL of IL-17A, Type 2 collagen expression was decreased and Type 10 collagen and *Runx2* levels were increased compared to that in the control group. In contrast, results with the 50-ng/mL concentrated achieved more significance. The level of *IHH* mRNA was increased in a dose-dependent fashion, even with 10 ng/mL IL-17A treatment,

compared to that in the control group (Figure 1A). Moreover, CCL2 and CCR2 expression were both upregulated after IL-17A stimulation in a dose-dependent manner compared with the control group (Figure 1B). Therefore, expression of CCL2 and its receptor, CCR2, was triggered when progression of CH began.

### CCR2 Deficiency Delayed CCL2-Induced CH

To determine the effects of CCL2 and CCR2 during CH, we supplied the CHs with active human CCL2 protein with and without CCR2 inhibition. The cells with no treatment were the control group. The efficiency of the inhibitors used in this study was analyzed at the protein level. After stimulation with Bin (50  $\mu$ M) for 24 h, expression of CCL2 and CCR2 proteins was synchronously decreased compared with the controls. Treatment with CAS (5 nM) for 24 h significantly suppressed CCR2 protein levels (Figure 2A). Expression of CCL2 and CCR2 mRNA was measured to determine the optimal time of supplementation of the active human CCL2 protein. During the treatment with 10 ng/mL of CCL2 protein, cellular expression of CCL2 mRNA gradually increased for 48 h and then was stable from 48 to 72 h. In addition, we pretreated chondrocytes with 5 nM of CAS for 1 day and then switched to CCL2 incubation. The CAS pretreatment did not alter the CCL2 mRNA expression (Figure 2B). Unlike CCL2, expression of CCR2 mRNA in chondrocytes kept increasing during treatment with CCL2 protein. However, CCR2 expression in the CAS-pretreated chondrocytes was at a low level before rapidly increasing 24 h after CCL2 stimulation (Figure 2C). Therefore, we chose a 24-h CCL2 stimulation time to simultaneously obtain significant CCR2 inhibition. After 24 h of CCL2 protein treatment, Type 2 collagen expression decreased, which was reversed by CCR2 suppression. Expression of *RUNX2* and *IHH* mRNA also was significantly increased due to the overexpression of CCL2, and the increment decreased when the chondrocytes were pretreated with CAS

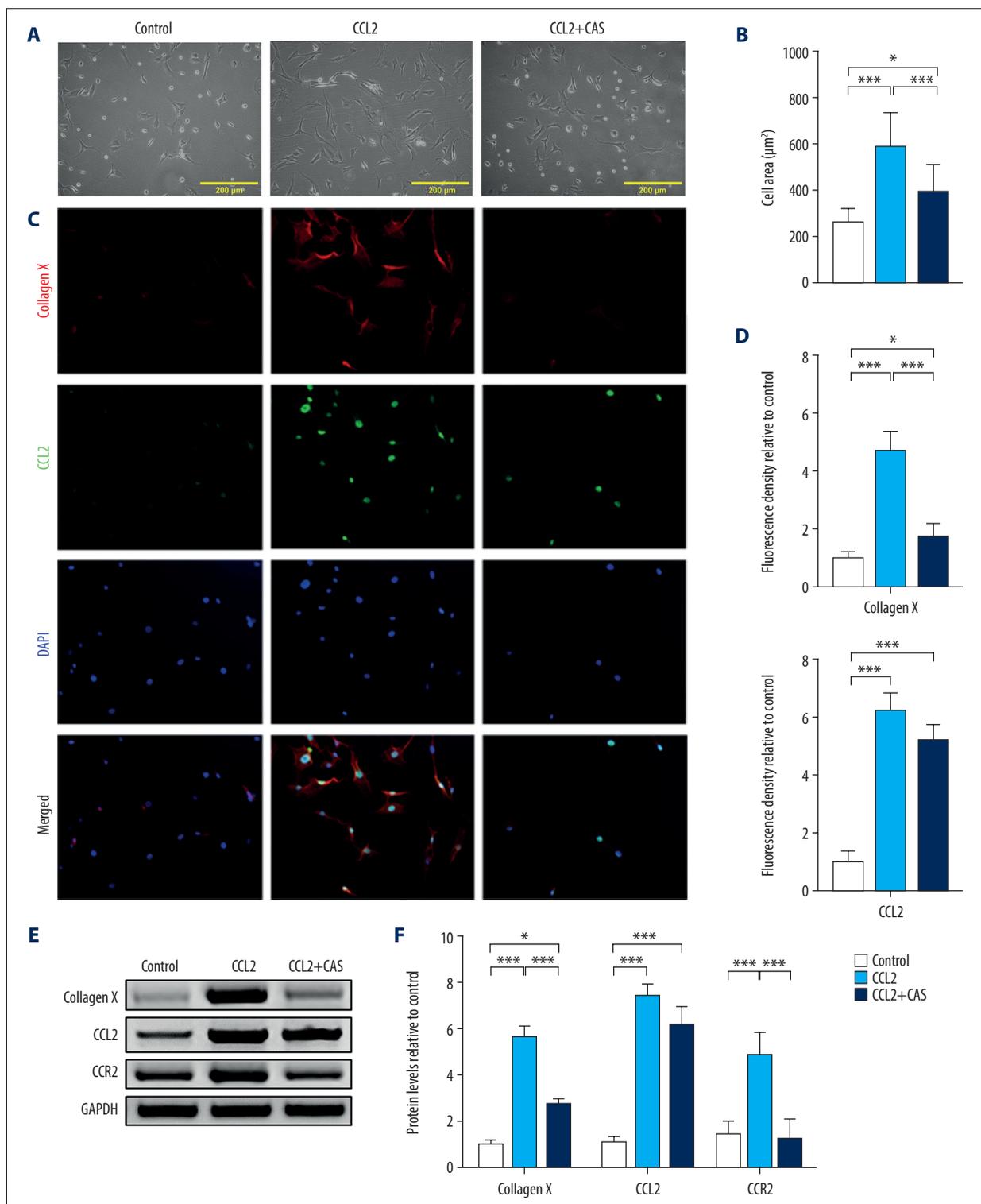


**Figure 2.** CCR2 deficiency delays chemokine (C-C motif) ligand 2 (CCL2)-induced chondrocyte hypertrophy (CH). **(A)** Protein expression of CCL2 and CCR2 was analyzed after treatment with bindarit or CAS and quantified. mRNA expression of **(B)** CCL2 and **(C)** CCR2 was analyzed after treatment with CCL2 protein with or without CAS pretreatment. **(D)** Expression of Type 2 collagen, *RUNX2*, and *IHH* mRNA in CH. **(E)** Cell proliferation was measured with an MTT assay. **(F)** Cell apoptosis was measured with flow cytometry. Data are shown as means±standard deviations vs the control group. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ .

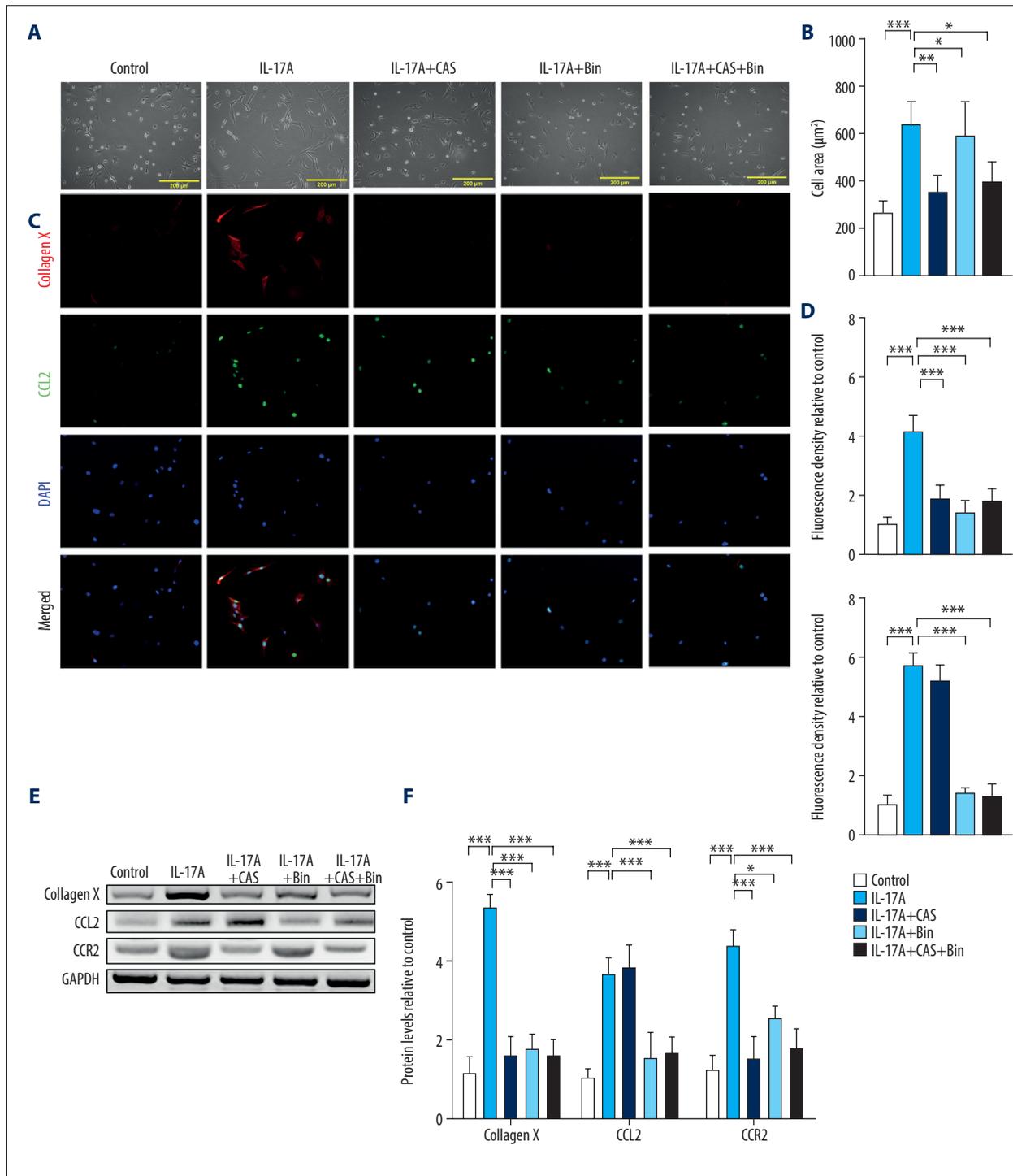
(Figure 2D). With the development of chondrocyte dedifferentiation, the proliferation decreased and the apoptosis usually increased. Given the results from MTT and FCM, CCL2 overexpression appeared to affect proliferation and promote apoptosis of the chondrocytes. Suppression of CCR2 by CAS, however, was integral to preventing the adverse effects caused by CCL2 (Figure 2E, 2F).

### CCR2 Deficiency Inhibited CCL2-Induced Type X Collagen Expression

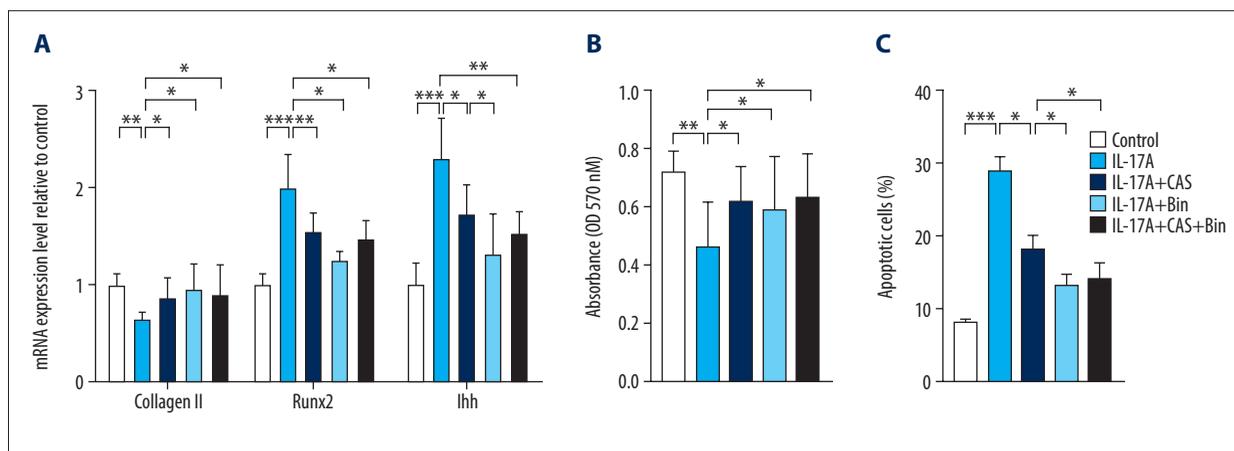
The cell area was measured to identify hypertrophic changes in chondrocytes. As shown in Figure 3A and 3B, CCL2 overexpression enlarged the cell area and resulted in a hypertrophic phenotype in CHs; pretreatment with a CCR2 inhibitor



**Figure 3.** CCR2 deficiency inhibits chemokine (C-C motif) ligand 2 (CCL2)-induced Type 10 collagen expression. **(A)** Representative images of cell morphology and **(B)** cell area measurement. **(C)** Protein expression of Type 10 collagen and CCL2 was determined with immunofluorescence (IF) (magnification: 200×). Red indicates Type 10 collagen, green indicates CCL2, and blue indicates the cell nucleus. **(D)** Quantification analysis of IF intensity. **(E)** Protein expression of Type 10 collagen, CCL2, and CCR2 and **(F)** quantification analysis. Data are shown as means±standard deviations vs the control group. \*  $P<0.05$ , \*\*\*  $P<0.001$ .



**Figure 4.** Blocking chemokine (C-C motif) ligand 2 (CCL2)/CCR2 inhibits interleukin-17A-induced Type 10 collagen expression. (A) Representative images of cell morphology and (B) cell area measurement. (C) Protein expression of Type 10 collagen and CCL2 was determined with immunofluorescence (IF) (magnification: 200×). Red indicates Type 10 collagen, green indicates CCL2, and blue indicates the cell nucleus. (D) Quantification analysis of IF intensity. (E) Protein expression of Type 10 collagen, CCL2, and CCR2 and (F) quantification analysis. Data are shown as means±standard deviations vs the control group. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .



**Figure 5.** Blocking chemokine (C-C motif) ligand 2/CCR2 delays interleukin-17A-induced chondrocyte hypertrophy. **(A)** mRNA expression of Type 2 collagen, *RUNX2*, and *IHH* was assessed with real-time polymerase chain reaction. **(B)** Cell proliferation was measured with an MTT assay. **(C)** Cell apoptosis was measured with flow cytometry. Data are shown as means±standard deviations versus the control group. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ .

prevented this process. To assess the level of the hypertrophy markers Type 10 collagen and CCL2 in chondrocytes, we analyzed their protein expression with IF and western blot. As shown in **Figure 3C and 3D**, CCL2 protein expression was up-regulated after supplementation with exogenous CCL2 protein, which was accompanied by increased Type 10 collagen protein production compared with controls. Moreover, suppression of CCR2 alleviated the CCL2-induced Type 10 collagen upregulation. However, CAS did not significantly affect CCL2 expression. The same trend was observed on the results of the western blots. CCL2 protein treatment increased the cellular expression of Type 10 collagen and CCL2 and pretreatment with CAS prevented upregulation of Type 10 collagen. CCL2 protein treatment also triggered upregulation of CCR2 protein expression, which was effectively suppressed by CAS (**Figure 3E, 3F**).

### CCL2/CCR2 Blockade Inhibited IL-17A-Induced Type X Collagen Expression

Because overexpression of CCL2 accelerated CH, which could be interrupted by CCR2 deficiency, we wanted to investigate whether suppressing CCL2 or CCR2 could inhibit IL-17A-induced hypertrophy and if its efficiency could be amplified by combined CCL2 and CCR2 inhibition. Therefore, we pretreated chondrocytes with CAS (5 nM), Bin (50  $\mu$ M), or a combination of CAS (5 nM) and Bin (50  $\mu$ M) for 24 h. The chondrocytes then were cultured for 24 h with IL-17A (50 ng/mL) to induce hypertrophy, as previously described. The untreated chondrocytes served as controls. The cell morphology measurement results indicated that IL-17A significantly increased the cell area and that pretreatment with CAS, Bin, or both prevented the increase in cell area (**Figure 4A, 4B**). The IF results showed that CAS did not significantly affect CCL2 protein levels, but Bin pretreatment

suppressed IL-17A-induced CCL2 upregulation. Furthermore, pretreatment with CAS, Bin, or the combination suppressed IL-17A-induced Type 10 collagen upregulation. The combination of CAS and Bin, however, was not more effective than treatment with either alone (**Figure 4C, 4D**). The inhibitory effects of CAS and Bin were verified using western blot, which suggested that suppression of CCL2 or CCR2 could alleviate IL-17A-induced Type 10 collagen upregulation, and that suppression of CCR2 resulted in a reduction in CCR2. Suppression of CCR2 did not affect the CCL2 level and combined treatment with CAS and Bin did not result in amplification (**Figure 4E, 4F**).

### CCL2/CCR2 Blocking Delayed IL-17A-Induced Chondrocyte Hypertrophy

Collagen II expression also was protected after pretreatment with CAS, Bin, or CAS and Bin combined, compared to the IL-17A group. The mRNA of *RUNX2* and *IHH* also was suppressed by inhibition of CCR2 or CCL2 (**Figure 5A**). Apart from this, suppression of CCR2 and CCL2 improved the proliferation and apoptosis triggered by IL-17A (**Figure 5B, 5C**). The efficiency of the destruction of *IHH* and apoptosis was more evident in the Bin-pretreated group than in the other 2 groups. However, the combination of CAS and Bin showed no advantage compared to treatment with either alone.

## Discussion

In the present study, we investigated the role of the CCL2/CCR2 axis in the process of chondrocyte hypertrophic differentiation. Cartilage degeneration is closely related to a variety of chronic bone and joint diseases, such as OA, rheumatoid arthritis, and intervertebral disc degeneration [18]. Because it

lacks blood vessels and nerve tissue, articular cartilage is difficult to repair once damaged. Therefore, prevention of cartilage degeneration as a way to forestall development of OA is an important goal [19]. As is well known, cartilage degeneration is dependent upon abnormal differentiation of chondrocytes, which is similar to the chondrocyte differentiation stage in the process of intra-chondral osteogenesis, both of which involve cell hypertrophy, terminal differentiation, mineralization, and finally death [20]. Therefore, we aimed to identify an efficient way to suppress CH and achieve a resulting chondrogenic phenotype.

First, we verified that CCL2 and CCR2 levels were increased when chondrocytes underwent hypertrophic differentiation caused by IL-17A. Moreover, exogenous supplementation of CCL2 also increased CCR2, which resulted in an enlarged cell area and hypertrophic markers in chondrocytes, which could be attenuated by blocking CCR2. Therefore, excessive CCL2 expression should be considered a risk factor for CH. In contrast to results from previous studies, we found that the effects of CCL2 on the morphology and hypertrophic differentiation of chondrocytes was related to upregulation of *IHH* and *RUNX2* expression. Abnormal activation of these signaling pathways plays an essential role in the development of cartilage degeneration. Amano et al [21] reported that the *IHH* signal triggers transcription and expression of Type 10 collagen in chondrocytes through the downstream transcription factor Gli combined with *RUNX2*, thereby causing calcification of cartilage. *RUNX2* is critical to endochondral bone formation and maturation of chondrocytes [22]. It promotes Type 10 collagen expression and mediates CH in association with Wnt/ $\beta$ -catenin signaling [23]. By pretreating chondrocytes with CCR2 inhibitor, the hypertrophic phenotype caused by CCL2 overexpression was alleviated by decreasing expression of Type 10 collagen, *IHH*, and *RUNX2*. Therefore, blocking CCR2 expression is a way to counter the effects of overexpression of CCL2 in chondrocytes.

Because CCL2 overexpression can induce CH, we explored the effects of inhibition of CCL2 on IL-17A-induced CH. CCL2 is a CC-type cell chemokine that induces the recruitment and activation of monocytes/macrophages by binding to CCR2 and it mediates most of the body's inflammatory response processes. CCL2 and CCR2 have been proven to play an essential role in OA pathogenesis by promoting an inflammatory response and ECM degradation [24]. The molecular mechanism, however, has not been fully elucidated. For instance, Xu et al reported

that CCL2 induced degradation of cartilage matrix and chondrocyte apoptosis by enhancing metalloproteinase [25]. Raghu et al stated that CCL2/CCR2 rather than the CCL5/CCR5 axis increased inflammation and tissue damage in OA [26]. In our study, we provided new insight related to the hypertrophy process in chondrocytes. Importantly, we found that pretreatment with an inhibitor of CCL2 or CCR2 resulted in significant resistance to IL-17A-induced hypertrophy, which means that suppressing the CCL2/CCR2 axis can prevent chondrocyte degeneration. This is in contrast to results from a previous study, which indicated that blocking CCL2 or CCR2 expression served as a treatment for inflammatory disease [27]. Our results also suggest that use of an CCL2/CCR2 axis inhibitor for prevention rather than treatment of OA is promising for alleviating pain and disability. In addition, we tested whether the combination of CAS and Bin could enhance the preventive effects, but no significant differences were observed. We speculate that: 1) CCL2 is the direct factor that induces CH and when it is inhibited, continued inhibition of CCR2 has no more influence; and 2) there might be a threshold value for inhibiting the CCL2/CCR2 axis that results in a delay in the hypertrophic process of chondrocytes.

The pathology of OA is complicated. Although has been confirmed to be a risk factor for it, our quantitative data elucidated the effects of the CCL2/CCR2 axis and its contribution to hypertrophy of chondrocytes. The significance of the present study is that it illuminated how pretreatment of chondrocytes with an inhibitor of CCL2 or CCR2 protects the chondrocytes from degeneration of CHs and revealed that the underlying mechanism is related to suppression of Type 10 collagen, *IHH*, and *RUNX2* expression.

## Conclusions

In conclusion, our work showed that the CCL2/CCR2 axis plays a crucial role in degeneration of chondrocytes. Blockade of the CCL2/CCR2 axis is meaningful to prevent the hypertrophic progress in chondrocytes, which is embodied in inhibition of Type 10 collagen, *RUNX2*, *IHH*, and apoptosis. Based on these findings, the CCL2/CCR2 axis could be a novel target for preclinical investigation in OA.

## Conflict of Interest

None.

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