Anti-inflammatory effects of aucubin in cellular and animal models of rheumatoid arthritis

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\[\text{ABSTRACT}\]
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease. It is known that aucubin (AU) exerts anti-inflammatory activity, but its effects and mechanisms in RA are unclear. This study investigated the anti-inflammatory effects and mechanisms of AU \textit{in vivo} and \textit{in vitro}. Human fibroblast-like synoviocyte cells from patients with RA (HFLS-RA), RAW264.7 cells, and MC3T3-E1 cells were used to evaluate the effects of AU on migration, invasion, apoptosis, osteoclast differentiation and production. Immunofluorescence was used to observe nuclear translocation of nuclear factor (NF)-\textit{κ}B, the double luciferase reporter gene method was used to observe NF-\textit{κ}B-p65 activity in AU-treated MC3T3-E1 cells. RT-qPCR was used to measure expression of bone metabolism and inflammation-related genes, and western blot was used to measure bone metabolism and NF-\textit{κ}B protein expression levels. Collagen-induced arthritis (CIA) rat model was used for pharmacodynamics study. Arthritis indexes were measured in the ankle and knee, histological staining and Micro-computed tomography were performed on the ankle joints. Also, inflammatory factor gene expression and the levels of NF-\textit{x}B-related proteins were detected \textit{in vitro}. AU significantly reduced the gene expression levels of three cell-related inflammatory factors and bone metabolism factors, effectively inhibited the expression of p-\textit{κ}αβ, p-\textit{κ}B, and p-p65 proteins. \textit{In vivo}, AU relieved joint inflammation, reduced related inflammatory factors, and inhibited NF-\textit{x}B signaling. It could be used to treat RA-related synovial inflammation and bone destruction through the NF-\textit{x}B pathway.

\[\text{KEY WORDS}\] Aucubin; Collagen-induced arthritis; Synovial inflammation; Bone erosion; NF-\textit{x}B signaling pathway

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Introduction
Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint lesions. The main pathological features are involvement of the hand and foot joints, including tenosynovitis and bone and cartilage destruction, which can impair joint function and cause severe disability. RA pathogenesis has not been fully elucidated, and treatment mainly focuses on anti-inflammatory effects and immune regulation \cite{1,2}. Modern pharmacological studies revealed that synovial hyperplasia and inflammation are significant features in disease development, as well as the pathological basis of other secondary lesions (bone and cartilage destruction) \cite{3}, in which fibroblast-like synoviocytes (FLS) play a key role \cite{4}. Following synoviosis, RA further erodes bone and cartilage tissue through the actions of two types of cells: osteoclasts (OCs) that absorb bone matrix and osteoblasts (OBs) that synthesize bone matrix \cite{5}. In RA, it is believed that OCs outnumber OBs, which leads to bone destruction when the tissue is absorbed by OCs \cite{6,7}.

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[⁎] These authors have no conflict of interest to declare.
Aucubin (AU, Fig. 1) is an iridoid component that is a secondary metabolite of widely distributed plants; it is the active ingredient of traditional Chinese medicine such as *Eucommia ulmoides* Oliv., *Plantago asiatica* L., and *Rehmannia glutinosa* Libosch. Recent studies reported that AU has anti-oxidation, anti-inflammatory, and anti-fibrosis effects [8, 9]. The anti-inflammatory effect of AU is related to its inhibition of nuclear factor (NF)-κB, which can inhibit the protein’s activity and reduce the expression of TNF-α by inhibiting IκBα phosphorylation [10]. AU has a protective effect on mouse articular cartilage and delays OA progression [11]. This protective effect may be related to the inhibition of chondrocyte apoptosis and excessive production of reactive oxygen species (ROS). AU can also significantly inhibit the expression of matrix metalloproteinase (MMP) and nitric oxide (NO) production in rat chondrocytes stimulated by interleukin (IL)-1β, and inhibit IL-1β-mediated p65 phosphorylation and nuclear translocation [12, 13]. AU is contained in the leaves, bark, and seeds of *E. ulmoides*. We previously reported that the alcohol extract and ethyl acetate part of *E. ulmoides* could improve ankle joint pathology in collagen-induced arthritis (CIA) rats, inhibit joint inflammation and bone destruction, reduce inflammatory factor release, and balance OC/OB metabolism [14, 15]. As the main component of *E. ulmoides*, whether AU itself has anti-RA effects remains to be proved. RAW264.7 cells are mouse mononuclear macrophage cell lines that can be differentiated into OCs [16], while MC3T3-E1 cells are mouse embryonic OB precursors and a very important model for studying OBs. We performed *in vitro* experiments to examine the effect of AU on the proliferation and apoptosis of human FLS cells from patients with RA (HFLS-RA), OC transformation of RAW264.7 cells, and bone metabolism in MC3T3-E1 cells. An *in vivo* CIA arthritis rat model was established by injecting bovine type II collagen into Wistar rats, which has similar immunological and pathologic characteristics to human RA [17], to observe the effect of AU on joint inflammation and bone destruction.

**Methods**

**Main reagents and antibodies**

AU was purchased from Yuanye Inc. (Shanghai, China), the purity of AU was more than 98% by HPLC. Tripterygium glycosides (TG) were purchased from Shanghai Fudan Fuhua Pharmaceutical Co., Ltd. (Shanghai, China; Z31020415). Human TNF-α was purchased from Peprotech (Rocky Hill, NJ, USA; 315-01A). DMEM was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China; MA0212-1). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA; 141). Anti-NF-κB-phospho-p65, anti-phospho-IκBα, and anti-phospho-IκBα receptor antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA; 3033, 2697, 2859). EZ-press RNA Purification Kits, 4 × Reverse Transcription Master Mix, and 2 × SYBR Green qPCR Master Mix were purchased from EZBioscience (EZBioscience, Roseville, MN, USA; B0004DP, A0010CGQ, A0012-R2). Glacial acetic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China; 20181008). Freund’s Adjuvant, Incomplete (FIA) was purchased from Sigma-Aldrich (St. Louis, MO, USA; F5506). M.Tuberculosis Des.H37 Ra (MTB) was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA; 0052519). Immunization Grade Bovine Type II Collagen, Lyophilized (CII) was purchased from Chondrex (Redmond, WA, USA; 2022). Type II Collagen, Lyophilized (CII) was purchased from Chondrex (Redmond, WA, USA; 2022).

**Cell culture**

HFLS-RA and MC3T3-E1 cells were purchased from Huatuo Biotechnology Co., Ltd. (Guangzhou, China). RAW264.7 cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

**Animals**

Twenty-four female rats (120 ± 10 g) were purchased from Beijing CRL Laboratory Animal Co., Ltd. (Beijing, China); the animal certificate number was SCXK (Beijing) 2016-0011. All animal procedures were performed according to the ethical guidelines of the Laboratory Animal Welfare and Animal Experimental Ethics Committee of Shanghai University of Traditional Chinese Medicine (Approval number: PZSHUTC19011101).

**CCK-8 assay**

HFLS-RA, RAW264.7, and MC3T3-E1 cells were pre-incubated for 24 or 48 h with AU at various concentrations (0, 10, 20, 40, 60, 80, 100, 120, 160, 200 μmol·L⁻¹). Then, 10% CCK8 medium was added to the 96-well plates, and a microplate reader was used to measure the absorbance at 450 nm. The relative cell viability rates are expressed as a proportion (%) of the control group.

**HFLS-RA cell migration and invasion assays**

An ibidi insert was placed in a 24-well culture plate. HFLS-RA cells were then inoculated into the insert and either left untreated (as negative controls) or supplemented with various concentrations of AU (20, 40, 80 μmol·L⁻¹) and TNF-α (20 ng·mL⁻¹) for 24 h. The scratches were photographed by inverted microscope at 0 h and 24 h respectively, and 3 places were randomly selected for each concentration. Image-pro software calculates the width of the scratch area. Relative mi-
gration distance of cells (scratch area) (μm) = [S (0 h) – S (24 h)] / S (0 h) × 100%.

Cells were also implanted in Transwell chambers and either left untreated (as negative controls) or supplemented with AU (20, 40, 80 μmol·L⁻¹) and TNF-α (20 ng·mL⁻¹) for 24 h. Following treatment, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The cells were then placed onto glass slides, and five fields from each group were selected for imaging under an inverted microscope. Cells were eluted with 33% acetic acid in 96-well plates, and the OD values was read at 570 nm. Cell invasion rate (%)= [OD (administration) – OD (background)] / [OD (Control) – OD (background)] × 100.

Flow cytometric analysis of HFLS-RA cell apoptosis

HFLS-RA cells were pre-incubated for 2 h with AU (20, 40, 80 μmol·L⁻¹) with or without TNF-α (20 ng·mL⁻¹). As recommended by the manufacturers of the Annexin V-FITC/PI apoptosis detection kit, cells were re-suspended with binding buffer and mixed with 5 μL of Annexin V-FITC and PI. After gaging, non-apoptotic cells were found in the lower left quadrant, mechanical injury cells were found in the upper left quadrant, early apoptotic cells were found in the lower right quadrant, and late apoptotic cells were found in the upper right quadrant (double positive).

Griess method to detect RAW264.7 cell NO release

RAW264.7 cells were pre-incubated for 2 h with AU (20, 40, 80 μmol·L⁻¹). All groups except the control group were stimulated with lipopolysaccharide (LPS, final concentration 1 μg·mL⁻¹) for 22 h. Supernatant (80 μL) was transferred to a fresh 96-well plate, mixed with Griess reagent (80 μL, 1:1), and incubated for 10 min. The absorbance at 540 nm was measured with a microplate analyzer. The relative rates of NO release are expressed as a proportion (%) of the control group.

TRAP staining to detect RAW264.7 cell differentiation into OCs

RAW264.7 cells were cultured in the 24-well plates. Next, 10% FBS + 1% penicillin and streptomycin + 50 ng·mL⁻¹ M-CSF + 100 ng·mL⁻¹ RANKL were used as the inducer in each group. The cells of treatment group were treated with AU (20, 40, 80 μmol·L⁻¹). The induction lasted for 7 days, and the solution was changed every 2 days. Then cells were processed with TRAP staining kits and observed microscopically. Red cells with more than three nuclei were identified as OCs.

Immunofluorescence to detect NF-κB-p65 nuclear translocation in RAW264.7 cells

RAW264.7 cells were cultured in the 24-well plates. Next, 10% FBS + 1% penicillin and streptomycin + 50 ng·mL⁻¹ M-CSF + 100 ng·mL⁻¹ RANKL were used as the inducer in each group. The cells of treatment group were treated with AU (20, 40, 80 μmol·L⁻¹). The induction lasted for 7 days, and the solution was changed every 2 days. Following treatment, the cells were fixed with 4% paraformaldehyde and 0.5% Triton X-100 for permeabilization at room temperature. Normal goat serum was added to the slide and closed at room temperature. A sufficient amount of diluted primary antibody (phosphorylated NF-κB p65) was added to each slide and placed in a wet box for overnight incubation at 4 °C. After blotting up the excess liquid on the slide with absorbent paper before adding diluted fluorescent secondary antibody. DAPI was added and incubated for 5 min without light. The specimens were sealed with solution containing anti-fluorescence quenching agent. Finally, images were collected under a fluorescence microscope.

Luciferase reporter gene assay of NF-κB-p65 gene activity in MC3T3-E1 cells

MC3T3-E1 cells were seeded in 48-well plates with NF-κB-p65 plasmid transfection solution for 24 h. Next cells were treated with TNF-α (20 ng·mL⁻¹) and/or AU (20, 40, 80 μmol·L⁻¹) for 24 h. According to the manufacturer instructions, 1 × PLB cells were lysed and mixed, and 20 μL was mixed with 100 μL luciferase substrate in a 1.5-mL Eppendorf tube before adding 100 μL stop solution. Luciferase reporter gene expression levels are expressed as a proportion (%) of the control group.

CIA model and treatment

CII was added to cooled 0.1 mol·L⁻¹ glacial acetic acid solution, mixed well, and incubated overnight at 4 °C. On the second day, an equal volume of FIA and M.Tuberculosis Des. H37 Ra was added to create a stable CII/FIA/MTB emulsion. After three days of adaptive feeding, rats were injected with CII/FIA/MTB emulsion in the tail (0.1 mL) and right hind paw (0.1 mL), except for the Blank group (n = 6). Seven days later, the model rats were injected with CII/FIA/MTB emulsion at the same sites to boost immunity. After successful modeling, the CIA rats were randomly divided into three groups. The CIA, AU, and TG groups were given intragastric administration with distilled water, 6 mg·kg⁻¹ of AU or up> of AU or 5.4 mg·kg⁻¹ of TG once a day for 4 weeks. The blank group received the same volume of distilled water.

Specimen collection

On day 49, the animals were anesthetized, and blood was taken from the abdominal aorta and centrifuged in a low-temperature refrigerated centrifuge at 4500 r·min⁻¹ for 15 minutes. The supernatant was taken and stored in a −80 °C freezer until analysis. The articular cartilage, synovium, and spleen were placed in liquid nitrogen and stored at −80 °C. The right hind ankle joints were collected in 10% neutral formaldehyde solution for fixation, and the left hind ankle joints were stored in absolute ethanol.

Body weight and paw swelling of CIA rats

Weight and paw volumes of CIA rats were recorded every 7 days by an independent observer without knowledge of the treatment protocol from day 0 (first immunization). Paw swelling was calculated as the mean value of both hind feet as determined with a toe volume measuring instrument.

AI of CIA rats

The AI in the ankle and knee joints was assessed by a macroscopic scoring system (0–4) of each limb: 0, no red-
ness or swelling; 1, mild redness and swelling; 2, moderate redness and swelling; 3, severe redness and swelling; 4, joint deformities. The AI was calculated as the average value of paws, measured every 7 day by an independent observer with no knowledge of the treatment protocol.

**Micro-CT**

Micro-CT was employed to evaluate the degree of bone damage. The left hind ankle joints were collected in absolute ethanol for scanning using the following parameters: scanning layer thickness 15 μmol·L\(^{-1}\), voltage 80 kV, current 114 μA, rotation angle 0°, and exposure time 500 ms. After scanning yielded a two-dimensional image, Mimics software was used to perform three-dimensional reconstruction processing to obtain a 3D image. The ankle joint was selected as the region of interest to perform threshold segmentation. The cortical bone and cancellous bone were segmented, and different tissue regions were extracted. BMD, BV/TV, and Tb.N, Tb.Sp were obtained by studying and analyzing these tissue regions.

**Pathological examination**

To assess synovial membrane inflammation and infiltration, the right hind ankle joint was collected in 10% neutral formaldehyde solution for fixation and then decalcified with 10% EDTA for 6 weeks. Sections of 5-μm thickness were prepared and stained with hematoxylin and eosin (H&E) as well as Safranin O-fast green staining. Lastly, it all be observed by light microscopy. Synovial inflammation pathological scores was evaluated by two blinded observers with the following system: 0−1, normal; 2−3, mild; 4−6, moderate; and 7−9, severe.

**RT-qPCR detection of mRNA levels of various inflammatory factors**

Cells were cultured in 6-well plates, and RNA was extracted with EZ-press RNA Purification Kits. RNA of spleen and ankle cartilage tissue of rats was extracted with Tissue RNA Purification Kit PLUS. The cDNA was synthesized with 4 × Reverse Transcription Master Mix. RT-qPCR was performed using 2 × SYBR Green qPCR Master Mix on an ABI Prism 7500 qPCR system (Thermo Fisher Scientific) with the following cycling conditions: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and a final extension at 72 °C for 90 s. The primers was shown in Table 1. Data were normalized to GAPDH expression using the 2\(^{-ΔΔCt}\) method. All experiments were repeated three times.

**Western blot**

Cells were cultured in 6-well plates for 12 h and then treated with TNF-α (20 ng·mL\(^{-1}\)) and AU (20, 40, 80 μmol·L\(^{-1}\)) for 24 h. Rat articular cartilage (40 mg) was ground in a homogenizer with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Protein concentrations were determined with bicinchoninic acid protein assay kits (Dalian Meilun Biotechnology Co., Ltd.). Equal concentrations of protein lysate from each group were then mixed with loading buffer, separated by SDS-

**Table 1: The Prime sequence of RT-qPCR**

<table>
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<tr>
<th>Gene</th>
<th>Mouse</th>
<th>Human</th>
<th>Rat</th>
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PAGES (EpiZyme, Cambridge, MA, USA), and then electro-transferred to nitrocellulose membranes. Next, membranes were blocked with 5% skimmed milk powder and incubated overnight with primary antibody at 4 °C. The following morning, the membrane was incubated with secondary antibody at room temperature, and immunoreactive bands were detected by enhanced chemiluminescence. The gray values of the protein bands were analyzed, and the ratio of the target protein to GAPDH was used to evaluate the expression level.

Statistical analysis

All the data are shown as mean ± SD. Statistical significance was evaluated by one-way analysis of variance with Bonferroni tests and Dunnett’s test by SPSS 26.0 (IBM Corp., Armonk, NY, USA). P < 0.05 was considered statistically significant.

Results

The anti-inflammatory effect and mechanism of AU on HFLS-RA cells

AU inhibits HFLS-RA cell migration and invasion

Cell Counting Kit-8 (CCK-8) assays were used to detect the effect of AU on HFLS-RA cell viability. After 24 and 48 h incubation, AU had a certain effect on cell viability at a concentration of 80 μmol·L⁻¹ (Fig. 2A). Therefore, 20/40/80 μmol·L⁻¹ concentrations were employed for subsequent experiments. The effect of AU on synovial cell migration and invasion was verified by scratch and Transwell assays. In the scratch healing assay (Fig. 2B), migration of the control and TNF-α groups was significantly higher than cells in the AU groups after 24 hours. The results of Transwell assays showed that the numbers of cells in AU groups were significantly lower than that in the TNF-α group (Fig. 2C). Collectively, we found that AU could inhibit HFLS-RA cell proliferation, migration, and invasion in a dose-dependent manner.

AU induces HFLS-RA cell apoptosis

After Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining, cell apoptosis was detected by flow cytometry. The apoptosis rate was calculated as the total ratio of early and late apoptotic cells and were as follows: control, 9.66%; TNF-α, 8.48%; 20 μmol·L⁻¹ AU, 13.29%; 40 μmol·L⁻¹ AU, 22.67%, and 80 μmol·L⁻¹ AU, 25.28%. AU significantly promoted HFLS-RA apoptosis in a dose-dependent manner compared with TNF-α (Fig. 2D).

AU downregulates inflammatory and vascular proliferation cytokines

Real time quantitative (RT-qPCR) was used to detect the effect of AU administration group on inflammatory cytokine expression in HFLS-RA cells (Fig. 3A). IL-1β, IL-6, MMP9, intracellular adhesion molecule 1 (ICAM1), and vascular endothelial growth factor (VEGF) mRNA levels were detected after 24-h treatment with AU. Compared with the control group, IL-1β, IL-6, MMP9, VEGF, and ICAM1 mRNA levels were significantly increased in the TNF-α group; meanwhile, levels in the AU (20, 40, 80 μmol·L⁻¹) groups were significantly decreased. We then explored the specific mechanism by which AU downregulated inflammatory cytokines.

AU inhibited NF-κB phosphorylation and activation

The expression of NF-κB-related proteins in HFLS-RA cells was detected by western blot. The ratio of target protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to evaluate expression level (Fig. 3B). Levels of the phosphorylated forms of IκBα, p65 protein were significantly increased in the TNF-α group compared with the control group. The expression level of IκBα phosphorylated protein in the TNF-α group was increased but there was no significant difference. The protein levels of p-IκBα, p-p65 in AU (20, 40, 80 μmol·L⁻¹) groups decreased significantly, while p-IκBα only decreased in the dosage of 40, 80 μmol·L⁻¹.

AU inhibited RAW264.7 cell differentiation into OCs

The role of AU in RAW264.7 cell differentiation

CCK-8 assays were used to detect the effect of AU on RAW264.7 cell viability. After 24 and 48 h, AU had an obvious effect on cell viability at a concentration of 40 μmol·L⁻¹ (Fig. 4A). The Griess method was used to detect NO release, which showed that AU could inhibit inflammation at 10 μmol·L⁻¹, and the inhibitory effect increased significantly at concentrations of 20 μmol·L⁻¹ and higher (Fig. 4B). Thus, 20/40/80 μmol·L⁻¹ treatments were selected for subsequent experiments. The effects of AU on RAW264.7 cell differentiation into OCs was observed by tartrate-resistant acid phosphatase (TRAP) staining. The color of OCs was purplish red. AU could inhibit RAW264.7 cell differentiation into OCs induced by receptor activator for NF-κB ligand (RANKL). Notably, the numbers of OCs were significantly decreased in all three AU groups (Fig. 4C).

AU inhibited the expression of bone metabolism-related proteins in RAW264.7 cells

mRNA levels of inflammatory and bone metabolism-related factors were detected by RT-qPCR. Compared with the
Fig. 2 Influence of AU on HFLS-RA viability, migration, invasion, and apoptosis. (A) Effects of AU on HFLS-RA synovial cell viability. (B) Effect of AU on scratch healing in HFLS-RA supplemented with various concentrations of AU (20, 40, 80 μmol·L⁻¹) and TNF-α (20 ng·mL⁻¹) for 24 h; images taken at 0 and 24 h, scale bar = 200 μmol·L⁻¹. Each image was measured three times. Data are presented as mean ± SD (n = 4), ***P < 0.001 vs TNF-α. (C) The invasion rate of HFLS-RA supplemented with AU (20, 40, 80 μmol·L⁻¹) and TNF-α (20 ng·mL⁻¹) for 24 h, scale bar = 200 μmol·L⁻¹. The experiment was repeated three times, and the OD value of crystal violet eluent was measured at 595 nm. Data are mean ± SD (n = 4), ***P < 0.001 vs TNF-α. (D) HFLS-RA cells supplemented with AU (20, 40, 80 μmol·L⁻¹) and TNF-α (20 ng·mL⁻¹) for 24 h were subjected to flow cytometry for apoptosis detection with Annexin V-FITC/PI. The experiment was repeated three times. Data are mean ± SD (n = 4, *P < 0.05, **P < 0.01, ***P < 0.001 vs TNF-α).
control group, VEGF, ICAM1, TNF-α, IL-6, IL-1β, MMP9, NF of activated T cells 1 (NFATC-1), cathepsin K (CTSK), TRAP, and c-FOS mRNA levels in the TNF-α group were significantly increased. Compared with the TNF-α group, mRNA levels were significantly decreased in the AU groups, except for NFATC-1, CTSK, and NFATC-1 in the 40 μmol·L⁻¹ group (Fig. 5A). The results showed that AU could inhibit inflammation- and bone metabolism-related factors. AU inhibited NF-xB phosphorylation and activation

We next investigated the effect of AU on NF-xB phosphorylation in the differentiation of RAW264.7 cells into OCs. Compared with the control group, cells in the TNF-α group were fused, and NF-xB-p65 transferred to the nucleus. In AU groups, cell fusion was reduced and nuclear translocalization of NF-xB-p65 protein was inhibited (Fig. 5B). The expression levels of NF-xB pathway and bone metabolism-related protein levels were detected by western blot. Protein levels of NF-xB pathway-related proteins p1-καβ, p1-xBa, p-p65 and OC production-related proteins RANKL, c-FOS, and NFATc-1 were significantly increased. Osteoclastogenesis inhibitory factor (OPG), a protein associated with OBs, was significantly decreased in TNF-α group. Compared with the TNF-α group, p-1καβ, p-1xBa, p-p65, RANKL, c-FOS, and NFATc-1 protein levels were significantly decreased in the AU groups, while OPG was significantly increased. AU inhibited NF-xB phosphorylation and activation

**Effects of AU on MC3T3-E1 cells**

The effect of AU on cell proliferation

CCK-8 assays were used to detect the influence of AU
on MC3T3-E1 cell viability. After treatment for 24 and 48 h, AU had a certain effect on cell viability at a concentration of 80 μmol·L⁻¹ (Fig. 6A), so 20/40/80 μmol·L⁻¹ concentrations were selected for subsequent experiments.

**AU inhibited NF-κB activation and the expression of bone metabolism-related protein**

After treatment with different doses of AU for 24 h, the expression of NF-κB-p65 plasmid was detected with double luciferase reporter gene kits. NF-κB-p65 reporter gene activity was significantly enhanced after induction by TNF-α (final concentration 20 ng·mL⁻¹), while the AU (20, 40, 80 μmol·L⁻¹) dose-dependently inhibited activity (Fig. 6B). The effects of AU on NF-κB pathway and bone metabolism-related protein levels in MC3T3-E1 cells were observed. Compared with the control group, p-IκBα, p-κBα and RANKL were significantly increased in the in the TNF-α group, the expression levels of bone morphogenetic protein 2 (BMP-2) and Smad1 related to OB differentiation were significantly decreased, and OPG was also decreased. Compared with the TNF-α group, p-IκBα, p-κBα, p-p65, and RANKL decreased significantly in the AU groups, while OPG, BMP-2, and Smad1 increased significantly (Fig. 6C).

**AU suppressed joint inflammation in CIA rats through the NF-κB pathway**

**AU attenuated joint inflammation**

Body weight and paw volume of the CIA rats were observed every 7 days, and the arthritis index (AI) was assessed. Compared with the blank group, the weight of CIA rats reached the lowest value on day 21, the rats were lethargic, and the fur appeared yellowed. There was no significant difference in body weight after 42 days. The weight of each AU administration group increased slowly compared with the control group, but there was no statistical difference in weight loss from day 28 (Fig. 7A). The mean paw swelling values were statistically compared. At day 21, there was no difference in paw swelling among the model, AU, and TG groups. The toes were red and swollen, and walking was difficult. From the second week after successful modeling, paw swelling in the AU groups was significantly decreased compared with the CIA group, and toe redness and swelling were improved (Fig. 7B). The AI of rats were evaluated. Compared with the blank group, the AI of the CIA group was significantly higher than that of the blank group on day 7, and the arthritis scores of the AU and TG groups were significantly higher than that of the control group from day 14. Compared with the model group, the AI in the TG group decreased significantly from the first week after administration, and the AU group decreased significantly from the second week after administration (Fig. 7C). The results demonstrate that AU could effectively improve inflammation in a rat RA model.

**AU effectively improved joint pathology in CIA rats**

Pathological examinations were performed to further investigate the beneficial effect of AU on joint inflammation in CIA rats (Fig. 7D). H&E and Safranin O-fast green cartilage staining were performed on the ankle joints (Fig. 7E, F). The microscopy results showed that cartilage in the blank group was well preserved without inflammation. Articular cartilage was severely damaged in the CIA group, and the residual cartilage tissue was red. The formation of old pannus included fibrous tissue hyperplasia, synovial plexus hyperplasia, articular cavity adhesions, subchondral bone tissue necrosis, and numerous OCs. AU administration reduced cartilage necrosis, fibrous tissue proliferation, and articular cavity adhesions. In the positive drug group, a small part of cartilage was destroyed and fibrous tissue hyperplasia was observed. Combined with synovial pathological scores (Fig. 7G), the results...
Fig. 5 Inhibitory effects of AU on proinflammatory cytokines, bone metabolism, NF-κB phosphorylation, and bone metabolism-related protein level activation. (A) mRNA expression of cytokines related to inflammation and bone metabolism in RAW264.7 cells treated with AU (20, 40, 80 μmol·L⁻¹). Data are mean ± SD (n = 4); *P < 0.05, **P < 0.01, ***P < 0.001 vs control; †P < 0.01, **†P < 0.001 vs + TNF-α. (B) Nuclear translocation of phosphorylated NF-κB-p65. (C) NF-κB-related protein (p-Iκκαβ, p-IκBα, p-p65) levels in RAW264.7 cells by AU (20, 40, 80 μmol·L⁻¹). Data are mean ± SD (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001 vs control; *P < 0.05, **P < 0.01, ***P < 0.001 vs + TNF-α. (D) Bone metabolism-related protein levels in RAW264.7 cells treated with AU (20, 40, 80 μmol·L⁻¹). Data are mean ± SD (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001 vs control; *P < 0.05, **P < 0.01, ***P < 0.001 vs + TNF-α.
showed that synovial hyperplasia was effectively improved in the AU group compared with the model group. Micro-computed tomography (CT) scanning was performed on the ankle joints (Fig. 7H), and relevant bone parameters were analyzed. The results showed that compared with the blank group, bone mineral density (BMD), the ratio of bone volume per tissue volume (BV/TV, which reflects changes in bone mass), trabecular number (Tb.N) all decreased significantly, Trabecular Separation (Tb.Sp.) also decreased but not statistically significant. All of this indicated that bone catabolism exceeded bone anabolism (Fig. 7I). Compared with the CIA group, BMD, BV/TV, and Tb.N were significantly increased in the AU administration group.

**AU inhibited inflammation and regulate bone metabolism**

Given the inflammation assessments and pathological results in rats, we used RT-qPCR to detected mRNA levels of related inflammatory- and bone metabolism-related factors. The results showed that compared with the blank group, mRNA levels of the inflammation factors IL-1β, VEGF, TNF-α, and IL-6 in the spleen were significantly increased (Fig. 8A), as were the cartilage mRNA levels of cytokines related to bone metabolism including NFATc-1, CTSK, TRAP, and c-FOS (Fig. 8B). Compared with the CIA group, mRNA levels in the AU group showed a significant decrease, indicating that AU influences the processes of inflammation and bone metabolism.

**AU inhibited NF-κB phosphorylation and activation in CIA rats**

Western blotting was performed to detect the effect of AU on NF-κB pathway activity. Compared with the blank
group, the protein expression levels of p-Iκαβ, p-IκBα, and p-p65 were significantly increased in the CIA group. Notably, levels of all three proteins decreased significantly in the AU group (Fig. 8C). These results indicate that the regulatory effect of AU on RA inflammation was closely related to the NF-κB pathway.

**Discussion**

RA is a chronic disease characterized by persistent synovial tissue hyperplasia, systemic inflammation, and irreversible joint damage \cite{13}. FLS cells is the main cell type in synovial tissues. Increased activity and decreased apoptosis of RA-FLS cells and their precursors (RA synovial fibroblasts) are the key causes of abnormal synovial hyperplasia in RA patients, which through a variety of mechanisms mediate damage to articular cartilage and bone tissue \cite{18, 19}. As we all known, the clinical manifestations of CIA model is multiple peripheral arthritis, local redness and swelling of joints, which will lead to deformities in severe cases. The pathological manifestations are proliferative synovitis and articular cartilage destruction. It is an ideal model for screening and studying the treatment of RA \cite{20}. According to research find-
ings, increasing the number of RA-FLS apoptosis could improve the pathological changes in arthritic animals caused by collagen [21]. The results of cell wound scratch and Transwell invasion assay shown that AU could effectively inhibit the scratch repair and invasion ability of HFLS-RA induced by TNF-α. Flow cytometry showed that AU could promote the apoptosis of HFLS-RA. In CIA rats treated with AU, hind paw swelling and redness were reduced. AU also downregulated the mRNA expression levels of inflammatory factors such as IL-1β and the production of bone metabolism-related factors such as VEGF in HFLS-RA cells and the spleen of CIA rats. VEGF is an important angiogenesis inducer that affects FLS proliferation and pannus formation [22]. It can activate cells to produce proteases that further degrade matrix membranes and promote vascular proliferation [23]. In the early stage of inflammation, ICAM1 can mediate the adhesion and infiltration of inflammatory cells and strongly promote synovium proliferation [24, 23]. IL-1β is a potent pro-inflammatory factor in RA that induces MMP production in the synovium and chondrocytes, thereby exacerbating inflammation and causing bone destruction. Reducing IL-1β expression in synovial tissue improved foot swelling and bone erosion in mice with RA. In addition, IL-1β gene polymorphism was associated with RA risk [26–28]. On one hand, IL-6 can promote the differentiation of Th17 cells; on the other hand, it can induce MMP production, stimulate OC differentiation, and promote articular cartilage destruction [29]. AU inhibited the proliferation, migration, and invasion of HFLS-RA while promoting the apoptosis and release of inflammatory factors both in vivo and in vitro. The net effect was to inhibit joint synovium proliferation and inflammation and prevent bone invasion.

Following inflammation of the synovial membrane, RA further erodes bone and cartilage tissue. OCs are an important observation indicator in the process of RA-mediated bone destruction [7]. RAW264.7 macrophages are involved in in-
Inflammatory response and could transform to OCs with the stimulation of macrophage colony-stimulating factor (M-CSF) and RANKL. Lipopolysaccharide (LPS) is a compound found in the cell wall of gram-negative bacteria that plays an important role in inducing inflammatory responses and causing a variety of inflammatory diseases. RAW264.7 macrophages is an important inflammatory cells involved in inflammatory response, and could transform to osteoclasts when stimulated by LPS, they will secrete a large number of pro-inflammatory factors such as NO. In our experiment, AU showed inhibitory effect on NO release, which proved that AU has a certain anti-inflammatory effect. Apart from this, we demonstrated that AU could inhibit RANKL-induced RAW264.7 cell differentiation and inhibit the fusion of OCs. In CIA rats, histological analysis (H&E staining and Safranin O-fast green staining) of the ankle revealed reduced cartilage necrosis and inflammatory cell infiltration in the AU group. The Micro-CT results further verified the pharmacological effect of AU on attenuating bone destruction. This was manifested in the fact that AU could increase the Percent

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**Fig. 8** AU effectively reduced the expression levels of related inflammatory factors and NF-κB phosphorylated protein in vivo. (A) Expression of pro-inflammatory cytokine mRNA levels (IL-1β, VEGF, TNF-α, IL-6). Data are mean ± SD (n = 6); \(^{\#}P < 0.05, \^{**}P < 0.01, \^{***}P < 0.001\) vs Blank; \(^{\dagger}P < 0.05, \^{\ddagger}P < 0.01, \^{\ddagger\ddagger}P < 0.001\) vs CIA. (B) Expression of bone metabolism cytokine mRNA levels (NFATc-1, CTSK, TRAP, c-FOS). Data are mean ± SD (n = 6); \(^{\#}P < 0.05, \^{**}P < 0.01, \^{***}P < 0.001\) vs Blank; \(^{\dagger}P < 0.05, \^{\ddagger}P < 0.01, \^{\ddagger\ddagger}P < 0.001\) vs CIA. (C) AU reduced phosphorylated NF-κB protein levels. Data are mean ± SD (n = 6); \(^{\#}P < 0.05, \^{**}P < 0.01, \^{***}P < 0.001\) vs Blank; \(^{\dagger}P < 0.05, \^{\ddagger}P < 0.01, \^{\ddagger\ddagger}P < 0.001\) vs CIA.
bone volume, the number of trabecular bones, and decrease the degree of trabecular bone separation. NFATc-1, CTSK, TRAP, and c-FOS are genes related to OC formation. Studies have shown that inhibiting NFATc-1 could inhibit OC overactive and prevent local bone erosion [10]. c-FOS and NFATc-1 are key transcription factors that can induce TRAP expression, and CTSK reflects the absorptive activity of OCs [13, 34]. We found that in RAW264.7 cells stimulated to differentiate into OCs with TNF-α, AU treatment could inhibit the mRNA expression of TNF-α, IL-6, IL-1β, MMP9, and other inflammatory factors. In the successfully induced OCs and cartilage tissues of CIA rats, NFATc-1, CTSK, TRAP, and c-FOS mRNA levels decreased significantly in the AU group. Collectively, the results show that AU could inhibit TNF-α induced OCs and precursor cell proliferation and differentiation and reduce bone cell apoptosis and OC overactivity by suppressing NFATc-1 to decrease bone resorption and increase production.

NF-κB signaling plays a key regulatory role in RA development by mediating the production of a variety of inflammatory factors. Phosphorylation of IkB kinase (IkK), IkB, and p65 in the NF-κB pathway affects RA pathogenesis [13]. Patients with RA have significantly higher NF-κB levels than healthy patients [30], and NF-κB-p65 is abundant in synovial tissue in CIA rats [37]. Once activated, p-NF-κB enters the nucleus and promotes the transcription of MMP-9, which further improves conditions for FLS cell invasion and migration [38]. Western blots showed that AU significantly reduced the protein levels of p-IκBα, p-IκBβ, and p-p65 in HFLS-RA cells induced by TNF-α, as well as the nuclear transfer of NF-κBα, NF-κBβ, and p65. In general, AU exerted its anti-inflammatory effect by inhibiting activation of the NF-κB pathway. At the same time, it had a positive regulatory effect on bone metabolism.

Comprehensive in vitro and in vivo experiments revealed that AU could effectively inhibit the development of synovial inflammation and inflammatory factor secretion in RA. At the same time, it has a negative effect on OCs with the net effects of regulating bone metabolism and improving the symptoms of arthritis in CIA model rats by ameliorating cartilage damage. Its bone protective effects are related to the inhibition of NF-κB pathway activation. These results could provide a direction for the development of drugs and other treatments for RA, for this, further research should be continued.

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