CKLF1 interference alleviates IL-1β-induced inflammation, apoptosis and degradation of the extracellular matrix in chondrocytes via CCR5

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Abstract. Osteoarthritis (OA) is a type of joint disease with a rising prevalence and incidence among the elderly across the global population. Chemokine-like factor 1 (CKLF1) is a human cytokine, which has been demonstrated to be involved in the progression of multiple human diseases. However, little attention has been paid to the impact of CKLF1 on OA. The present study was designed to identify the role of CKLF1 in OA and to clarify the regulatory mechanism. The expression levels of CKLF1 and its receptor CC chemokine receptor 5 (CCR5) were examined by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. A Cell Counting Kit-8 assay was used to estimate cell viability. The levels and expression of inflammatory factors were determined by ELISA and RT-qPCR, respectively. Apoptosis was investigated by TUNEL assays and the protein levels of apoptosis-related factors were analyzed by western blotting. RT-qPCR and western blotting were used to examine the expression of extracellular matrix (ECM) degradation-associated proteins and ECM components. Dimethylmethylene blue analysis was used to analyze the production of soluble glycosamine sulfate additive. A co-immunoprecipitation assay was used to confirm the protein interaction between CKLF1 and CCR5. The results revealed that CKLF1 expression was increased in IL-1β-exposed murine chondrogenic ATDC5 cells. Furthermore, CKLF1 silencing enhanced the viability of IL-1β-induced ATDC5 cells, while inflammation, apoptosis and degradation of the ECM were reduced. Additionally, CKLF1 knockdown led to decreased CCR5 expression in IL-1β-challenged ATDC5 cells, and CKLF1 bound with CCR5. The enhanced viability, as well as the suppressed inflammation, apoptosis and degradation of the ECM, following CKLF1 knockdown in the IL-1β-induced ATDC5 cells were all restored after CCR5 was overexpressed. In conclusion, CKLF1 might serve a detrimental role in the development of OA by targeting its receptor CCR5.

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Introduction

Osteoarthritis (OA) is the most common form of arthritis and accounts for pain and disability all over the world (1). OA is defined as a degenerative joint disease contributing to the damage of cartilage and its surrounding tissues (2). It is generally acknowledged that OA is a severe and debilitating disease resulting in a poor quality of life, and imposes a great burden on healthcare systems and costs to society due to the ageing population (3,4). Notably, patients with OA are at an elevated risk of cardiovascular mortality (5). The extracellular matrix (ECM) is regarded as a complex network comprising secreted extracellular molecules, and its degradation is deemed a central hallmark of OA (6,7). A number of studies have highlighted that inflammatory responses and the apoptosis of chondrocytes are associated with the pathology of OA (8-10). Despite much progress being made, the pathogenesis behind OA remains uncertain, as the molecular mechanism implicated in the degradation of cartilage matrix and the progression of OA is complicated (11,12). Therefore, more efforts need to be made to further investigate the underlying mechanism.

Chemokine-like factor 1 (CKLF1) is an unusual member of the chemokine family that was cloned in 2001, with two successive cysteine residues in the sequence (13,14). Mounting evidence has verified that CKLF1 functions as a crucial modulator in human diseases (15,16). For instance, the findings of a study by Liu *et al* (17) suggest that CKLF1 serves an oncogenic role in the tumorigenesis and metastasis of hepatocellular carcinoma. CKLF1 contributes to neointimal hyperplasia via the activation of the NF-κB/vascular cell adhesion molecule 1 signaling pathway (18). Additionally, Pan *et al* (19) proposed that CKLF1 exhibits higher expression in necrotic cartilage tissues compared with normal cartilage tissues. However, the relationship between CKLF1 and OA remains elusive.

CC chemokine receptor 5 (CCR5) is a receptor of CKLF1 (20). Extensive research has revealed that CCR5 participates in the initiation and progression of breast,

gastric, colorectal and pancreatic cancer, in which CCR5 upregulation may elicit pro-tumor effects while CCR5 downregulation may elicit the opposite effects (21-25). Furthermore, the CKLF1/CCR5 axis has been reported to promote neutrophil migration in cerebral ischemia (20). However, whether CKLF1 exerts its functions in OA by mediating CCR5 expression still requires investigation.

The aim of the present study was to evaluate the significance of CKLF1 and to determine the relationship between CKLF1 and CCR5 in OA.

Materials and methods

Cell culture and treatment. The culture medium for the murine chondrogenic ATDC5 cell line (RIKEN BioResource Center) was DMEM/F12 (HyClone; Cytiva) with 5% FBS (Cytiva), 100 U/ml penicillin and 100 μg/ml streptomycin, and cells were routinely maintained in a humidified incubator with 5% CO_2 at 37˚C. To induce inflammatory injury, untransfected or transfected ATDC5 cells were exposed to 10 ng/ml IL-1β for 24 h at 37˚C (26).

Plasmid transfection. The specific small interfering RNAs (siRNAs) targeting CKLF1 (siRNA-CKLF1-1 sense, 5'-UUCACAAAGCAUUUCAGAGUA-3' and antisense, 5'-CUCUGAAAUGCUUUGUGAAGU-3'; and siRNA-CKLF1-2 sense, 5'-UCAUAGAUGUCACAGUUACCA-3' and antisense, 5'-GUAACUGUGACAUCUAUGAUC-3') were provided by Shanghai GenePharma Co., Ltd., with non-targeting siRNA [siRNA-negative control (NC) sense, 5'-AUCGCAACAUAGACAGCUAACAG-3' and antisense, 5'-CUGUUAGCUGUCUAUGUUGCGAU-3'] also from Shanghai GenePharma Co., Ltd., used as an NC. CCR5 overexpression vector (Ov-CCR5) and the empty overexpression vector were constructed by Sangon Biotech Co., Ltd., using the pBluescript vector. The aforementioned plasmids (20 nM) were transfected into ATDC5 cells using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. Cells were harvested at 48 h post-transfection for subsequent experiments.

Cell Counting Kit-8 (CCK-8) assay. In brief, following IL-1β stimulation, 10 μl CCK-8 solution (Bioswamp; Wuhan Bienle Biotechnology Co., Ltd.) was added to the transfected cells (5,000 cells/well) that had been plated into a 96-well plate. After incubation for an additional 1 h at 37˚C, the optical density (OD) value was recorded at 450 nm using a microplate reader (BioTek Instruments, Inc.). Cell viability (%)=(OD treatment-OD blank)/(OD control-OD blank) x100.

TUNEL. Apoptosis was assessed using an *in situ* cell death detection kit (Chemicon International; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Briefly, ATDC5 cells were immobilized with 4% paraformaldehyde for 15 min at room temperature, followed by PBS washing. Then, cells were immersed in 50 μl TUNEL reaction mixture for 1 h at 37˚C and incubated with 0.5 μg/ml DAPI (Beyotime Institute of Biotechnology) for 10 min at room temperature. Finally, after the addition of Antifade Mounting Medium (Beyotime Institute of Biotechnology), the images were captured under a fluorescence microscope (Olympus Corporation) in six random fields of view. The apoptotic rate was calculated as follows: Apoptosis rate=(average number of apoptotic cells/average number of total cells) x100.

ELISA. Following indicated transfection or treatment, the cells were centrifuged at 1,000 x g for 5 min at 4˚C. The supernatant was collected and used for ELISA. Levels of inflammatory cytokines TNF-α and IL-6 were examined using mouse TNF-α ELISA kit (cat. no. ab208348) and mouse IL-6 ELISA kit (cat. no. ab222503) (both Abcam) according to the manufacturer's protocol. The OD value at 450 nm was detected using a microplate reader (BioTek Instruments, Inc.).

Detection of sulfated glycosaminoglycan (sGAG). The production of sGAG was assessed using the dimethylmethylene blue (DMMB) method (27). Cell suspension (20 μl) at the density of $4x10^6$ cells/cm^2 was mixed with 200 μl DMMB reagent (Sigma-Aldrich; Merck KGaA), and the absorbance at 525 nm was recorded using a FlexStation 3 MultiMode Microplate Reader (Molecular Devices, LLC). Total sGAG was normalized to total protein for cell division with the application of the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Extraction of total RNA from cells was carried out using TRI Reagent® (Molecular Research Center, Inc.), and cDNA was synthesized using the SuperScript RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR analysis was conducted with FastStart Universal SYBR Green Master (Roche Diagnostics GmbH) and a 7500c Real-Time PCR Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 10 min, followed by 37 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 1 min, then extension for 10 min at 65˚C. The following primers were used in the present study: CKLF1 forward, 5'-GGCCTTTGCTTATCTCCTTTCC-3' and reverse, 5'-AGCCTAGCAATCTGCTGTCC-3'; TNF-α forward, 5'-ACCCTCACACTCACAAACCA-3' and reverse, 5'-ACCCTGAGCCATAATCCCCT-3'; IL-6 forward, 5'-GCCTTCTTGGGACTGATGCT-3' and reverse, 5'-TGTGACTCCAGCTTATCTCTTGG-3'; MMP3 forward, 5'-CATCCCCTGATGTCCTCGTG-3' and reverse, 5'-CTTCTTCACGGTTGCAGGGA-3'; MMP13 forward, 5'-ACCCAGCCCTATCCCTTGAT-3' and reverse, 5'-TCTTCCATGTGGTTCCAGCC-3'; type II collagen forward, 5'-ATGAGGGAGCGGTAGAGACC-3' and reverse, 5'-GCCCTAATTTTCGGGCATCC-3'; a disintegrin and metalloproteinase with thrombospondin motifs type 4 (ADAMTS-4) forward, 5'-CCTACCTGGATCAGGCGTTC-3' and reverse, 5'-CTCCCAGAAGGAGCCTTGAC-3'; a disintegrin and metalloproteinase with thrombospondin motifs type 5 (ADAMTS-5) forward, 5'-GCAGGGAACATAGGCAGGTT-3' and reverse, 5'-ACCAAACTATTCGGTTAGGCTGA-3'; aggrecan forward, 5'-CATGCTTATGCCTTCCGAGC-3' and reverse, 5'-CTTTCTTCTGCCCGAGGGTT-3'; CCR5 forward, 5'-AGCCGGGAAGGTAGTCTCAT-3' and reverse, 5'-GGGGCGTTCTCCAAAACAAC-3'; and GAPDH forward, 5'-GCCTCCTCCAATTCAACCCT-3' and reverse, 5'-CTCGTGGTTCACACCCATCA-3'.

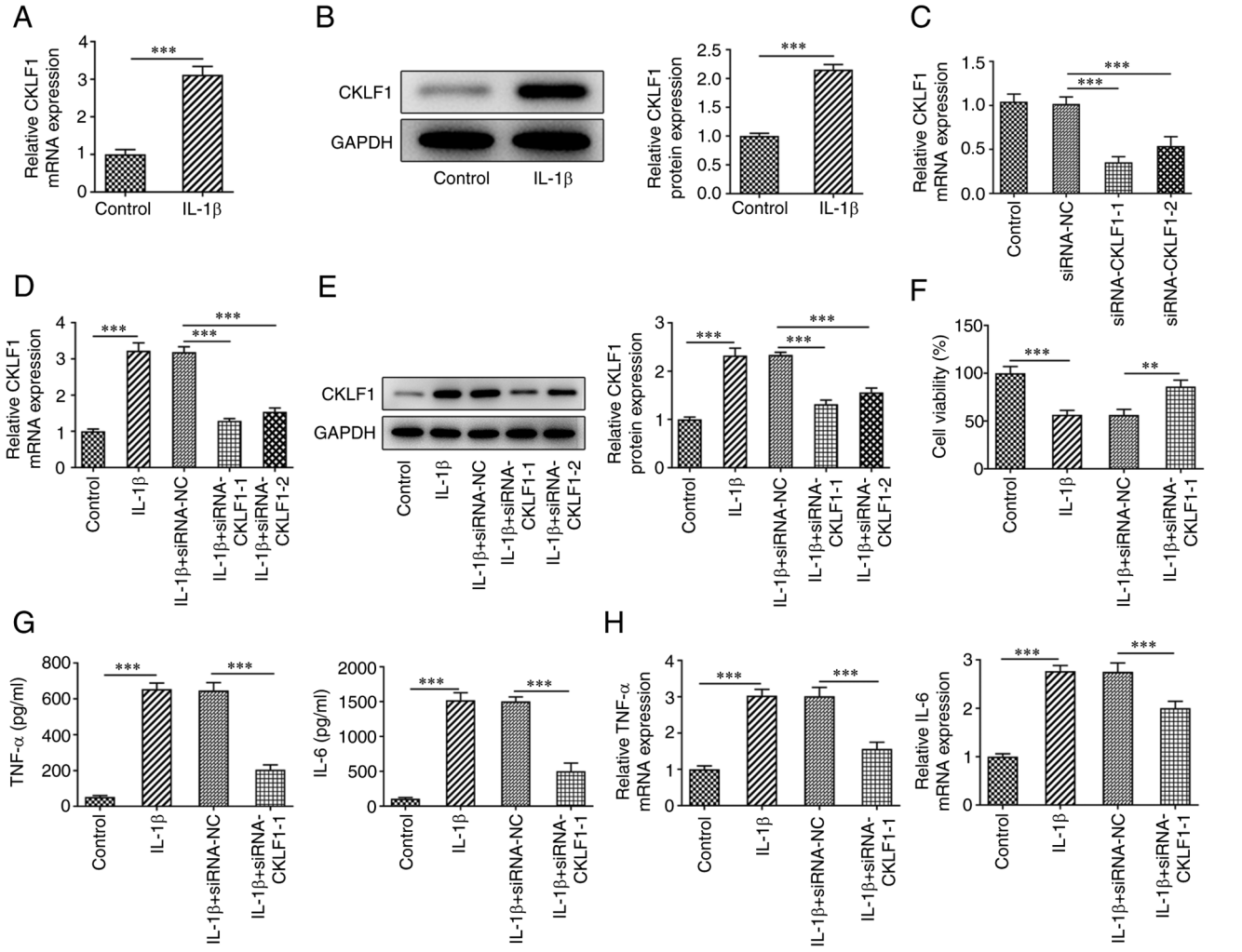

Figure 1. Knockdown of CKLF1 enhances the viability while alleviating inflammation in IL-1β-insulted chondrocytes. (A) RT-qPCR and (B) western blot analysis were performed to examine CKLF1 expression in IL-1β-treated ATDC5 cells. (C) CKLF1 expression as evaluated by RT-qPCR in untreated ATDC5 cells after transfection. The transfection efficiency of siRNA-CKLF1-1/2 in treated cells was also tested by (D) RT-qPCR and (E) western blotting. (F) Viability of IL-1β-treated ATDC5 cells was assessed using a Cell Counting Kit-8 assay. (G) ELISA was performed to determine the levels of TNF-α and IL-6 in IL-1β-exposed ATDC5 cells. (H) RT-qPCR was performed to determine the expression levels of TNF-α and IL-6 in IL-1β-exposed ATDC5 cells. **P<0.01 and ***P<0.001. CKLF1, chemokine-like factor 1; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; NC, negative control.

Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (28) and GAPDH acted as the endogenous control.

Western blotting. The collection and quantification of total protein from cells were performed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and a BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.), respectively. Next, protein samples (25 μg per lane) were electrophoresed on 10% gels using SDS-PAGE and transferred to PVDF membranes (Merck KGaA). After the membranes were incubated with 5% non-fat milk for 2 h at room temperature, non-specific binding was impeded. Subsequently, the membranes were probed with primary antibodies at 4˚C overnight. Anti-CKLF1 (1/1,000; cat. no. ab180512), anti-Bcl-2 (1/1,000; cat. no. ab182858), anti-Bax (1/1,000; cat. no. ab32503), anti-cleaved caspase 3 (1/5,000; cat. no. ab214430), anti-caspase 3 (1/2,000; cat. no. ab184787), anti-MMP3 (1/1,000; cat. no. ab52915), anti-MMP13 (1/3,000; cat. no. ab39012), anti-type II collagen (1/1,000; cat. no. ab34712), anti-ADAMTS-4 (1/1,000; cat. no. ab185722), anti-ADAMTS-5 (1/250; cat. no. ab41037), anti-CCR5 (1/1,000; cat. no. ab65850) and anti-GAPDH (1/2,500; cat. no. ab9485) antibodies were all purchased from Abcam, while the anti-aggrecan (1/1,000; cat. no. bs-1223R) antibody was obtained from BIOSS. On the following day, the membranes were incubated with HRP-conjugated secondary antibody (1/2,000; cat. no. ab6721; Abcam) for 2 h at room temperature. ECL Prime Western Blotting Detection Reagent (Amersham; Cytiva) was employed to visualize the protein bands, and band intensity was determined using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). GAPDH was used as the loading control.

Co-immunoprecipitation (Co-IP). ATDC5 cells were rinsed with pre-cooled PBS at 4˚C for 2 h and dissolved in RIPA lysis buffer (Beyotime Institute of Biotechnology) on ice for 30 min. After centrifugation at 13,000 x g for 10 min at 4˚C, the supernatant of cell lysate (500 μg) was collected and probed with 2 μg IgG antibody, CKLF1 antibody (cat. no. abs138894; Absin Bioscience, Inc.) or CCR5 antibody (cat. no. AM20421PU-N; OriGene Technologies, Inc.,) overnight at 4˚C, followed by

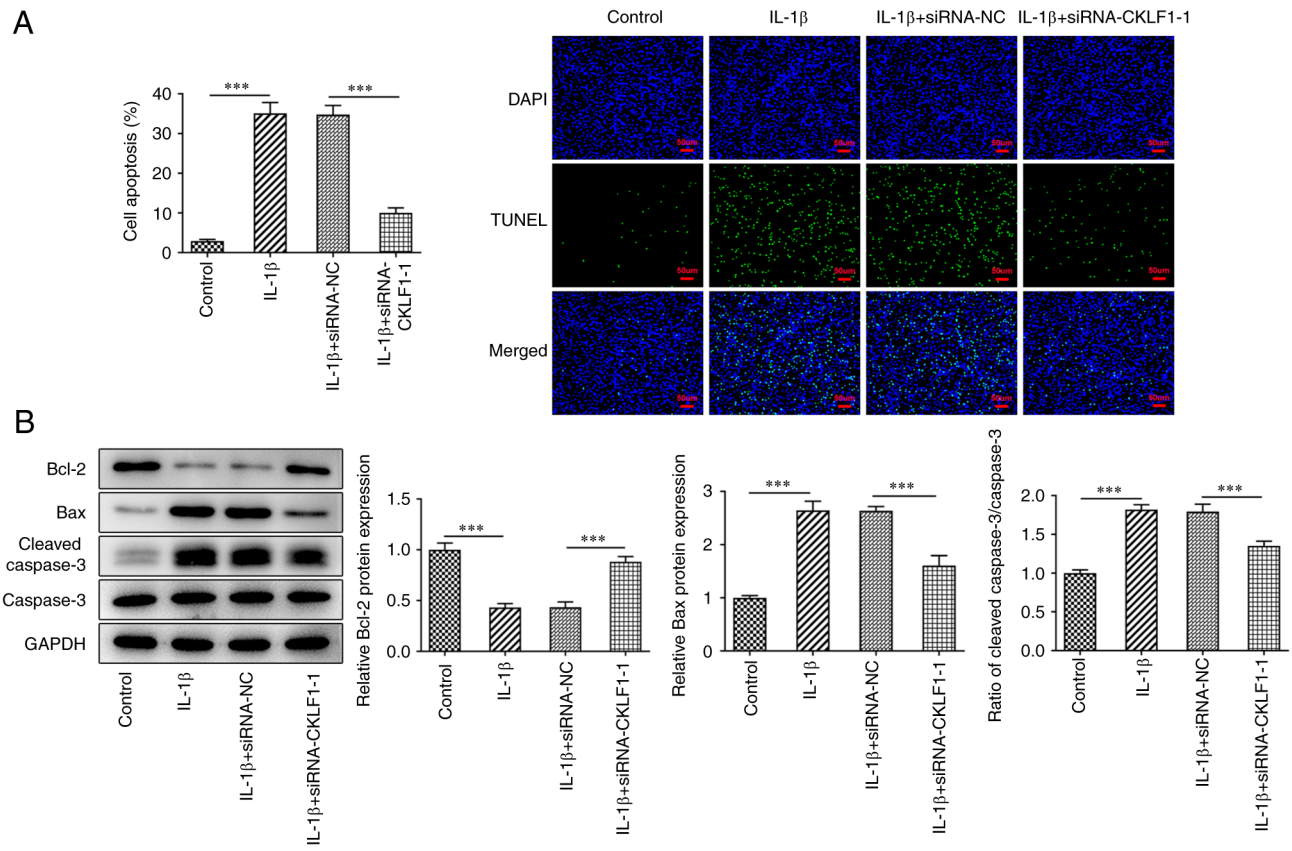

Figure 2. CKLF1 knockdown protects chondrocytes against IL-1β-triggered apoptosis. (A) TUNEL assays were used to estimate the apoptosis of IL-1β-stimulated ATDC5 cells. (B) Protein levels of apoptosis-related factors were analyzed by western blotting. ***P<0.001. CKLF1, chemokine-like factor 1; siRNA, small interfering RNA; NC, negative control.

incubation with 0.2 mg protein A agarose beads (Pierce; Thermo Fisher Scientific, Inc.) at room temperature for an additional 2 h. IgG was used as a negative control. After the IP reaction, agarose beads were centrifuged at 1,000 x g for 3 min at 4˚C to the bottom of the tube. The supernatant was then carefully absorbed, and the agarose beads were washed three times with 1 ml lysis buffer. A total of 15 μl 2X SDS sample buffer was finally added for boiling at 100˚C for 5 min, followed by western blotting as aforementioned.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software; Dotmatics) and data are presented as the mean ± SD of three independent experiments. Unpaired Student's t-test was used for comparisons between two groups, while one-way ANOVA followed by Tukey's test was applied for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of CKLF1 enhances viability while alleviating inflammation in IL-1β-insulted chondrocytes. A previous study has reported that CKLF1 exhibits higher expression in necrotic cartilage tissues compared with normal cartilage tissues (19). To investigate the role of CKLF1 in IL-1β-stimulated chondrocyte injury, CKLF1 expression was analyzed by RT-qPCR and western blotting after ATDC5 cells were stimulated with IL-1β. The results suggested that IL-1β treatment elevated the expression levels of CKLF1 in ATDC5 cells (Fig. 1A and B). To explore the role of CKLF1 in IL-1β-induced ATDC5 cells, CKLF1 was silenced by transfection with siRNA-CKLF1-1 or siRNA-CKLF1-2 and the interference efficiency was examined by RT-qPCR. As shown in Fig. 1C, CKLF1 expression was significantly downregulated in the siRNA-CKLF1-1 and siRNA-CKLF1-2 groups compared with the siRNA-NC group. Further, the elevated CKLF1 expression in IL-1β-treated chondrocytes were decreased following transfection of siRNA-CKLF1-1/2 plasmids, and siRNA-CKLF1-1 was selected for the subsequent loss-of-function experiments due to an improved interference efficiency (Fig. 1D and E). In a CCK-8 assay, the viability of the ATDC5 cells was notably reduced following IL-1β treatment and the suppressed cell viability was restored when CKLF1 was downregulated (Fig. 1F). TNF-α and IL-6 are proinflammatory cytokines (29). The experimental results of ELISA and RT-qPCR analysis demonstrated that TNF-α and IL-6 levels were significantly increased in IL-1β-exposed ATDC5 cells, while the levels were significantly decreased after transfection with siRNA-CKLF1-1 (Fig. 1G and H). In summary, CKLF1 silencing enhanced the viability and decreased inflammation in IL-1β-challenged chondrocytes.

CKLF1 knockdown protects chondrocytes against IL-1β-triggered apoptosis. Dysregulation of apoptosis occurring in osteoarthritic cartilage is responsible for the progression

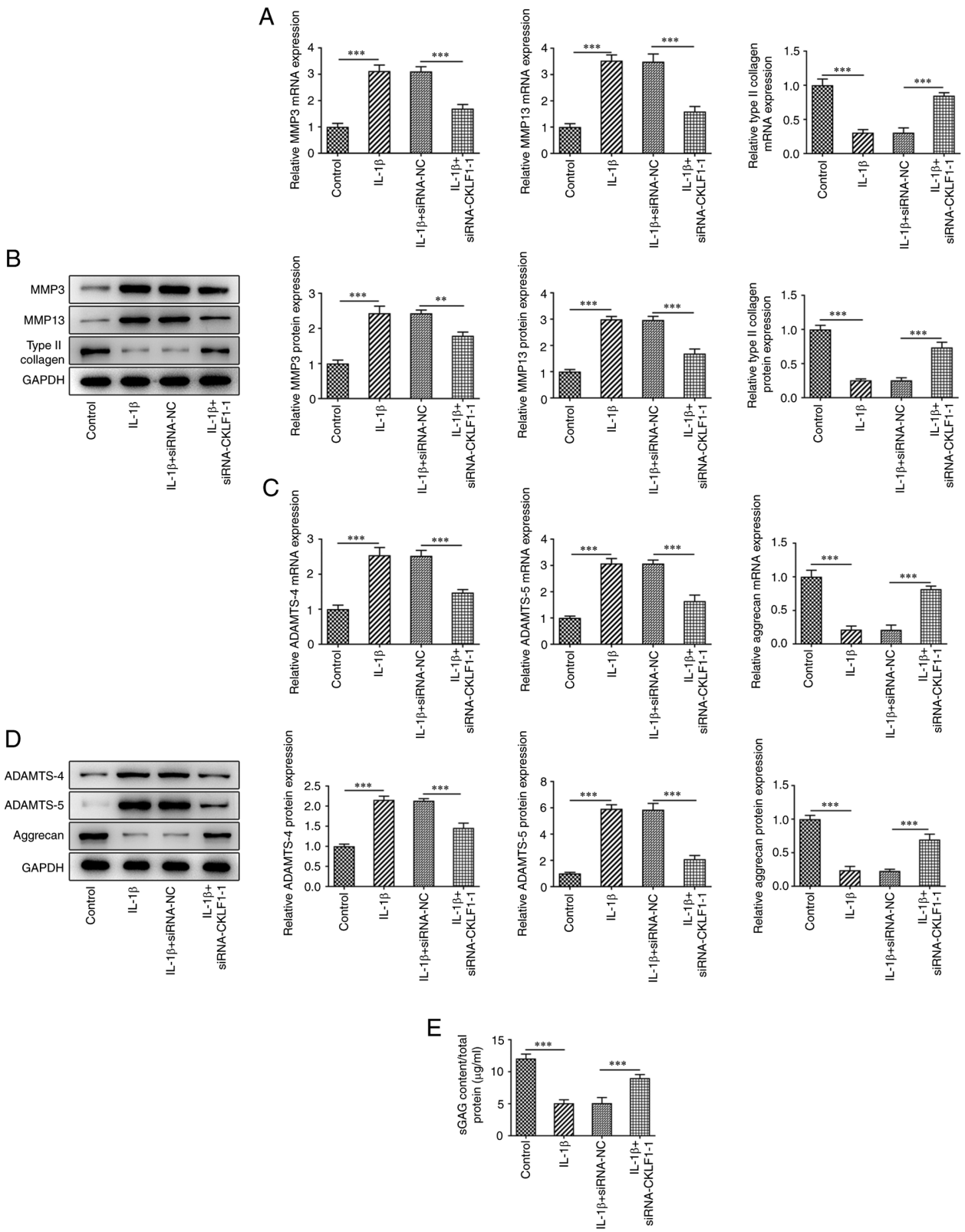

Figure 3. CKLF1 knockdown alleviates IL-1β-induced chondrocyte extracellular matrix degradation. (A) RT-qPCR and (B) western blotting were performed to examine MMP3, MMP13 and type II collagen expression in IL-1β-treated ATDC5 cells. (C) RT-qPCR and (D) western blotting were performed to examine ADAMTS-4, ADAMTS-5 and aggrecan expression in IL-1β-treated ATDC5 cells. (E) sGAG content was analyzed using the dimethylmethylene blue method. **P<0.01 and ***P<0.001. CKLF1, chemokine-like factor 1; RT-qPCR, reverse transcription-quantitative PCR; ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs type 4; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs type 5; sGAG, soluble glycosamine sulfate additive; siRNA, small interfering RNA; NC, negative control.

of OA (10). Therefore, apoptosis was subsequently examined using TUNEL assays and western blot analysis to explore the effect of CKLF1 silencing on IL-1β-induced apoptosis in ATDC5 cells. As shown in Fig. 2A, it was observed that

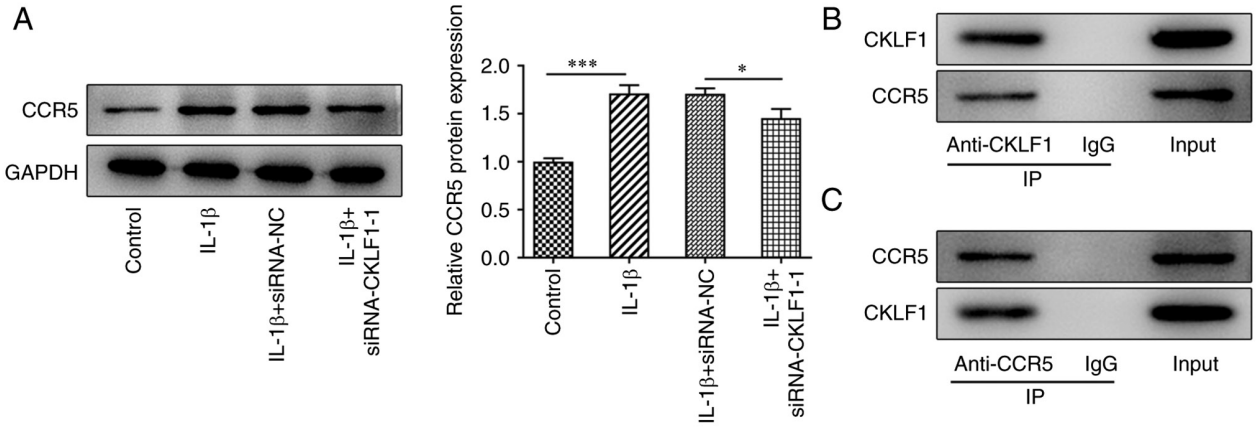

Figure 4. CKLF1 binds to its receptor CCR5. (A) Western blotting was performed to analyze the protein levels of CCR5 in IL-1β-treated ATDC5 cells after CKLF1 was silenced. (B and C) Association between CKLF1 and CCR5 was validated using a co-immunoprecipitation assay. *P<0.05 and ***P<0.001. CKLF1, chemokine-like factor 1; CCR5, CC chemokine receptor 5; siRNA, small interfering RNA; NC, negative control; IP, immunoprecipitation.

IL-1β-challenged ATDC5 cells possessed a higher apoptotic rate compared with the control group. However, CKLF1 knockdown impeded the IL-1β-induced apoptosis in ATDC5 cells. A similar result was also obtained in western blot analysis, which indicated that the downregulated protein levels of Bcl-2 and the upregulated levels of Bax and cleaved caspase3/caspase3 in IL-1β-exposed ATDC5 cells were reversed after CKLF1 was silenced (Fig. 2B). Overall, CKLF1 interference served a suppressive role in IL-1β-triggered chondrocyte apoptosis.

CKLF1 depletion alleviates IL-1β-induced chondrocyte ECM degradation. Type II collagen and aggrecan are the main components of cartilage ECM (30). Furthermore, MMP3, MMP13, ADAMTS-4 and ADAMTS-5 are essential for the degradation of the ECM (31). To determine whether CKLF1 participates in ECM degradation in OA, the expression of MMP3, MMP13, type II collagen, ADAMTS-4, ADAMTS-5 and aggrecan at the mRNA and protein levels was assessed by RT-qPCR and western blot analysis, respectively. The results revealed that IL-1β treatment significantly increased the expression levels of MMP3, MMP13, ADAMTS-4 and ADAMTS-5, while it significantly reduced the expression levels of type II collagen and aggrecan. However, these effects were all counteracted after CKLF1 was knocked down (Fig. 3A-D). In addition, using the DMMB method, sGAG content was revealed to be significantly reduced in ATDC5 cells following IL-1β treatment and to be significantly increased again after transfection with siRNA-CKLF1 (Fig. 3E). To conclude, all of these results suggested that CKLF1 knockdown weakened the IL-1β-induced ECM degradation in chondrocytes.

CKLF1 binds to its receptor CCR5. CCR5 is a receptor of CKLF1 (19). Therefore, subsequent experiments investigated whether CKLF1 could regulate the IL-1β-induced damage in chondrocytes by binding to its receptor CCR5. Western blotting demonstrated that CCR5 was upregulated in IL-1β-stimulated ATDC5 cells. After CKLF1 was silenced, the protein levels of CCR5 were significantly reduced (Fig. 4A). Furthermore, a Co-IP assay verified the interaction between CKLF1 and CCR5 (Fig. 4B and C). Overall, CKLF1 bound with its receptor CCR5.

Silencing CKLF1 reverses the IL-1β-stimulated viability decrease and inflammatory response in chondrocytes by downregulating CCR5 expression. To test the hypothesis that CKLF1 was involved in the development of OA by binding CCR5, CCR5 was first upregulated by transfection with Ov-CCR5 (Fig. 5A). CCK-8 assays revealed that the increased viability of IL-1β-treated ATDC5 cells induced by CKLF1 knockdown was reduced again after CCR5 was overexpressed (Fig. 5B). ELISA and RT-qPCR analysis also demonstrated that CCR5 overexpression reversed the reduced levels of TNF-α and IL-6 that were induced by the knockdown of CKLF1 (Fig. 5C and D). In summary, CCR5 elevation restored the impacts of CKLF1 interference on the viability injury and inflammation in IL-1β-treated chondrocytes.

CKLF1 depletion mitigates the IL-1β-evoked apoptosis in chondrocytes by inhibiting CCR5 expression. Whether CKLF1 could regulate IL-1β-induced apoptosis in chondrocytes by binding to its receptor CCR5 was studied in the following experiments. TUNEL assays revealed that CKLF1 knockdown reduced the apoptosis of IL-1β-treated ATDC5 cells, while this influence was counteracted by CCR5 overexpression (Fig. 6A). As expected, CCR5 overexpression reversed the increased Bcl-2 protein level and the decreased Bax and cleaved caspase3/caspase3 levels caused by CKLF1 knockdown (Fig. 6B). Overall, CKLF1 interference suppressed CCR5 expression to attenuate the IL-1β-enhanced chondrocyte apoptosis.

CKLF1 knockdown alleviates ECM degradation in IL-1β-treated chondrocytes via the suppression of CCR5. To observe the change of ECM degradation in IL-1β-treated chondrocytes with CKLF1 knockdown and CCR5 overexpression, the expression levels of MMP3, MMP13, type II collagen, ADAMTS-4, ADAMTS-5 and aggrecan were determined by RT-qPCR and western blotting. CKLF1 silencing resulted in reduced MMP3, MMP13, ADAMTS-4 and ADAMTS-5 expression, and increased type II collagen and aggrecan expression in IL-1β-exposed chondrocytes; however, this was reversed by overexpression of CCR5 (Fig. 7A-D). Furthermore, the increased sGAG content following CKLF1 knockdown in

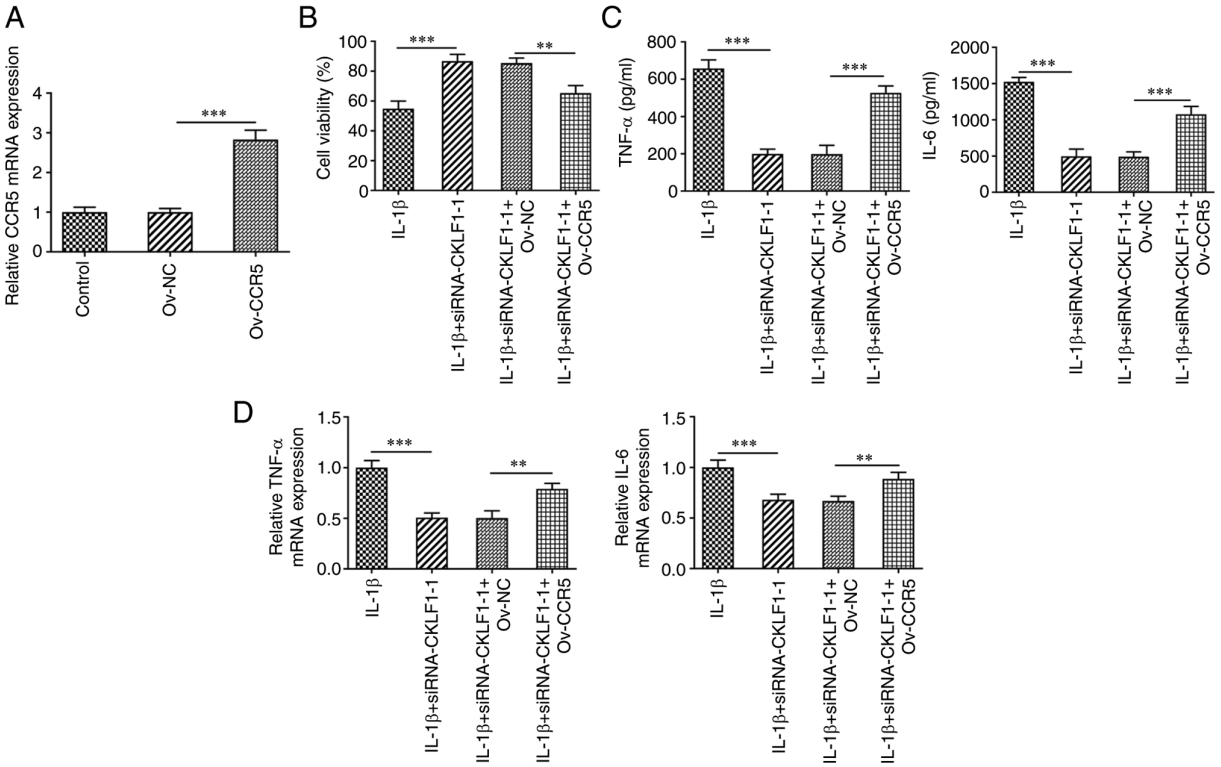

Figure 5. Silencing CKLF1 reverses the IL-1β-stimulated viability decrease and inflammatory response in chondrocytes by downregulating CCR5 expression. (A) Transfection efficiency of the Ov-CCR5 plasmid was examined by RT-qPCR. (B) Viability of IL-1β-treated ATDC5 cells was assessed by Cell Counting Kit-8 assays. (C) ELISA was performed to determine the levels TNF-α and IL-6 in IL-1β-exposed ATDC5 cells. (D) RT-qPCR was performed to determine the expression levels of TNF-α and IL-6 in IL-1β-exposed ATDC5 cells. **P<0.01 and ***P<0.001. CKLF1, chemokine-like factor 1; CCR5, CC chemokine receptor 5; Ov-CCR5, CCR5 overexpression vector; Ov-NC, empty overexpression vector; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

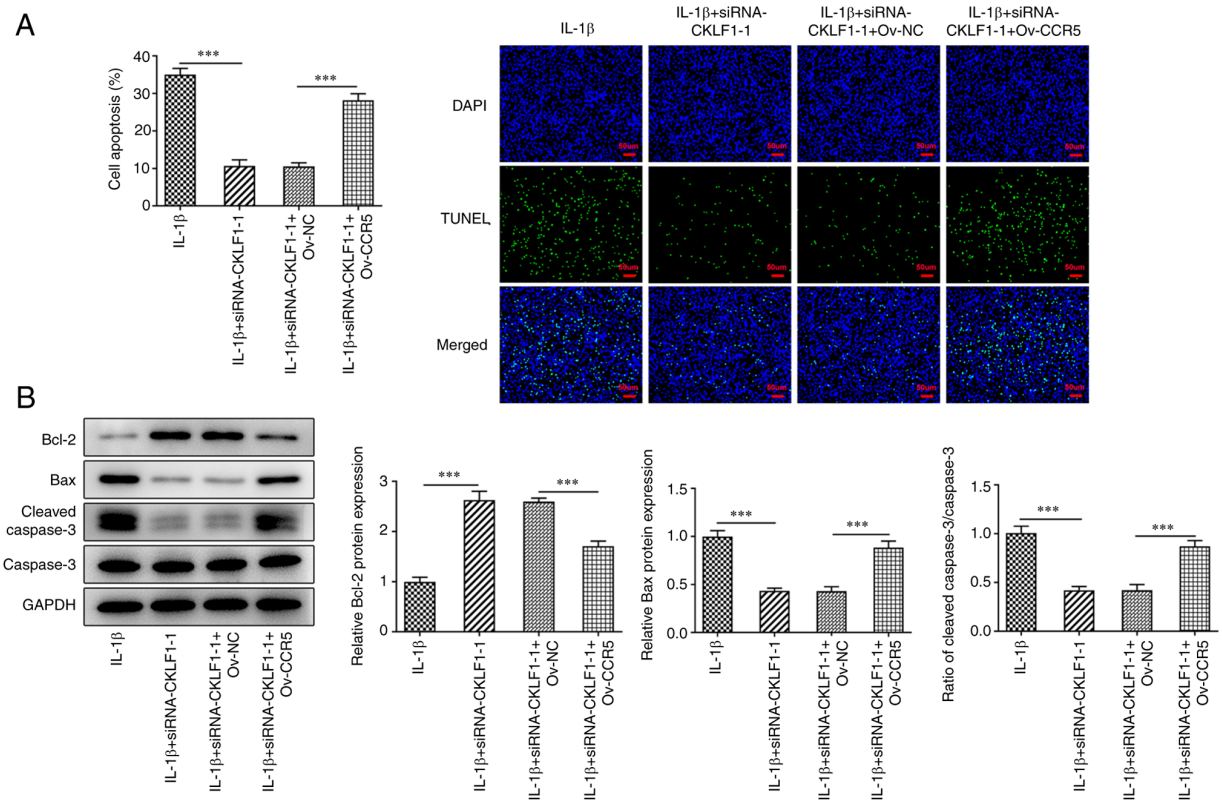

Figure 6. CKLF1 knockdown mitigates the IL-1β-evoked apoptosis in chondrocytes by inhibiting CCR5 expression. (A) TUNEL assays estimated the apoptosis of IL-1β-stimulated ATDC5 cells. (B) Protein levels of apoptosis-related factors were analyzed by western blotting. ***P<0.001. CKLF1, chemokine-like factor 1; CCR5, CC chemokine receptor 5; Ov-CCR5, CCR5 overexpression vector; Ov-NC, empty overexpression vector; siRNA, small interfering RNA.

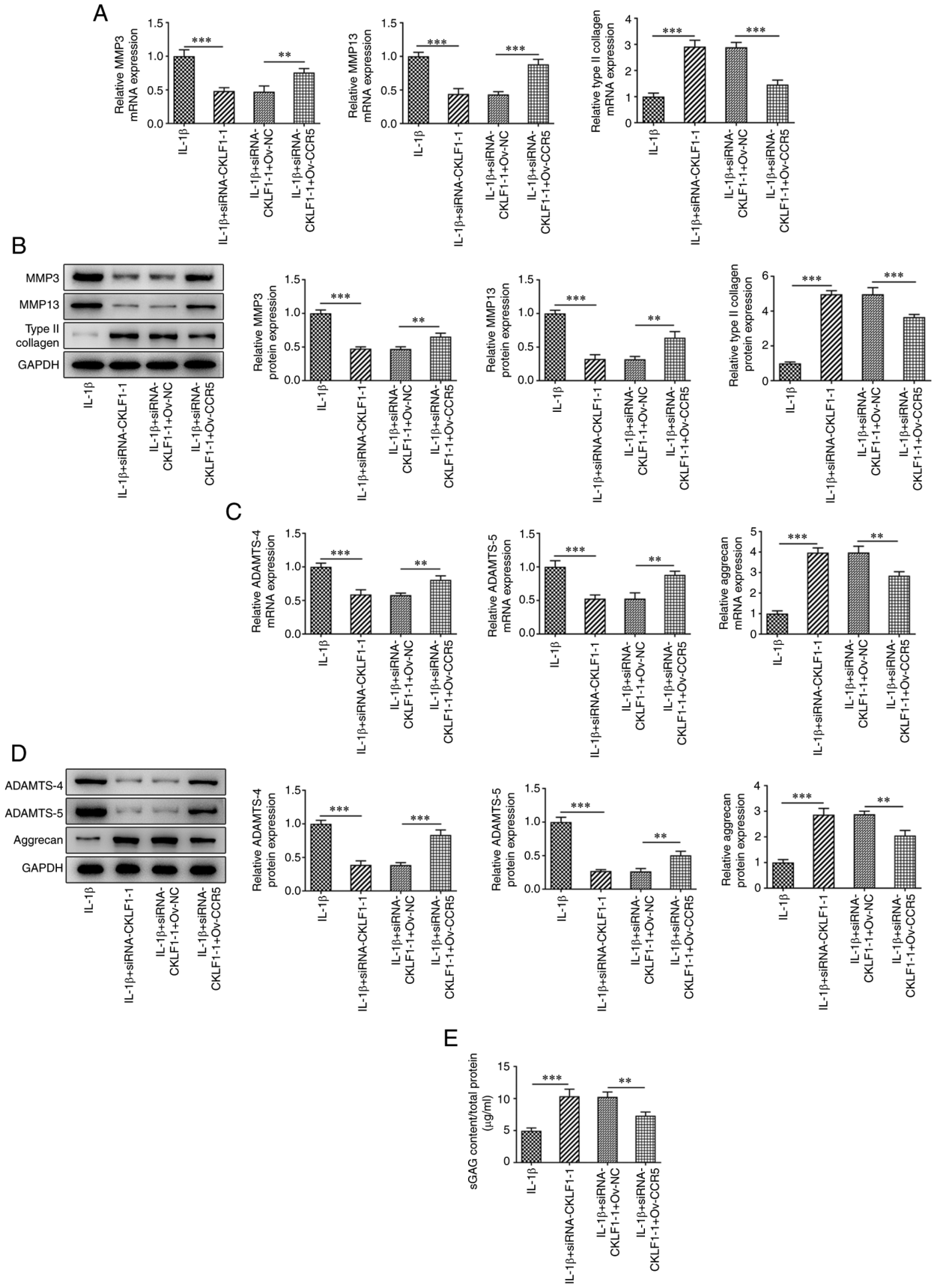

Figure 7. CKLF1 knockdown alleviates extracellular matrix degradation in IL-1β-treated chondrocytes via the suppression of CCR5. (A) RT-qPCR and (B) western blotting were performed to examine MMP3, MMP13 and type II collagen expression in IL-1β-exposed ATDC5 cells. (C) RT-qPCR and (D) western blotting were performed to examine ADAMTS-4, ADAMTS-5 and aggrecan expression in IL-1β-exposed ATDC5 cells. (E) sGAG content was examined using the dimethylmethylene blue method. **P<0.01 and ***P<0.001. CKLF1, chemokine-like factor 1; CCR5, CC chemokine receptor 5; ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs type 4; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs type 5; Ov-CCR5, CCR5 overexpression vector; Ov-NC, empty overexpression vector; RT-qPCR, reverse transcription-quantitative PCR; sGAG, soluble glycosamine sulfate additive; siRNA, small interfering RNA.

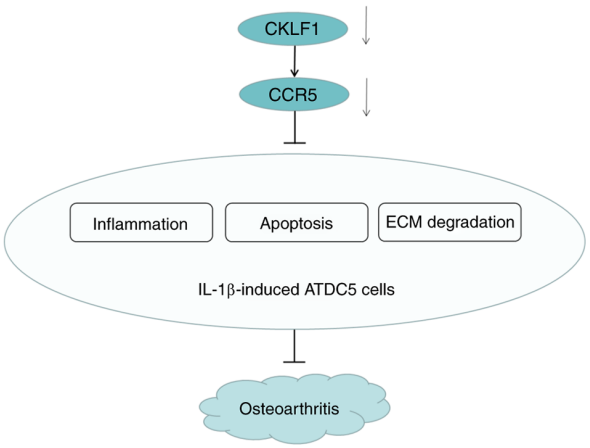

Figure 8. Proposed mechanism of CKLF1/CCR5 in osetoarthritis. The main finding of the present study was that CKLF1 silencing alleviated the IL-1β-induced inflammation, apoptosis and degradation of the ECM in chondrocytes via binding to its receptor CCR5. CKLF1, chemokine-like factor 1; ECM, extracellular matrix; CCR5, CC chemokine receptor 5.

IL-1β-exposed chondrocytes was decreased by CCR5 overexpression (Fig. 7E). Collectively, CKLF1 contributed to ECM degradation in IL-1β-induced chondrocytes by interacting with CCR5.

Discussion

OA has long been acknowledged as a highly prevalent degenerative joint disease, and it is the most commonly diagnosed condition of the musculoskeletal system (11,12). At present, pain management or joint replacement remain the only available treatments for OA due to the complicated factors involved in its onset and development, and the incomplete understanding of the molecular mechanisms (32). There are increasing reports regarding the pathogenesis of OA in terms of the inflammatory response (33,34). The interplay between inflammation and OA has been highlighted by cartilage injury in OA; thus, exploring effective treatments for inflammation has also become a research area in OA therapy (33). Furthermore, dysregulation of apoptosis occurring in osteoarthritic cartilage is also responsible for the progression of OA (10). The ECM is an intricate and specialized three-dimensional macromolecular network (7), and the degradation of the ECM is viewed as a pivotal hallmark of OA (6). IL-1β is recognized as the major catabolic factor in the pathogenesis of OA (34,35), and is frequently utilized to stimulate OA models *in vitro* (26). Therefore, 10 ng/ml IL-1β was employed in the present study to induce an OA model in ATDC5 cells, and then viability, inflammation, apoptosis and degradation of the ECM in OA were evaluated.

CKLF1 is a cytokine that is widely expressed in the human body (13). A growing body of evidence has demonstrated that CKLF1 possesses broad-spectrum biological functions in human diseases (15,16). Furthermore, Tao *et al* (36) demonstrated that CKLF1 has a distinctly increased expression level in patients with OA. Pan *et al* (19) proposed that CKLF1 exhibits higher expression in necrotic cartilage tissues compared with normal cartilage tissues. Accumulating research has revealed that CKLF1 is involved in the inflammation response in hepatocellular carcinoma (16), cerebral ischemia injury (37), psoriasis (38) and renal injury (39). CKLF1 facilitates the degradation of the ECM to exacerbate the process of abdominal aortic aneurysms (40). In the present study, CKLF1 was revealed to be upregulated in IL-1β-treated ATDC5 cells. Functional experiments demonstrated that the attenuated viability of IL-1β-challenged ATDC5 cells was improved again after CKLF1 was knocked down. Additionally, the levels and expression of proinflammatory cytokines (TNF-α and IL-6) were elevated in ATDC5 cells following IL-1β treatment, but restored by CKLF1 interference, which indicated that the IL-1β-induced inflammation in chondrocytes was suppressed by the silencing of CKLF1. Furthermore, the apoptosis of ATDC5 cells triggered by IL-1β exposure was hampered when CKLF1 was knocked down, and was accompanied by elevated Bcl-2 protein levels and decreased Bax and cleaved caspase3/caspase3 levels in the IL-1β + siRNA-CKLF1-1 group compared with those in the IL-1β + siRNA-NC group.

Proinflammatory cytokines, including type II collagen and aggrecan, have been reported to stimulate the degradation of the ECM (41). Additionally, MMPs, including MMP3 and MMP13, which have been determined as main contributors of ECM degradation, are in charge of type II collagen degradation (42). ADAMTS proteases are zinc-dependent metalloproteinases that are connected with the degradation of the ECM, including aggrecan degradation by ADAMTS-4 and ADAMTS-5 (43,44). Furthermore, MMP3, MMP13, ADAMTS-4 and ADAMTS-5 are produced in activated chondrocytes (45). Consistent with these findings, the present study revealed that the expression levels of MMP3, MMP13, ADAMTS-4 and ADAMTS-5 were increased, while the expression levels of type II collagen and aggrecan were decreased in IL-1β-treated chondrocytes. However, these effects were all mitigated by the knockdown of CKLF1. It has been reported that sGAG can bind with ECM-related proteins to direct cellular processes (46). The present study also revealed that the inhibited sGAG content in IL-1β-exposed chondrocytes was restored by CKLF1 knockdown.

CCR5, which was first identified as a human immunodeficiency virus type 1 coreceptor, has been recognized as a receptor of CKLF1 in transient cerebral ischemia (19,47). Furthermore, CCR5 is highly expressed in chondrocytes in the inflammatory environment (48-50). Based on these findings, the present data confirmed that the increased expression levels of CCR5 in IL-1β-induced chondrocytes were decreased after CKLF1 was silenced. Additionally, the present study confirmed the interaction of CKLF1 with its receptor CCR5. In addition, after CCR5 was overexpressed, the stimulated viability and the attenuated inflammation and apoptosis in IL-1β-treated chondrocytes, that had been caused by CKLF1 knockdown, were all reversed. Furthermore, the decreased expression levels of MMP3, MMP13, ADAMTS-4 and ADAMTS-5, and the increased expression levels of type II collagen and aggrecan in IL-1β-treated chondrocytes due to CKLF1 interference, were also restored by CCR5 overexpression.

However, there are a number of limitations of the present study. Only the regulatory effect of CKLF1 and CCR5 on the damage to chondrocytes exposed to IL-1β *in vitro* was discussed. Further *in vivo* experiments will need to be performed in future investigations to support the conclusions

obtained in the present study. Additionally, future studies are required to clarify the expression levels of CKLF1 in OA tissues and the impacts of CKLF1 overexpression on OA.

In summary, the present study revealed that silencing of CKLF1 bound to its receptor CCR5 to ameliorate IL-1β-induced inflammation, apoptosis and degradation of the ECM in chondrocytes, therefore hindering the progression of OA (Fig. 8). To the best of our knowledge, this was the first time that the role of CKLF1 in OA was assessed and the present study was the first to demonstrate an association between CKLF1 and CCR5 in OA. Overall, the findings of the present study may have potential implications for OA therapy. However, future studies are required to further clarify the expression levels of CKLF1 in OA tissues and the role of CKLF1 in OA *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HW and ZW designed the study. HW, ZW and KX performed the experiments and analyzed the data. HW drafted the manuscript and interpreted the data. ZW and KX revised the manuscript for important intellectual content. All authors have read and approved the final manuscript. HW and ZW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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