Chemerin promotes MAPK/ERK activation to induce inflammatory factor production in rat synoviocytes

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Abstract. Chemerin is a chemokine found in adipose tissue that specifically binds to the G protein-coupled receptor, chemokine-like receptor 1, and acts as a chemoattractant for macrophages and dendritic cells. Chemerin levels in the synovial fluid are associated with disease severity in patients with osteoarthritis (OA). However, to the best of our knowledge, the specific mechanism through which chemerin exerts its effects in OA remains unclear. The present study aimed to investigate the underlying mechanism of chemerin-associated synoviocyte inflammation. A Cell Counting Kit-8 assay was used to determine the optimal concentration of chemerin that exerted an effect on the viability of normal rat synoviocytes. The expression levels of MEK/ERK signaling pathway-related marker genes, including MEK, ERK, MMP-3 and MMP-13, were detected using reverse transcription-quantitative PCR. In addition, chemerin-induced phosphorylation of MEK, ERK1/2 and p38 MAPK was analyzed using western blotting, and the production of inflammatory factors following chemerin treatment was determined using ELISA. For the *in vivo* assessment of the effect of chemerin, Sprague Dawley rats underwent knee surgery to establish an arthritis model. The knee joints were then injected with normal saline or recombinant chemerin, and the synovium and knee joint tissues were harvested for H&E histological observations after 3 weeks. In addition, synovial tissue was analyzed for the production of inflammatory factors by ELISA. The results of the present study revealed that chemerin enhanced the viability of synoviocytes in a dose-dependent manner. The stimulatory effect of chemerin on synoviocytes was shown to be accompanied by the activation of MEK, ERK1/2 and p38 MAPK, which was associated with the production of MMP-13, MMP-3, TNF-α, IL-6 and IL-1β by synoviocytes. Inhibition of the ERK1/2 signaling pathway significantly reduced chemerin-induced MMP-13, MMP-3, TNF-α, IL-6 and IL-1β production. H&E staining showed that synovial hyperplasia and articular cartilage wear were more severe in chemerin treated rats after knee surgery than in knee surgery alone and saline controls. In addition, the articular cartilage surface was damaged, and the synovial tissue showed inflammatory cell infiltration. In Sprague Dawley rats that underwent surgery, but did not receive chemerin treatment, a slight raise in inflammatory cell infiltration and increased levels of inflammatory factors were observed compared with rats that did not undergo surgery; however, Secretion of downstream inflammatory cytokines IL-6, MMP-3, MMP-13, and IL-1β was significantly increased in chemerin treated groups compared with control and chemerin + PD98059 groups. In conclusion, the findings of the present study suggested that chemerin may enhance the production of inflammatory factors in synoviocytes by activating the MEK/ERK signaling pathway.

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Introduction

Osteoarthritis (OA) is a chronic degenerative disease involving the presence of synovial lesions, which may be indicative of an underlying inflammatory response, that precedes or eludes identification by X-ray imaging or MRI (1,2). The pathological process of OA involves inflammation of the synovium, which may manifest during the initial stages of synovitis as debilitating pain. Even if patients do not outwardly display clinical symptoms of synovitis, the diseased joints usually exhibit local synovitis, and this inflammatory reaction is most apparent in the adjacent articular cartilage injury area (3,4). It was originally suggested that obese patients were prone to OA due to cartilage destruction caused by biomechanical stress (5). However, evidence has indicated that the large amount of adipose tissue that accumulates in obese patients, which stores energy and produces endocrine response factors, can release a number of cytokines and adipokines that participate in detrimental physiological and pathophysiological processes, such

as immune and inflammatory responses, insulin resistance and tumorigenesis (6). Similarly, a previous study revealed that increased calorie intake in animal models led to both a joint and systemic inflammatory response by promoting the release of adipokines, cytokines and chemokines (7). These findings highlight the importance of inflammatory responses in determining the occurrence and outcome of OA.

Chemerin is a recently identified chemotactic protein that guides macrophages and dendritic cells that express its receptor, chemokine-like receptor 1 (CMKLR1) (8), to inflammatory sites, and is involved in both adaptive and innate immunity (9). Chemerin is expressed in liver cells, white adipose tissue, monocyte-derived macrophages and immature dendritic cells (10). In adipose tissues, chemerin is widely distributed, displays endocrine activity, and has been discovered to play a role in metabolic diseases, such as obesity, type 2 diabetes and cardiovascular disease (11-13). Chemerin levels in human synovial fluid have been found to correlate with OA severity using the Kellgren-Lawrence classification as a criterion (14). Furthermore, CMKLR1 is expressed on articular synoviocytes (15). Chemerin receptors in rats, called G protein-coupled receptors-DEZ, are expressed in hepatocytes, white adipose tissue, monocyte-derived macrophages, immature dendritic cells and synoviocytes (16), and their expression has been reported to be associated with the production of a variety of inflammatory mediators that are released into synovial lesions in OA and affect articular cartilage. Previous evidence has suggested that the MEK/ERK signaling pathway may mediate the inflammatory responses in OA, and MAPKs have been evaluated as potential therapeutic targets (17,18). However, to the best of our knowledge, the mechanisms through which chemerin promotes inflammatory factor production in synoviocytes have not been evaluated. Therefore, the present study aimed to investigate whether chemerin could activate the ERK/MEK signaling pathway, and to determine the profile of inflammatory mediators released by joint synoviocytes in response to chemerin treatment. The results may provide a potential novel approach for the clinical treatment of OA via the targeting of the chemerin-associated MAPK signaling pathway.

Materials and methods

Materials and equipment. Tanon 5200 multi automatic chemiluminescence/fluorescence image analysis system was purchased from Tanon Science and Technology Co., Ltd. The fluorescence inverted microscope was obtained from Olympus Corporation. High-glucose (95%) DMEM was acquired from Beijing Solarbio Science & Technology Co., Ltd. Recombinant murine chemerin was purchased from R&D Systems, Inc. Penicillin/streptomycin and PBS solutions were acquired from Beijing Solarbio Science & Technology Co., Ltd. High performance RIPA lysis buffer solution and western and immunoprecipitation cell lysis solution were purchased from Beyotime Institute of Biotechnology. BCA protein quantification and ECL kits were obtained from MultiSciences (Lianke) Biotech Co., Ltd. The PVDF membrane (0.45 μm) was obtained from MilliporeSigma. Rat MMP-3 (cat. no. SEKR-0067-96T), MMP-13 (cat. no. SEKR-0035-96T), TNF-α (cat. no. SEKR-0009-96T), IL-1β (cat. no. SEKR-0002-96T) and IL-6 (cat. no. SEKR-0005-96T) ELISA kits were acquired from Beijing Solarbio Science & Technology Co., Ltd.

Cell culture. Rat synoviocytes (RSC-364) were purchased from Shanghai Zeye Biotechnology Co., Ltd. and cultured at 37˚C in 95% glucose DMEM supplemented with 10% FBS (Beijing Solarbio Science & Technology Co., Ltd.) and 1% penicillin/streptomycin in a 5% CO_2 incubator. The cells were sub-cultured by rinsing twice with sterile PBS, followed by digestion using Trypsin-EDTA solution (0.25% pancreatin +0.02% EDTA; without phenol red) (Beijing Solarbio Science & Technology Co., Ltd.), which was evenly applied under observation using an inverted microscope. At the point at which 70-80% of the cells had begun to shrink and become round and detached, 95% high-glucose DMEM was rapidly added to the cells under gentle agitation using a sterile Papanicolaou dropper. The cells were subsequently sub-cultured at a ratio of 1:2 or 1:3. The present study was approved by the Ethics Committee of Guangxi Medical University (approval no. 20150303-12; Nanning, China).

For cryopreservation, the cells were transferred into centrifuge tubes and centrifuged to remove the supernatants. The cell pellets were subsequently resuspended in an appropriate amount of cryopreservation solution (containing 20% FBS, 10% DMSO and 70% DMEM). The cells were then dispensed into 1.5 ml cryopreservation tubes, placed in a cryopreservation box at -80˚C, and then transferred to liquid nitrogen for long-term preservation. To recover the cryopreserved cells, cryotubes containing the cells were quickly placed in a 37˚C water bath for re-warming. After thawing, the tubes were placed in a centrifuge (300 x g; 5 min; 37˚C) to remove the supernatant. The cells were then re-suspended in DMEM and transferred to a culture flask. The rat synoviocytes were evaluated experimentally within two to five passages.

Cell Counting Kit-8 (CCK-8) assay. After RSC-364 synoviocytes were passaged three times, the cells were seeded into two 96-well plates at a density of $5x10^3$ cells/well and incubated in a 5% CO_2 atmosphere at 37˚C for 24 h. The plates were subsequently divided in half, and half of each plate was treated with a MEK inhibitor (PD98059; 10 μM; cat. no. 9900S; Cell Signaling Technology, Inc.) at 37˚C for 30 min. Then, 100 μl of 0, 0.25, 0.5 or 1.0 μg/ml Reconstituted Chemerin's medium was added, and the plate was placed back into the incubator. After 24 h of incubation, 10 μl CCK-8 solution was added to each well, according to the instructions of the CCK-8 assay kit (Dojindo Molecular Technologies, Inc.), and further incubated for 4 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Multiskan™ FC; Thermo Fisher Scientific, Inc.). The experiment was repeated three times. The CCk-8 control group was the group without chemerin, and recombinant chemerin was dissolved in distillation-distillation H_2O.

Reverse transcription-quantitative PCR (RT-qPCR). Synoviocytes passaged to the third generation were seeded into a 6-well plate at a density of $4x10^4$ cells/well and cultured for 24 h. The cells were subsequently divided into two groups: The control group and the chemerin group (0.5 μg/ml recombinant murine chemerin). After continuous culture for 48 h, total

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ERK	AACGGTCAGAAAGTGGCGAT	ACGTTCTTTCGGCAGGTCAT
MEK	TCTGCAGTTAACGGGACCAG	AGCTCTAGCTCCTCCAGCTT
MMP-3	CTGGGCTATCCGAGGTCATG	TCCGCTGAAGAAGTAAAGAAACC
MMP-13	CAAGCAGCTCCAAAGGCTAC	TGGATGTGACCGTTTTCGGT
GAPDH	AAGCCCATCACCATCTTCCAGGAG	ATGAGCCCTTCCACAATGCCAAAG

RNA was extracted using an Cell RNA rapid extraction kit (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed using a fluorescence quantitative PCR kit (SYBR Green Master mix; Roche Diagnostics), according to the manufacturer's instructions, on an ABI 7500 Real-Time PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used for GAPDH, MMP-3, MMP-13, MEK and ERK detection are listed in Table I. GAPDH was used as the internal reference gene using the $2^{-\Delta\Delta Cq}$ method (19). All experiments were repeated in triplicate.

Western blotting. Cells passaged for three generations were divided into the control and chemerin groups and seeded into 6-well plates at a density of $4x10^4$ cells/well. After 24 h of attachment, the cells were stimulated with 0.5 μg/ml chemerin for 10 min at 37˚C, prior to the addition of RIPA lysis buffer (containing protease inhibitors). After incubation on ice, the cells were lysed via sonication and centrifuged to obtain the supernatant. The protein concentration was determined using a BCA assay and equal amounts of protein were mixed with loading buffer in a water bath for 5 min. Proteins were separated via electrophoresis, which was initially performed at 80 V and then at 120 V until the bromophenol blue dye had migrated to ~1 cm away from the lower edge of the gel. The proteins were subsequently transferred to PVDF membranes, blocked at room temperature for 1 h under gentle agitation and washed three times with TBS-Tween 20 (TBST) for 5 min each. The membranes were then incubated with the following primary antibodies at room temperature for 2 h or overnight at 4˚C: Rabbit anti-ERK1/2 (cat. no. ab184699; Abcam), rabbit anti-phosphorylated (p)-ERK1/2 (cat. no. 4377T; Cell Signaling Technology, Inc.), rabbit anti-p38 MAPK (cat. no. 8690T; Cell Signaling Technology, Inc.), rabbit anti-p-p38 MAPK (cat. no. 4511T; Cell Signaling Technology, Inc.) and mouse HRP-conjugated GAPDH (cat. no. HRP-6004; ProteinTech Group, Inc.). Following the primary antibody incubation, the membranes were washed three times with TBST for 5 min each time and incubated with HRP-conjugated anti-mouse (cat. no. 7076P2; Cell Signaling Technology, Inc.) or anti-rabbit (cat. no. 7074S; Cell Signaling Technology, Inc.) secondary antibodies for 1 h at room temperature. Protein bands were visualized using ECL reagent and densitometric analysis was performed using ImageJ software (National Institutes of Health). Multiple preliminary experiments were repeated with the conclusion that the best phosphorylation reaction time was when chemerin was added for 10 min, and the performance was most evident at this time point.

Animal studies. The present study was approved by the Ethics Committee of Guangxi Medical University (approval no. 20150303-12; Nanning, China). In total, 30 Sprague Dawley rats were divided into three groups: Groups a, b and ch. Osteoarthritis models were created by modified Huths anterior cruciate ligament transection in groups b and ch (17); anesthesia was achieved with 2% pentobarbital sodium (40 mg/kg). Following surgery, the rats were intraperitoneally injected with 200,000 units of penicillin for 3 consecutive days and permitted to roam freely in the cage. Subsequently, 1 week after modeling, 20 rats were randomly divided into two groups: In group ch, each knee joint was injected with ~0.1 ml solution containing recombinant chemerin once every 3 days for 3 weeks, while rats in group b were injected with the same volume of normal saline. Rats in group a did not undergo surgical modeling, and the knee joints were injected with the same volume of normal saline at the same time as groups b and ch. The housing environment for each group of rats was identical.

Modeling test. After routine disinfection and draping, a longitudinal incision was made in the medial parapatellar region of the knee of the rat, followed by incision of the muscle (along the direction of the incision) to avoid opening the joint capsule. After cutting the muscle, a scalpel was used to separate the muscles and subcutaneous tissues on both sides of the patella and push the patella laterally. The purpose was to separate the patella from the knee joint, destroy their fitted binding, and dislocate the knee joint. Subsequently, the tissue near the joint capsule was separated with ophthalmic scissors, and a transverse incision perpendicular to the skin incision was made at the joint capsule after fully exposing the capsule. After finding the anterior cruciate ligament, the anterior cruciate and medial collateral ligaments were cut. The joint capsule was opened, the medial meniscus of the knee was located, and the muscles and tissues around the medial meniscus were dissected with a scalpel and ophthalmic scissors. Then, the cruciate ligament that fixes the medial meniscus was cut off and the medial meniscus was removed. Subsequently, the drawer test was performed. If the drawer test was positive, it was considered that the model was successfully established. The wound was washed with normal saline, and finally each layer was sutured. Because the muscle at the joint capsule is particularly weak, muscle sutures were made above and below it. The surgical incision was disinfected with iodophor.

H&E staining. A total of 3 weeks post final chemerin or saline injection, the rats were injected with an overdose of 1% sodium pentobarbital (150 mg/kg) for euthanasia. The skin was incised along the middle of the knee joint to expose the entire knee joint. Then, an incision was made from the upper edge of the patella to the femur, and the soft tissue was separated from the tibia with ophthalmic scissors along both sides of the patella. The knee joint cavity was opened, and forceps were used to excise the patella and surrounding tissues. The synovial tissue of the patella that continued downwards under the patella was removed and carefully cut with ophthalmic scissors. Subsequently, the tissues were embedded in paraffin, deparaffinized in xylene and rehydrated in a descending series of ethanol [xylene (I) for 5 min, xylene (II) for 5 min, 100% ethanol for 2 min, 95% ethanol for 1 min, 80% ethanol for 1 min, 75% ethanol for 1 min and distilled water for 2 min]. Then, using a H&E staining kit (cat. no. G1120; Beijing Solarbio Science & Technology Co., Ltd.) the sections were stained with hematoxylin solution for 10 min and rinsed with autoclaved water. Following hematoxylin staining, the sections were incubated with differentiating solution for 30 sec, washed with warm water (~50˚C) for 5 min and stained with eosin dye solution for 2 min, prior to undergoing a final rinse with autoclaved water. Conventional ethanol dehydration was performed step by step [95% ethanol (I) for 1 min, 95% ethanol (II) for 1 min, 100% ethanol (I) for 1 min, 100% ethanol (II) for 1 min, two Toluene carbolic acid (3:1) for 1 min, xylene (I) for 1 min and xylene (II) for 1 min], and the sections were subsequently sealed with neutral resin, covered with a coverslip and observed under a microscope. The above method refers to relevant kits.

ELISA. Sprague Dawley rat synoviocytes were cultured for three passages and then seeded into a 6-well plate at a density of $4x10^4$ cells/well, which were cultured in a 37˚C constant temperature incubator for 24 h. Following the incubation, the cells were divided into three groups: i) Control group (group N); ii) chemerin group (0.5 μg/ml chemerin; group C); and iii) inhibitor group (pretreatment with 10 μM PD98059 for 1 h, then treatment with 0.5 μg/ml chemerin; group P). Following 48 h of treatment, the supernatant was collected (300 x g; 5 min; 37˚C) to determine the levels of MMP-3, MMP-13, TNF-α, IL-1β and IL-6 secreted by the synoviocytes in each group using the corresponding ELISA kits, according to the manufacturer's protocols.

In addition, the synovial tissue was excised from rats according to the aforementioned method, added to sterile PBS (1 g tissue per 10 ml PBS) and mashed. The suspension was centrifuged at 1,000 x g for 10 min to obtain the supernatant, which was used to measure the levels of MMP-3, MMP-13, TNF-α, IL-1β and IL-6 according to the instructions of the respective ELISA kits.

Statistical analysis. Statistical analyses were performed, and data are presented as the mean ± SD. Statistical differences between more than two groups were determined using one-way ANOVA with Tukey's post hoc test, while differences between two groups were determined using unpaired Student's t-test. SPSS software v25.0 (IBM Corp.) was used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

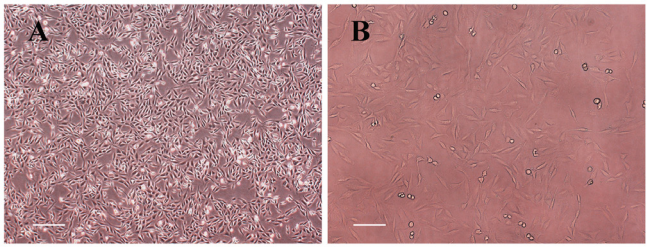

Figure 1. Morphology of RSC-364 synoviocytes. (A) Magnification, x40. Scale bar, 200 μm. (B) Magnification, x100. Scale bar, 80 μm.

Results

Chemerin regulates the viability of synoviocytes in a dose-dependent manner. To determine the effect of chemerin on synoviocytes, RSC-364 cells were used. The morphology of the cells was observed and as expected, the cells were found to be elongated and polygonal in shape (Fig. 1). Subsequently, a CCK-8 assay was performed following 24 h of treatment with different concentrations of recombinant mouse chemerin. The results revealed that after 24 h, chemerin increased viability, which could be prevented by pretreating the cells with the MEK/ERK inhibitor, PD98059 (Fig. 2). The difference in the effects of chemerin at 24 h suggested that the effects of chemerin may be time- and dose-dependent. In addition, the results supported the use of 0.5 μg/ml chemerin as the optimal dose to affect synoviocytes in a 24-h period to use in subsequent experiments, and further indicated that the effect of chemerin may be related to the MEK/ERK signaling pathway.

Chemerin promotes the activation of the MAPK signaling pathways in synoviocytes. Based on the aforementioned findings and given the important role of the MAPK signaling pathways in the pathogenesis of OA (17,18), the present study next sought to determine whether chemerin may regulate the expression of MAPKs. After 48 h of incubation, the mRNA expression levels of MEK and ERK were significantly upregulated in synoviocytes that were cultured in the presence of 0.5 μg/ml recombinant mouse chemerin compared with those observed in the control group (Fig. 3A and B). Furthermore, the mRNA expression levels of MMP-3 and MMP-13 were also upregulated (Fig. 3C and D). MMP-3 and MMP-13 were examined by RT-qPCR assays to investigate whether inflammatory factors were expressed at the gene level and provide a corresponding basis for subsequent ELISA assays.

MAPK activity is regulated by phosphorylation (18). Therefore, the current study also evaluated the levels of total and p-MAPK proteins after exposing the synoviocytes to chemerin for 10 min. Western blotting analysis demonstrated that the expression levels of ERK1/2, p-ERK1/2, MEK, p-MEK and p-p38 MAPK were upregulated following chemerin treatment, while the expression levels of total p38 MAPK were unaltered (Fig. 4). These findings are consistent with the mRNA expression levels observed via RT-qPCR analysis and indicated that chemerin may regulate cell viability by inducing ERK1/2 expression and p38 MAPK phosphorylation.

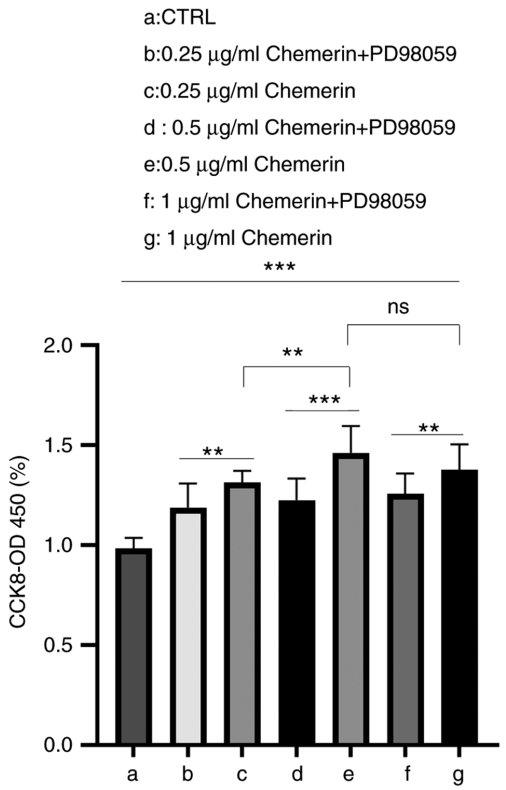

Figure 2. Chemerin affects the viability of synoviocytes in rats. Viability was determined using the CCK-8 assay. Cells were pretreated with the MEK/ERK pathway inhibitor, PD98059, and then treated with chemerin for 24 h. **P<0.01 and ***P<0.001. CCK-8, Cell Counting Kit-8; OD, optical density; CTRL, control; ns, not significant.

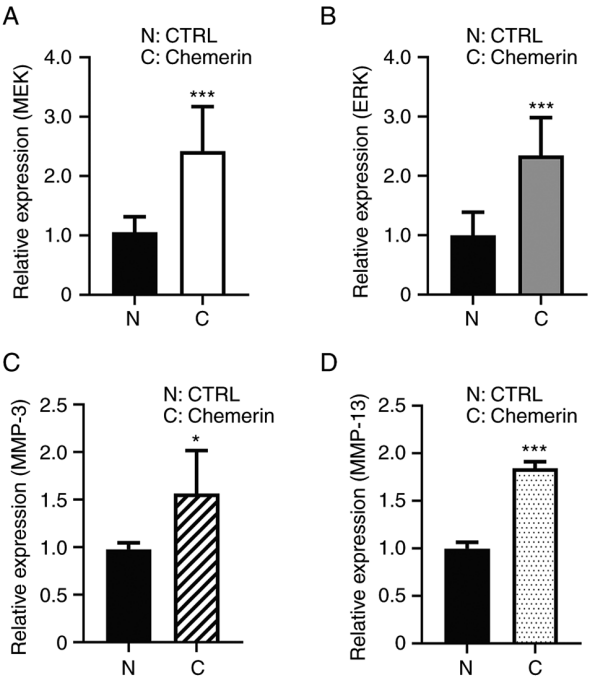

Figure 3. Chemerin upregulates the expression levels of mRNAs involved in the MAPK signaling pathway in RSC-364 synoviocytes. The expression levels of MAPK signaling pathway-related mRNAs were measured using reverse transcription-quantitative PCR and normalized to the expression of GAPDH in group N and group C. (A) Relative MEK, (B) ERK, (C) MMP-3 and (D) MMP-13 mRNA expression. *P<0.05 and ***P<0.001 vs. group N. CTRL, control; N, control synoviocytes, C, synoviocytes exposed to 0.5 μg/ml chemerin for 48 h.

Chemerin upregulates the expression of inflammatory cytokines in a MAPK-dependent manner. Given the role of synovial tissue inflammation in OA progression (3,4), whether chemerin could modulate the production of inflammatory mediators in synoviocytes and whether MAPKs may be involved in this process was further investigated. Cells were stimulated with chemerin for 48 h in the presence or absence of the MEK/ERK pathway inhibitor, PD98059, and ELISAs were performed to evaluate the concentrations of MMP-3, MMP-13, TNF-α, IL-1β and IL-6 in the cell culture supernatants. The results demonstrated that chemerin significantly increased the levels of each of these inflammatory modulators (Fig. 5A-E), and that the increase in MMP-3 and MMP-13 was reduced in synoviocytes pretreated with PD98059 (Fig. 5D and E). The levels of TNF-α, IL-1β and IL-6 were also significantly reduced by PD98059 compared with the chemerin group (Fig. 5A-C). These results suggested the potential role of the MEK/ERK signaling pathway in mediating the activation of MMPs, and potentially other inflammatory mediators, in synoviocytes.

Chemerin promotes synoviocyte inflammatory hyperplasia and the release of inflammatory factors in rats. To determine whether the effects of chemerin could be observed in an *in vivo* model, rats were divided into three groups: Group a (the blank group) did not receive surgery and received injections of saline; group b (the control group) underwent knee surgery to induce arthritis, followed by saline injections; and group ch (the chemerin group) received knee surgery, followed by chemerin injections. Observation of H&E-stained synovial tissue under a microscope revealed that the tissue from the blank group contained one to two layers of synoviocytes arranged regularly, which were mostly flat in shape (Fig. 6). The tissue was relatively loose, with a small number of capillaries and no evident inflammatory cell infiltration; the cartilage surface was smooth and flat, without damage or bone destruction, and no inflammatory cell infiltration could be observed under the microscope. For the control group (Fig. 7), the synovial tissue was more hyperplasic compared with that of the blank group. A small amount of synovial fibroblast proliferation was observed, and inflammatory cell infiltration was identified in the intercellular space. There was also slight cartilage destruction, but no evident bone destruction. Finally, in the chemerin group (Fig. 8), the synovial tissue showed obvious signs of proliferation and an increased infiltration of inflammatory cells (mostly lymphocytes) compared with that of the control group. There was also obvious vascular proliferation, and the continuity of the articular cartilage surface was interrupted, with obvious cartilage defects.

The results of the ELISAs revealed that recombinant mouse chemerin also promoted the production of inflammatory mediators in the synovial tissue. Compared with the control group, each of the assayed inflammatory factors in the chemerin group was significantly increased. In addition, when the blank and control groups were compared, synovial inflammation was observed in the control group; however, the degree of lesions and the levels of inflammatory factors were decreased compared with the chemerin group (Fig. 9). These results suggested that chemerin may activate MMPs

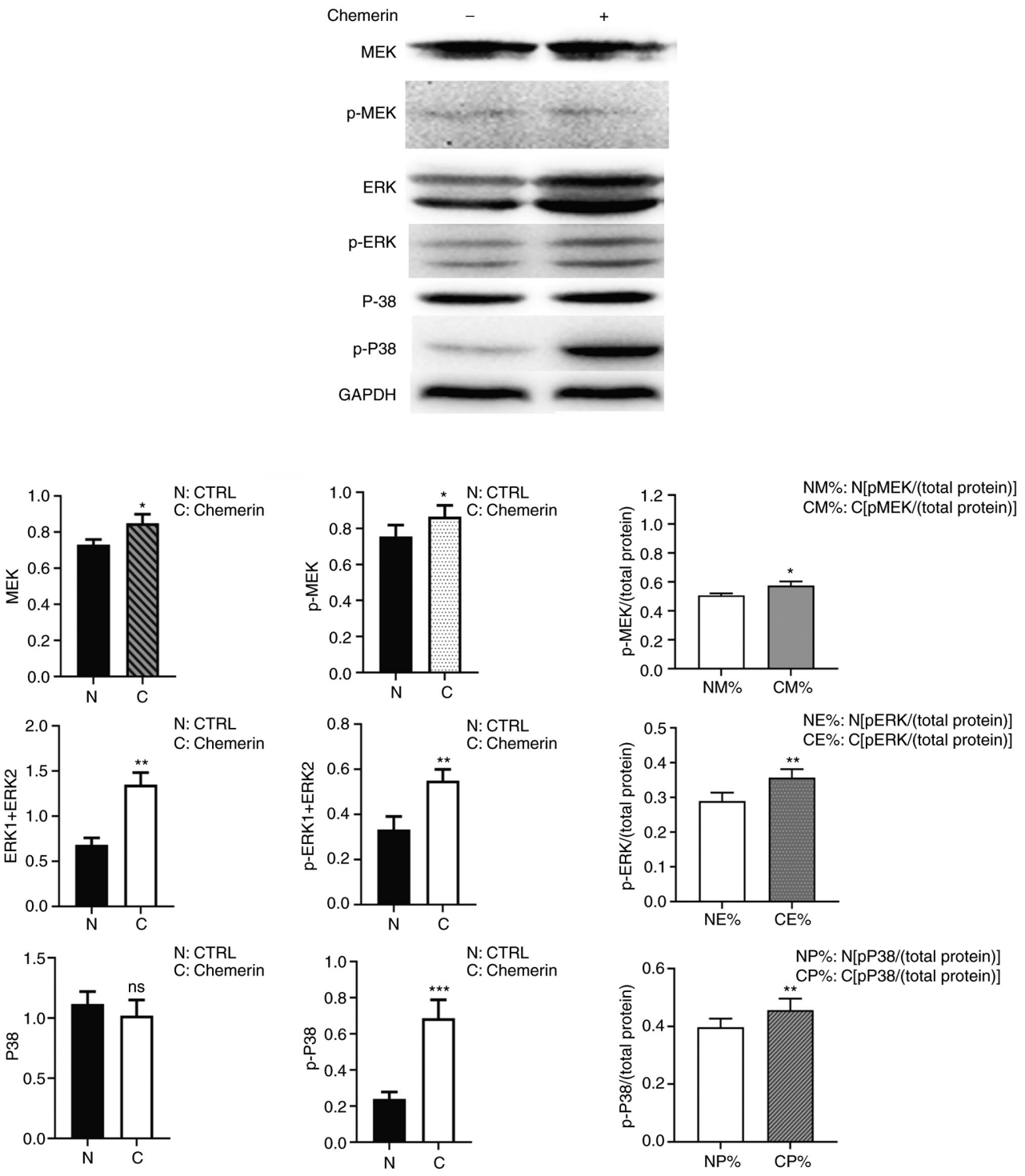

Figure 4. Chemerin upregulates the expression and phosphorylation of MAPK signaling pathway-related proteins in RSC-364 synoviocytes. Western blotting analysis was performed using synoviocytes that were untreated or stimulated with 0.5 μg/ml chemerin for 10 min (indicated by - and + symbols). Semi-quantification of the expression levels is shown. *P<0.05, **P<0.01 and ***P<0.001 indicate that the control group (N, NM%, NE% and NP%) was compared with the respective treated group (C, CM%, CE% and CP%). CTRL, control; p, phosphorylated; ns, not significant; N, control synoviocytes; C, synoviocytes exposed to 0.5 μg/ml chemerin for 10 min.

and potentially other inflammatory mediators in synoviocytes.

Discussion

It has been demonstrated that chemerin is an adipokine with chemotactic activity and its levels, as well as those of other inflammatory factors, have been found to be higher in obese individuals (20). Chemerin was discovered to promote the secretion of TNF, IL-1β, IL-6, MMP-1 and MMP-8 by chondrocytes, and these factors are known to play an important role in the development of OA (21). Furthermore, chemerin has been detected in the synovial fluid of patients with OA, and its expression was found to be associated with the levels

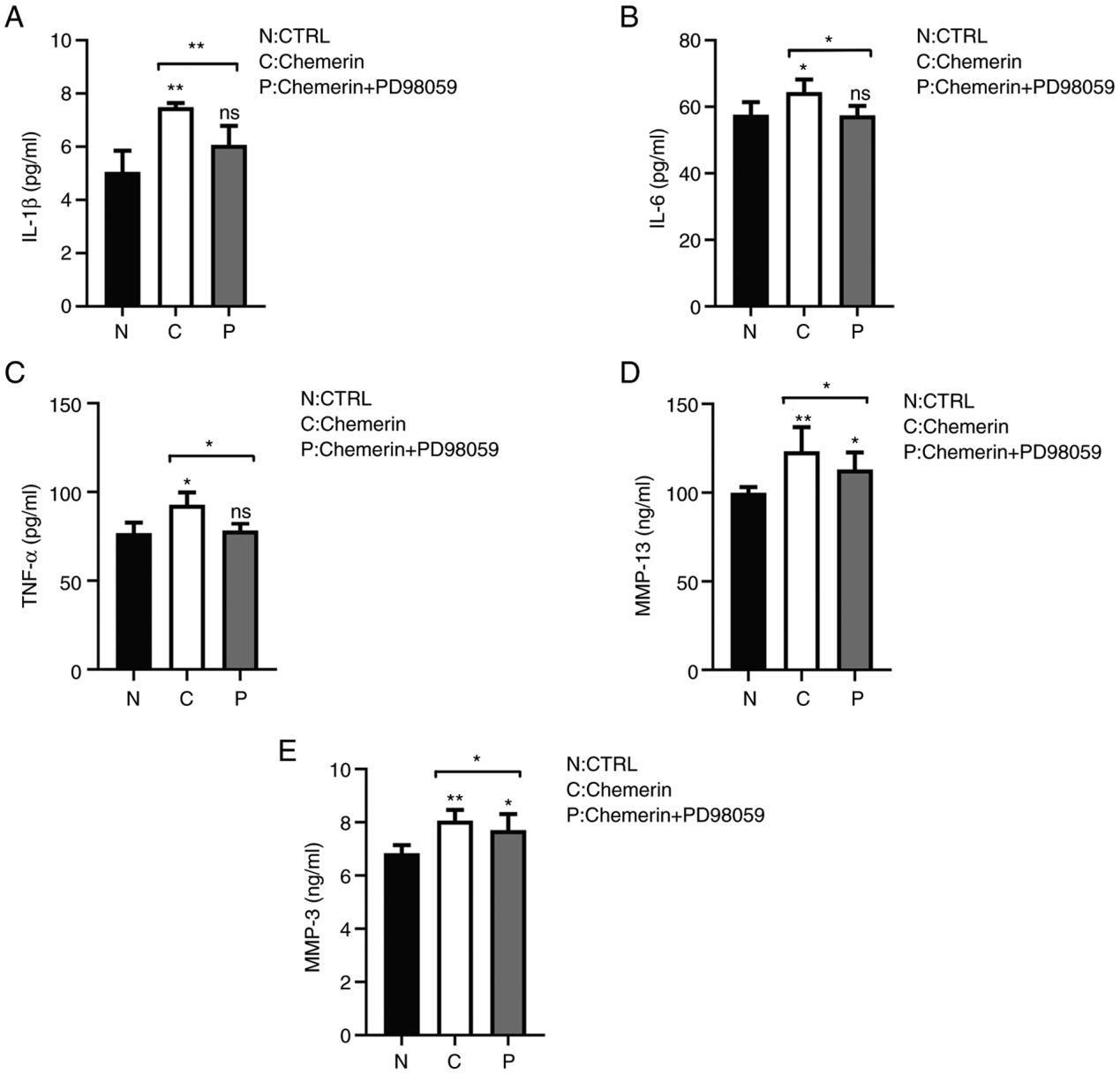

Figure 5. Chemerin enhances the secretion of inflammatory factors in RSC-364 synoviocytes in a MAPK-dependent manner. The levels of MMP-3, MMP-13, TNF-α, IL-1β and IL-6 in synoviocyte supernatants were determined using ELISA after 48 h of culture in the presence or absence of chemerin and the MEK inhibitor, PD98059. Secretory levels of (A) IL-1β, (B) IL-6, (C) TNF-α, (D) MMP-13 and (E) MMP-3 were increased compared with the N group, and decreased following the addition of the inhibitor. Semi-quantification of the expression levels is shown. *P<0.05 and **P<0.001. CTRL, control; ns, not significant; N, blank control group; C, chemerin group; P, chemerin + inhibitor PD98059 group.

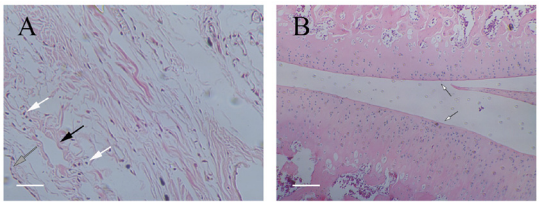

Figure 6. Microscopic observation of synovial tissue and cartilage from the blank group of rats. Rats in the blank group did not undergo surgery and were injected with saline. (A) Microscopic observation of the synovial tissue revealed that the tissue was relatively loose (magnification, x40; scale bar, 200 μm). (B) Cartilage microscopic observation revealed that the cartilage surface was smooth (magnification, x40; scale bar, 200 μm). In Fig. 6A, the arrows point to macrophages, hyperplastic synovial tissue and blood vessels, while in Fig. 6B, the arrows point to the cartilage surface injury. White arrows point to immune cells, black arrows point to vascular tissue and gray arrows point to synovial tissue.

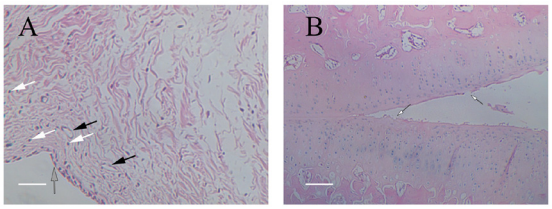

Figure 7. Microscopic observation of synovial tissue and cartilage from the control group of rats. Rats in the control group underwent knee surgery and were then injected with saline. (A) Microscopic observation of the synovial tissue revealed that there were more fibroblast synovial cells compared with the blank group (magnification, x40; scale bar, 200 μm). (B) Cartilage microscopic observation demonstrated that the cartilage surface was slightly damaged (magnification, x40; scale bar, 200 μm). In Fig. 7A, the arrows point to macrophages, hyperplastic synovial tissue and blood vessels; in Fig. 7B, the arrows point to the cartilage surface injury. White arrows point to immune cells, black arrows point to vascular tissue and gray arrows point to synovial tissue.

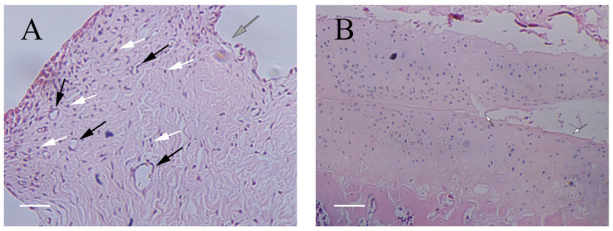

Figure 8. Microscopic observation of synovial tissue and cartilage from the chemerin group of rats. Rats in the chemerin group underwent knee surgery and were then injected with chemerin. (A) Microscopic observation of the synovial tissue revealed the proliferation of fibroblasts and the presence of lymphocyte infiltration (magnification, x40; scale bar, 200 μm). (B) Cartilage microscopic observation revealed cartilage surface defects (magnification, x40; scale bar, 200 μm). In Fig. 8A, the arrows point to macrophages, hyperplastic synovial tissue and blood vessels; in Fig. 8B, the arrows point to the cartilage surface injury. White arrows point to immune cells, black arrows point to vascular tissue and gray arrows point to synovial tissue.

of inflammatory factors (22). Therefore, we hypothesized that chemerin may regulate the secretion of inflammatory factors in synoviocytes.

The results of the present study provided direct evidence that chemerin may promote the production of inflammatory mediators in synoviocytes using ELISAs to determine the secretory levels of a variety of inflammatory cytokines and chemokines, including MMP-3, MMP-13, TNF-α, IL-1β and IL-6. A previous study suggested that systemic MMP-13 may play a role in knee OA and may be regulated by inflammatory signaling (23), thus supporting the relevance of the findings of the present study. Furthermore, TNF-α, IL-1β and IL-6 have each been shown to increase the expression levels of the receptor for chemerin, CMKLR1 (24), which suggested the existence of a positive feedback loop that may exacerbate inflammation in OA and other inflammatory conditions. In addition, the current *in vivo* experimental results demonstrated that chemerin promoted synovial inflammatory hyperplasia and cartilage damage, and also increased the number of inflammatory cells and inflammatory factors secreted. While the pathogenesis of OA involves the degeneration of fragments and particles of cartilage, inflammatory responses in the synovium, including the release of inflammatory mediators by synovial lesions, are known to aggravate articular cartilage destruction, contributing to a vicious cycle that leads to disease progression (25). Therefore, the results of the present study provided evidence supporting a model in which chemerin functionally interacts with inflammatory factors and amplifies their ability to mediate cartilage degeneration.

MAPK signaling is also known to play an important role in the pathogenesis of OA (25). In the present study, the results indicated that chemerin activated MAPK expression in synoviocytes at both the transcriptional and post-transcriptional level. In addition, pretreatment with a MAPK pathway-specific inhibitor attenuated the induction of MMP-3, MMP-13, TNF-α, IL-1β and IL-6 by chemerin. These results indicated that chemerin may induce or exacerbate the development of OA by regulating the MEK/ERK signaling pathway, thereby affecting the secretion of inflammatory factors by synoviocytes. Furthermore, animal osteoarthritis models were established via the modified Huths anterior cruciate ligament transection modeling method, which has been traditionally used for the modeling of large animals and subsequently, small mammals, including rabbits, rats and mice, which are now considered to be the most widely used models (26,27). This method has been found to induce events associated with OA in the knee joint of animals, including synovial inflammation, changes in chondrocyte physiology and destruction of the cartilage and osteophyte formation. Using this model, the anterior cruciate ligament is completely transected after direct observation through arthrotomy (medial or lateral) or arthroscopic surgical methods, causing joint instability, thereby leading to the occurrence of OA (28).

In conclusion, the findings of the present study suggested that chemerin may play an important role in obesity-induced OA, and that it may exert its effect via the activation of the MAPK signaling pathway in synoviocytes, which subsequently leads to the production of inflammatory mediators that cause cartilage degeneration. These results support the potential of chemerin to serve as a biomarker of disease severity. In animal experiments, preliminary experiments were repeated several times, and it was finally concluded that chemerin injection should be optimally performed every 3 days, while the third injection response was the most pronounced. Since synovitis is most apparent in the early stages of osteoarthritis, if the injection time is prolonged and the joint damage is severe, synovitis will be relatively attenuated. Furthermore, in the modified Huths model, in order to make the joints wear and tear normally, the mice are usually allowed to move after the model is completed. This is done to model the wear and tear of joints in humans due to factors associated with walking in daily life.

However, the current study has certain limitations. Firstly, the study used surgical modeling to establish a rat OA model, which to a certain extent differs from the pathogenesis of human OA, therefore the effect of surgery-induced trauma cannot be excluded. Secondly, the present study used Sprague Dawley rats as the animal model, whose joints are small; thus, biomechanical factors, such as body weight, will cause varying extents of joint destruction, thereby leaving the potential for certain errors to occur in the experimental results. Thirdly, regarding the experimental design, the current study only focused on the effect of MEK/ERK in the MAPK signaling pathway and the inflammatory response in synoviocytes; therefore, the findings may only relate to part of the mechanism of action of chemerin on the bone and joints. The inflammatory factors measured were the main inflammatory factors associated with synovitis, which have certain hallmarks. However, these inflammatory factors are not comprehensive, and subsequent related studies are required. In addition, the upstream and downstream mechanisms affecting chemerin expression were not investigated. Therefore, future studies will aim to explore the upstream and downstream mechanisms affecting chemerin and whether chemerin exerts an effect in OA through other signaling pathways. Moreover, cartilage differentiation of stem cells has been studied in regenerative medicine; however, whether chemerin has an effect on this process currently remains unknown, which is another direction of further research. Finally, following the expansion of research into

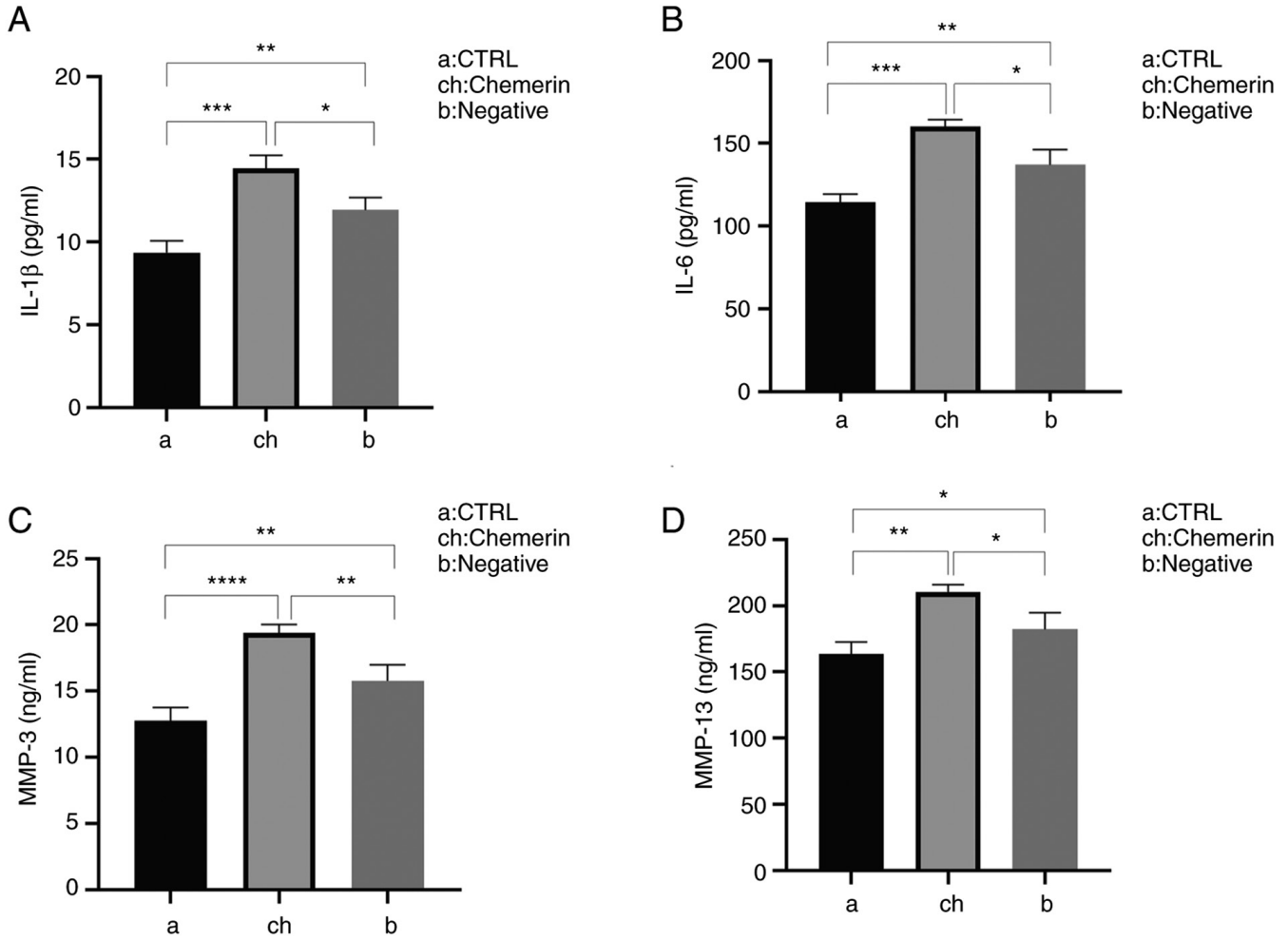

Figure 9. Inflammatory factor levels in rats after knee surgery and/or chemerin injection, determined using ELISA. Secretory levels of (A) IL-1β, (B) IL-6, (C) MMP-3 and (D) MMP-13 were significantly increased in the ch group compared with the a and b groups, but to the greatest extent compared with the a group. *P<0.05, **P<0.01, ***P<0.001 and ****P < 0.001. CTRL, control; a, blank (saline) group; ch, model (surgery + chemerin) group; b, control (surgery + saline) group.

adipokines, it was found that they may play an important role in obesity-induced OA; therefore, adipokines may have the potential to be used as a biomarker to reflect the severity of the disease, as well as help the clinical monitoring and intervention of early OA in obese patients. Altogether, adipokines may play an active role in improving the current diagnosis and treatment of OA, which provides a novel insight into the potential treatment strategies to render the early prevention of OA more achievable, thereby prolonging the mechanics of human autologous joints.

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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

SL conceived the study. CW and SZ designed the study. CW, SZ, LH and JL performed the data analysis. SL acquired the funding, and SL and GD supervised the study. CW and SZ confirm the authenticity of all the raw data. SL, CW, SZ, LH, JL, QZ and GD helped to interpret the data, write and review the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Ethics Committee of Guangxi Medical University (approval no. 20150303-12; Nanning, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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