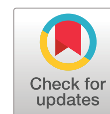
BIOCELL
2023 47(6): 1307-1316

Tech Science Press



Scutellarin alleviates complete freund's adjuvant-induced rheumatoid arthritis in mice by regulating the Keap1/Nrf2/HO-1 pathway

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Key words: Scutellarin, Rheumatoid arthritis, Th1/Th2 balance, Keap1/Nrf2/HO-1 pathway, Immunosuppression

Abstract: Scutellarin (SCU) is a herbal flavonoid glucuronide with multiple pharmacological activities, including anti-oxidant, anti-inflammation, vascular relaxation, anti-platelet, and myocardial protection. However, the effect of SCU on complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) had not been studied. In this study, we investigated the beneficial effects of SCU in the CFA-induced RA mice model and the anti-arthritic activity was evaluated by paw edema. Enzyme-linked immunosorbent assay (ELISA) was carried out to evaluate the plasma levels of immunoglobulin (Ig)G, IgE, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, receptor activator of nuclear factor-κB ligand (RANKL), and osteoprotegerin (OPG). Histological slides were prepared from the harvested paws of mice to determine the pathological changes in the joints. The proportions of T helper type 1 (Th1) and T helper type 2 (Th2) cells of CD4+ T lymphocyte subsets were analyzed by flow cytometry. The expression of Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1) was analyzed using real-time quantitative PCR (RT-qPCR) and western blotting assays. The present study demonstrated that SCU prevented CFA-induced RA, and inhibited the expression of inflammation factors, IgG, IgE, TNF-α, IL-1β, and IL-6. While SCU also reduced the RANKL level, it increased OPG expression in RA mice. The Th1/Th2 ratio was significantly lower in mice treated with SCU. Additionally, HO-1 expression was reduced while the expression of Keap1 and Nrf2 was elevated following SCU treatment. Results provide preliminary evidence to employ SCU in arthritis treatment which might be related to the regulation of Th1/Th2 balance and the Keap1/Nrf2/HO-1 pathway.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints, which causes joint deformation, cartilage erosion, and functional limitation (Firestein, 2003; Jiang *et al.*, 2021). RA is a globally distributed disease with a prevalence of approximately 0.5%–2% (Wu *et al.*, 2022). As a disabling disease, RA patients require chronic, if not lifetime treatments to alleviate the symptoms. Disease-modifying antirheumatic drugs (DMARDs) are conventional drugs used to treat RA. RA can be treated with a single drug or a combination of more than one drug (Zhao *et al.*, 2022). A lot of studies contribute to exploring the pathogenesis of RA and finding effective drugs to cure RA patients. Although non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to prevent RA progression and maintain joint function, prolonged use of NSAIDs may lead to upper gastrointestinal bleeding, nephrotoxicity, and other adverse effects (Crofford, 2013; Sostres *et al.*, 2010; Wynne and Long, 1996). Thus, more and more researchers have focused on developing novel therapeutic agents. Currently, some biological DMARDs are also being recommended. For example, natural bioactive ingredients are expected to work as reliable complementary approaches for RA treatment due to their good safety profile and medicinal synergy effects. In recent decades, several bioactive molecules have been discovered for RA control (Zhang *et al.*, 2020; Funk *et al.*, 2009).

Scutellarin (SCU) is a flavonoid extracted from *Erigeron breviscapus*, which has anti-oxidative and anti-inflammatory properties and can protect the cardiovascular system (Wang and Ma, 2018). Previous studies reported that SCU showed

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Received: 04 January 2023; Accepted: 06 February 2023; Published: 22 May 2023

Doi: 10.32604/biocell.2023.028714

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a protective role in cartilage destruction. For example, Luo et al. (2020) demonstrated that SCU could prevent osteoarthritis (OA) progression by inhibiting inflammation. In another study, SCU was found to ameliorate OA by inhibiting the Wnt/β-catenin and mitogen-activated protein kinase (MAPK) signaling pathways (Liu *et al.*, 2020). The nuclear factor (NF)-κB and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways were also reported to be involved in the protective effects of SCU in OA (Wang *et al.*, 2019). In addition, SCU could inhibit collagen-induced arthritis (CIA) by lowering toll-like receptor 4 (TLR4)/NF-κB mediated inflammation (Zhang *et al.*, 2017). Recent evidence demonstrated that SCU could activate the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway and increase the expression of the antioxidant protein heme oxygenase-1 (HO-1) (Liu *et al.*, 2019). In hepatocytes, SCU could inhibit kelch ECK associating protein 1 (Keap1), an endogenous inhibitor of Nrf2 (Mukerjee *et al.*, 2022), and upregulate the expression of Nrf2 and HO-1 (Wu and Jia, 2019). However, to our knowledge, few studies have reported the relationship between SCU and CFA-induced RA.

In the present study, we examined the potential effects of SCU on CFA-induced RA by evaluating inflammation and osteoclast differentiation. Moreover, the underlying mechanism of SCU against CFA-induced RA involving the Keap1/Nrf2/HO-1 pathway was also probed. This can provide a direction for the action mechanism and drug research of the anti-RA effect of SCU. Therefore, this study may provide a new treatment strategy for RA and a reference for clinical application and development of research on SCU.

Materials and Methods

Rheumatoid arthritis model construction and treatment

The experimental protocol and animal ethics procedures were approved by the Institutional Animal Care and Use Committee, Ningbo No.6 Hospital. The animal use license number is SYXK (Jiangsu) 2018-0027. A total of 24 male C57BL/6 mice weighing 25–30 g were purchased from the Academy of Zhejiang Province Medical Sciences and kept on a 12 h light/dark cycle at a constant temperature (25°C ± 2°C) and humidity (60%–70%). Mice were provided a standard diet and water ad libitum. Mice were arbitrarily divided into four groups (n = 6 each). Group I mice served as the control. The model was constructed as previously described (Wilhelm *et al.*, 2021). The mice were injected with 0.1 mL of complete Freund's adjuvant (CFA) by the intraplantar route in the right hind paws to induce RA. After seven days of injection, mice in group II (Model) were administered with 0.9% sterile saline by gavage for 21 days. Further, mice in group III (CFA + SCU) were treated with 20 mg/kg SCU and group IV (CFA + leflunomide) were treated with 4 mg/kg leflunomide (positive control). At the end of the experimental period (28 days), all mice were anesthetized and sacrificed by chloroform anesthesia. The serum, spleen, and synovium samples were obtained for further biochemical and histopathological experiments.

Bodyweight and footpad volume measurements

The body weight of mice was measured with a precision balance every 3 days and the alterations were calculated by comparing them to the initial body weight. The hind footpad volumes were measured using the footpad volume measuring instrument (Muromachi Kikai, Japan) every 3 days. The swelling rate was calculated by the following formula: swelling rate (%) = (footpad volume after treatment – footpad volume before treatment)/footpad volume before treatment × 100%.

Spleen index assessment

After the mice were euthanized, the spleens were expunged immediately and processed in phosphate buffer saline (PBS). The spleens were weighed and the indexes were calculated using the expression: organ weight/body weight × 10. After weighing, the spleens were digested into single cells for subsequent experiments.

Inflammatory marker assessment

The collected blood samples were centrifuged at 6,000 rpm for 15 min to obtain sera. The levels of immunoglobulin (Ig) IgG (mlbio, ml037601-J), IgE (mlbio, ml037602), tumor necrosis factor (TNF)-α (mlbio, ml002095), interleukin (IL)-1β (mlbio, ml063132), IL-6 (mlbio, ml063159), receptor activator of NF-κB ligand (RANKL; mlbio, ml037743), and osteoprotegerin (OPG; Abcam, ab203365) were measured with corresponding enzyme-linked immune sorbent assay (ELISA) kits according to the instructions. The synovium tissues were homogenized for measuring the levels of RANKL and OPG.

Histopathological analysis

The joints of the hind paw tissues were processed with 4% paraformaldehyde for 24 h and decalcified with 10% formic acid solution. After being embedded in paraffin and sliced into the 5 μm thick sections, the samples were stained with hematoxylin for 5 min and eosin for 2 min. After being dehydrated with gradient alcohol, the samples were permeated and sealed with xylene and neutral gum, respectively. The whole field views of hematoxylin and eosin (H&E)-stained slices were captured using a light microscope (Olympus, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labeling staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to study apoptosis in synovium tissue. After routine dewaxing and rehydration, the sections were treated with proteinase K for 15 min at 37°C and then incubated with a TUNEL mixture (Beyotime, C1086) for 60 min in the dark according to the manufacturer's protocol. 4′,6-diamidino-2-phenylindole (DAPI) staining (Beyotime, 1 μg/mL) was applied for nuclear staining. The TUNEL-positive cells were measured under the microscope (Nikon C1 System, Nikon Corporation).

Flow cytometric analysis

The percentages of IFN-γ+ T cells and IL-4+ T cells and the Th1/Th2 ratio were determined by flow cytometry with a

TABLE 1

Primer sequences for RT-qPCR

Gene	Primer sequences
Nrf2	Forward: 5′-3′GCAGGCTGAGACTACCACTG
	Reverse: 5′-3′GGACAGATCACAGCCCTCAAT
HO-1	Forward: 5′-3′GGAAATCATCCCTTGCACGC
	Reverse: 5′-3′CCTGAGAGGTCACCCAGGTA
Keap1	Forward: 5′-3′GTAGGCCGCCTCATCTACAC
	Reverse: 5′-3′ACCATTGCTCGGGTTGTAGG
GAPDH	Forward: 5′-3′TCTCTGCTCCTCCCTGTTCC
	Reverse: 5′-3′ACTGTGCCGTTGAATTTGCC

FACS Canto II (BD Biosciences). The following antibodies were used for flow cytometry: Fluorescein isothiocyanate (FITC)-anti-CD4, PerCP-Cy5.5-IFNγ, and IL-4-APC. Isolated spleen cells were treated with ionomycin (750 ng/ml, Sigma), phorbol 12-myristate 13-acetate (200 ng/ml, Sigma), and BD GolgiPlug (BD Biosciences) for 5 h in a cell incubator (37°C, 5% CO_2). Then, the cells were incubated with the aforementioned antibodies in the dark for 30 min. The data were analyzed by the Flowjo software (Tree Star, Korea).

Reverse transcription-quantitative polymerase chain reaction
Real-Time Quantitative Reverse Transcription PCR (RT-qPCR) analysis was performed as previously reported (Xie *et al.*, 2020). Total RNA was extracted from mice synovial tissue using Trizol reagent (Invitrogen, CA, USA). The RNA was reverse transcribed into cDNA using FastKing First-strand cDNA Synthesis Mix (Tiangen, Beijing, China). RT-qPCR was performed using SYBR Green PCR Master Mix (Lifeint, Xiamen, China) on the MX3000P Real-Time system (Stratagene, Carlsbad, CA, USA). The composition of the PCR reaction mixture is presented in Table 1. The qPCR reaction conditions applied were: 95°C for 3 min, followed by 40 cycles of 95°C for 12 s and 62°C for 40 s. The primer sequences for RT-qPCR are shown in Table 1. GAPDH was used as an internal reference gene, and relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis
Western blot analysis was carried out according to a previously published method (Shen *et al.*, 2021). Total protein was extracted from tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein concentration was detected using a BCA protein assay kit (Beyotime). Then, 25 µg of protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE: 10% separating gel and 5% concentrating gel). The separated protein was transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 5% skim milk for 1 h at room temperature. The PVDF membrane was incubated with the corresponding primary antibodies overnight at 4°C. The primary antibodies used in this study are listed below: Nrf2 (1:1,000; cat. no. ab137550; Abcam), HO-1 (1:1,000; cat. no. ab52947; Abcam), and Keap1 (1:2,000; cat. no. ab227828; Abcam). Following that, the PVDF membrane was incubated with goat anti-rabbit IgG H&L secondary

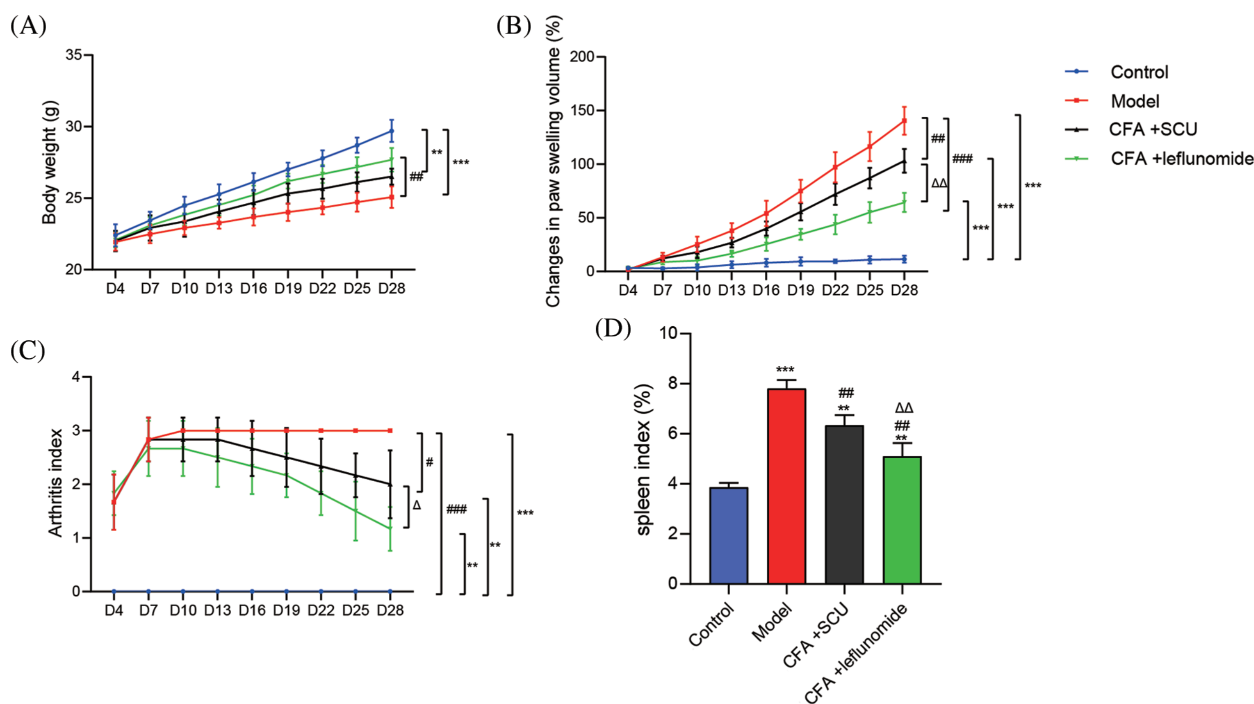

FIGURE 1. Scutellarin (SCU) improved clinical responses in rheumatoid arthritis (RA) mice. (A) Effects of SCU on the body weight. (B) Effects of SCU on the paw swelling volume. (C) Effects of SCU on the arthritis index. (D) Effects of SCU on the spleen index. The average content in each group was expressed as the mean ± square deviation (SD) ($n = 6$). **$p < 0.01$ and ***$p < 0.001$ *vs.* the control group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ *vs.* the model group; Δ$p < 0.05$ and ΔΔ$p < 0.01$ *vs.* the complete Freund's adjuvant (CFA) + SCU group.

antibodies (1:10,000; cat. no. ab6721; Abcam) for 60 min at room temperature. Protein bands were visualized using ECL (Thermo, MA, USA) and subsequently analyzed with ImageJ software v 1.8.0 (National Institutes of Health, MD, USA).

Statistical analysis

All of the statistical examinations were analyzed using GraphPad Prism 8.0 software (GraphPad Software, CA, USA). Data were illustrated as mean ± standard deviation (SD). A one-way ANOVA was used to statistically compare differences among more than two groups, followed by Tukey's test. Data were regarded as statistically significant differences if $p < 0.05$.

Results

SCU improved clinical responses of RA mice

To explore the effect of SCU on regulating the occurrence and development of RA *in vivo*, the RA mouse model was successfully constructed by CFA induction and administrated with SCU or leflunomide. Firstly, we measured the volume of the paw and the body weight of the mice and evaluated the arthritis index according to the degree of joint redness and swelling. As shown in Figs. 1A–1C, the body weights of the RA model mice were significantly lower than that of control mice ($p < 0.001$), and the paw volume and arthritis index were significantly increased ($p < 0.001$). Leflunomide, a Food and Drug Administration (FDA)-approved medication indicated for the treatment of RA, was employed as a positive control. Compared with the model mice, RA mice treated with SCU or leflunomide showed higher body weight, and significantly decreased paw swelling volume and arthritis index ($p < 0.05$; Figs. 1A–1C).

The spleen is an important immune organ, which can systematically reflect the immune functional status. As shown in Fig. 1D, a significantly higher spleen index was found in the RA model mice compared to the control mice ($p < 0.001$). The treatment of SCU or leflunomide significantly reduced the spleen index of RA mice, indicating SCU could exert a certain degree of immunosuppression ($p < 0.01$; Fig. 1D).

SCU relieved the histological damage and chondrocyte apoptosis in RA mice

The morphology of joint tissues harvested from mice was detected by HE staining. In the control group, the articular

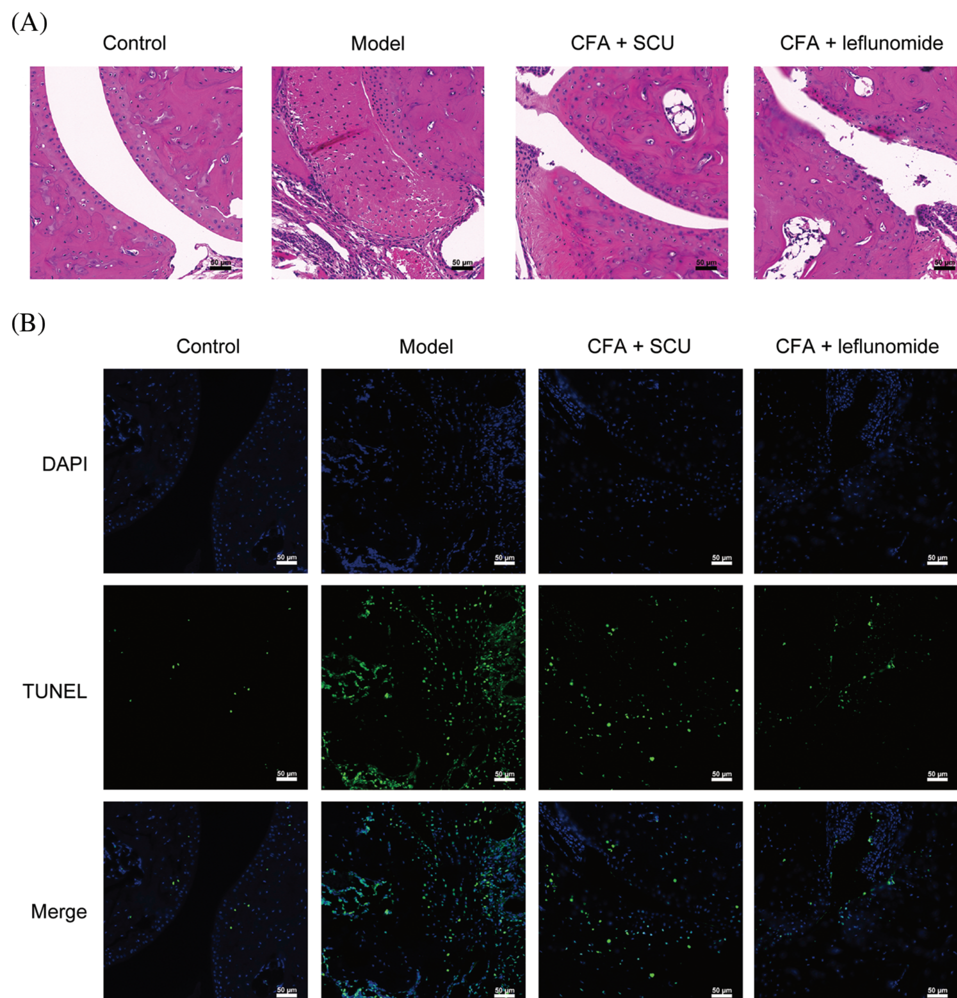

FIGURE 2. Scutellarin (SCU) relieved the histological damage and chondrocytes apoptosis in rheumatoid arthritis (RA) mice. (A) Effects of SCU on histological damage. (B) Chondrocyte apoptosis in RA mice was detected by the TUNEL assay (scale bar = 50 μm); CFA: complete Freund's adjuvant.

surface of the mice was smooth and intact. Obvious histopathological changes were found in the RA model group. Rough and degenerated cartilage surfaces and abnormal hyperplastic synovium were observed accompanied by abundant inflammatory cell infiltration in the joint cavity in RA mice. Treatment with SCU or leflunomide reduced the degree of cartilage destruction, inflammatory cell infiltration, and synovial epithelium hyperplasia of RA mice (Fig. 2A). In addition, chondrocyte apoptosis was evaluated by TUNEL staining after the mice were sacrificed. As shown in Fig. 2B, only a few apoptotic cells were observed in the joint tissue of control mice while the RA model mice showed a substantial number of apoptotic cells. Upon treatment using SCU or leflunomide, chondrocyte apoptosis was significantly suppressed in the RA mice.

SCU inhibited serum immunoglobulins and cytokines in RA mice

Then the serum inflammatory factors of RA mice were detected by ELISA as discussed. As shown in Figs. 3A–3E, the concentrations of immunoglobulins (IgG and IgE) and inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the serum samples of RA mice were significantly higher than that of control mice ($p < 0.001$). However, these immunoglobulins and inflammatory cytokines levels in the serum samples of RA mice remarkably dropped after SCU or leflunomide treatment ($p < 0.01$; Figs. 3A–3E). It was noticed that SCU showed a similar inhibitory effect on immunoglobulin levels like with leflunomide. Results revealed that SCU could inhibit the inflammatory responses in RA mice.

SCU improved the balance of Th1 and Th2 cells in spleens of RA mice

Th1 and Th2 cells play important roles in the pathogenesis of RA (Piazza *et al.*, 2022). We hence analyzed the Th1 and Th2 cell profiles in mouse spleens by flow cytometry. Results showed that the percentages of IFN-γ+ T cells and IL-4+ T cells were higher in the RA model group than those in the control group. This was also observed for the Th1/Th2 ratio ($p < 0.001$; Figs. 4A–4D). Compared with the RA model mice, the frequency of IFN-γ+ T cells and IL-4+ T cells were significantly decreased in RA mice treated with SCU or leflunomide ($p < 0.01$) with SCU showing better efficiency in inhibiting IFN-γ+ T cells ($p < 0.01$). In addition, the Th1/Th2 ratio in the CFA + leflunomide group was significantly higher than the CFA + SCU group ($p < 0.01$), which was similar to the RA model group. However, SCU treatment significantly reduced the Th1/Th2 ratio (Fig. 4D).

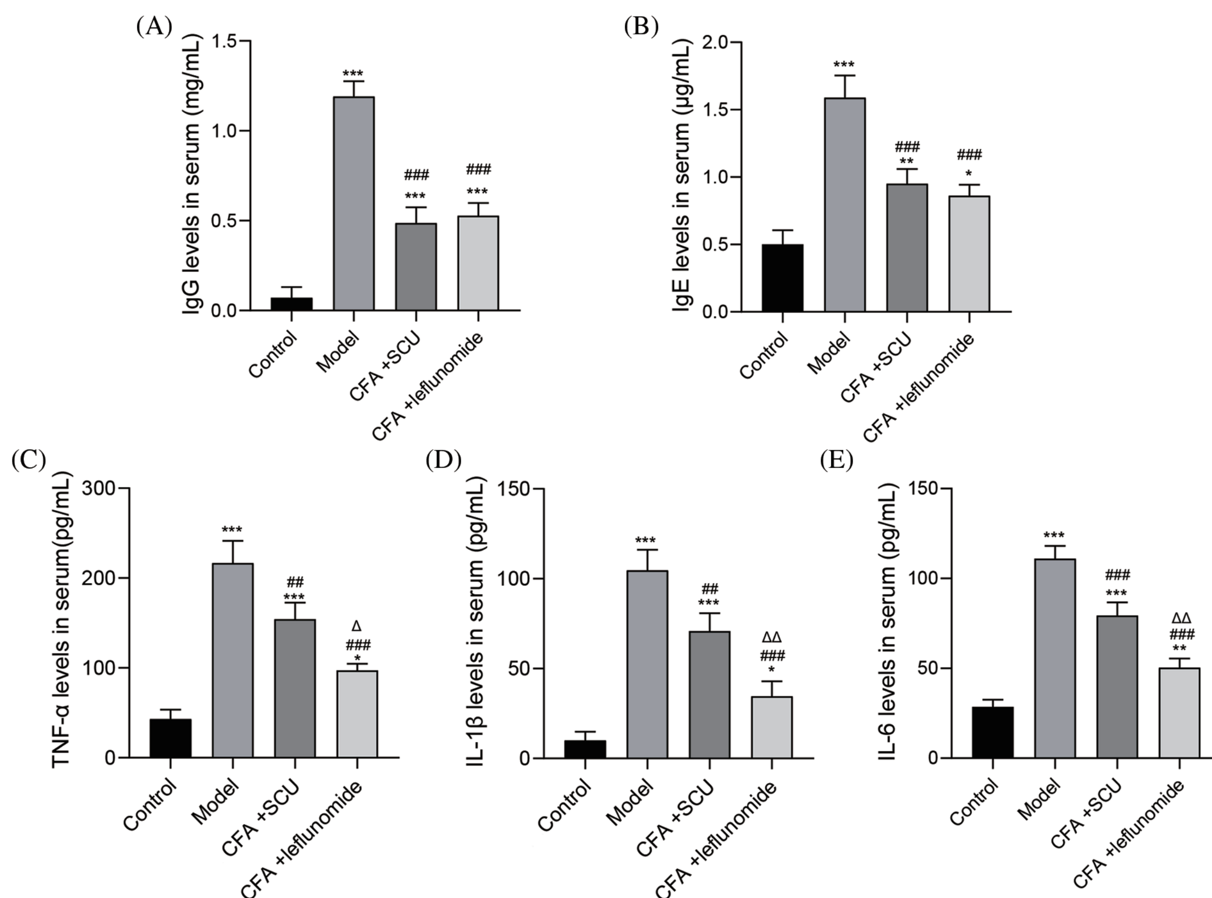

FIGURE 3. Scutellarin (SCU) inhibited serum immunoglobulins and cytokines in rheumatoid arthritis (RA) mice. (A–E) Immunoglobulin (Ig) G, IgE, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 levels in each group. The average level in each group was expressed as the mean ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs.* the control group; ##$p < 0.01$ and ###$p < 0.001$ *vs.* the model group; Δ$p < 0.05$ and ΔΔ$p < 0.01$ *vs.* the complete Freund's adjuvant (CFA) + SCU group.

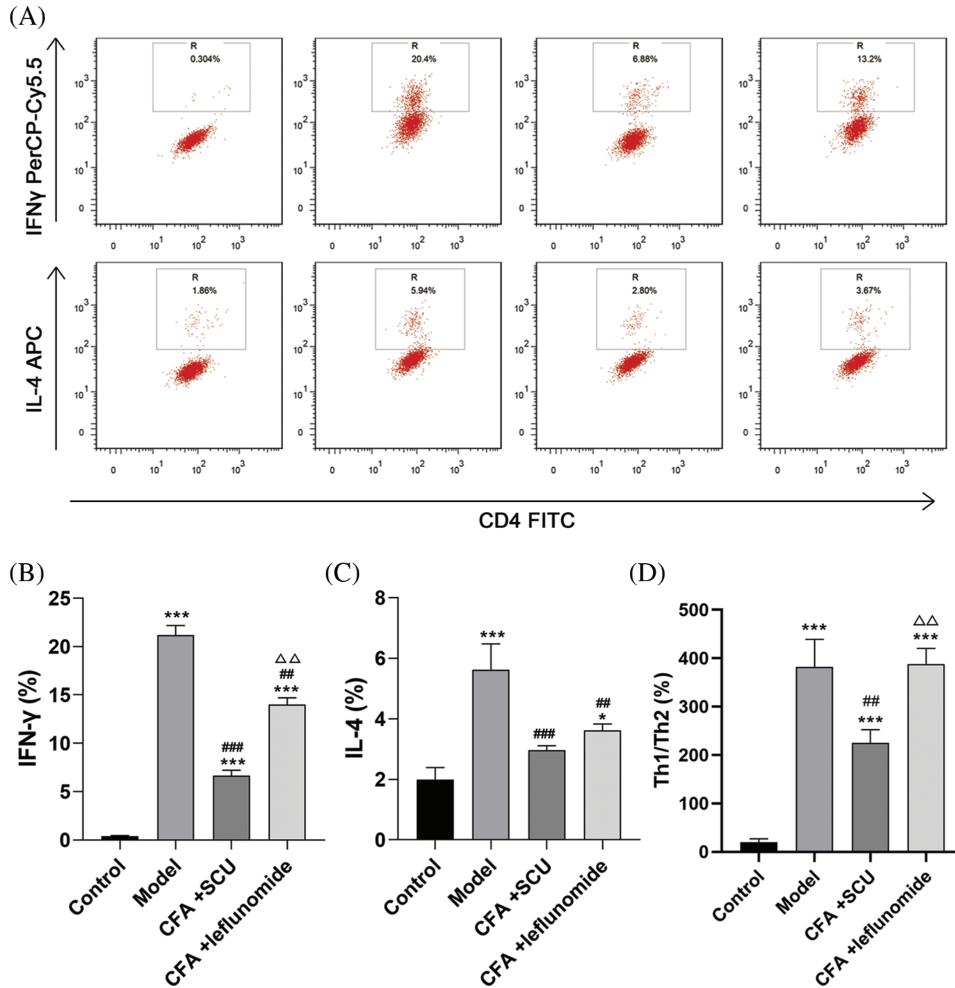

FIGURE 4. The effects of scutellarin (SCU) on the percentage of T helper type 1 (Th1) and T helper type 2 (Th2) cells in mouse spleens. (A) CD4 antibody was used to isolate T-helper (Th) cells. (B–D) Expression of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) and the Th1/Th2 ratio were analyzed. The average content in each group was expressed as the mean ± SD ($n = 6$). *$p < 0.05$ and ***$p < 0.001$ *vs.* the control group; ##$p < 0.01$ and ###$p < 0.001$ *vs.* the model group; ΔΔ$p < 0.01$ *vs.* the complete Freund's adjuvant (CFA) + SCU group.

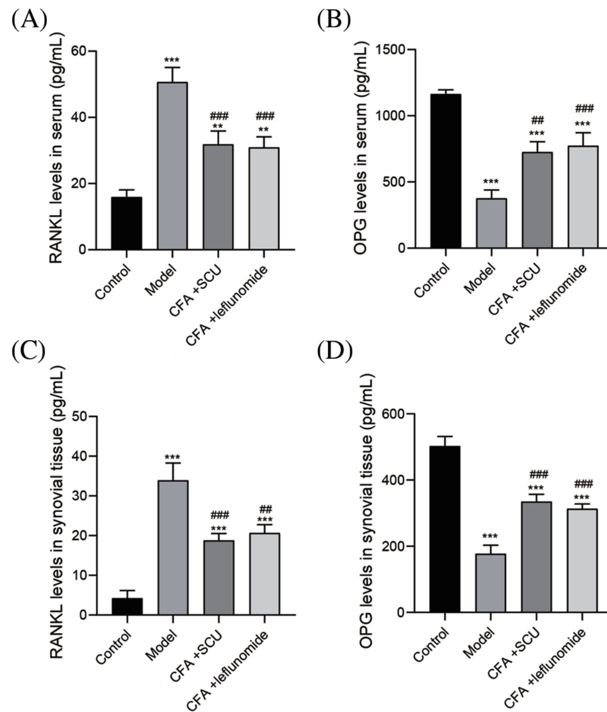

FIGURE 5. Scutellarin (SCU) inhibited receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) levels in RA mice. (A and B) Effects of SCU on the serum levels of RANKL and OPG. (C and D) Effects of SCU on the tissue levels of RANKL and OPG. The average content in each group was expressed as the mean ± SD ($n = 6$). **$p < 0.01$ and ***$p < 0.001$ *vs.* the control group; ##$p < 0.01$ and ###$p < 0.001$ *vs.* the model group.

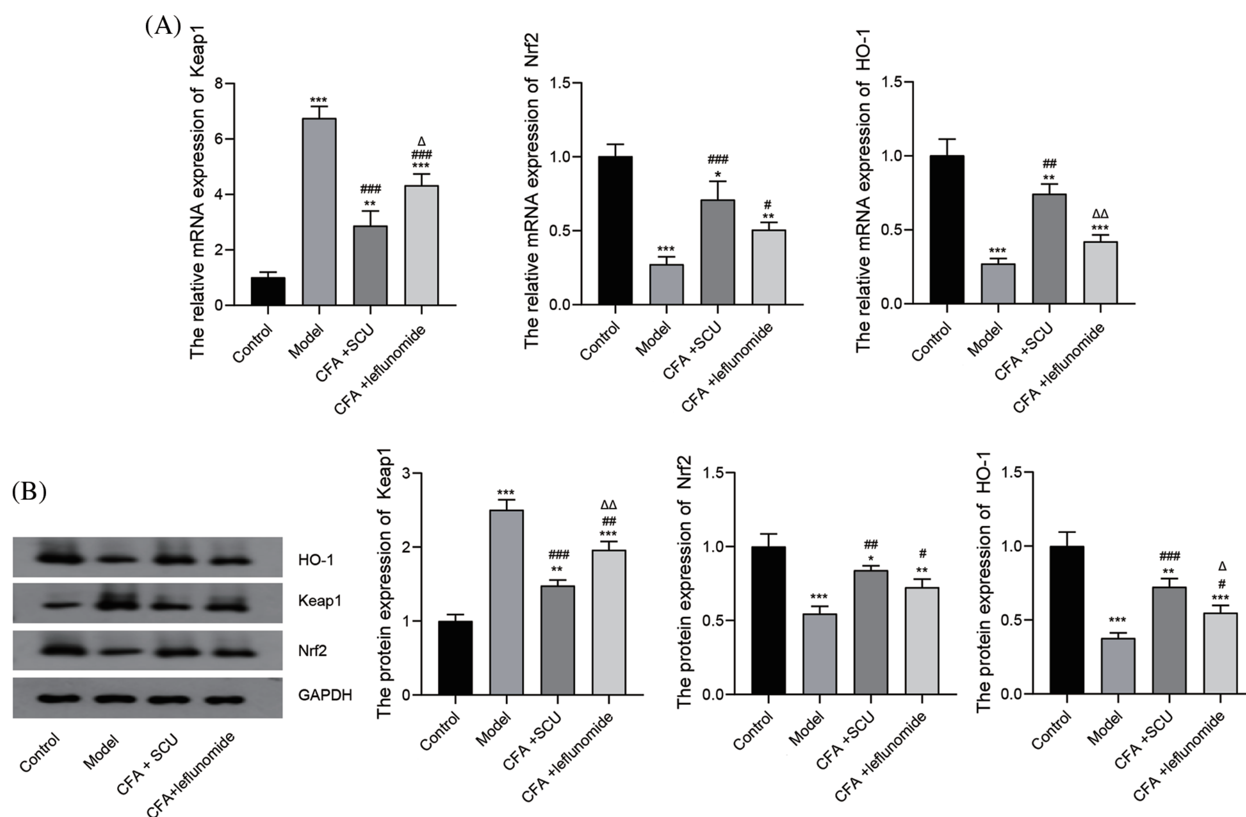

FIGURE 6. Analyzing the mechanism of the anti-arthritis effects of scutellarin (SCU). (A) Relative mRNA expressions of Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1) were detected by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). (B) Protein expression levels of Keap1, Nrf2, and HO-1 were detected by western blotting. The average content in each group was expressed as the mean ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs.* the control group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ *vs.* the model group; $\Delta p < 0.05$ and $\Delta\Delta p < 0.01$ *vs.* the complete Freund's adjuvant (CFA) + SCU group.

SCU inhibited RANKL and OPG levels in RA mice

The vital involvement of cytokines RANKL and OPG in osteoclast differentiation and activation is known (Lacey *et al.*, 1998). To further investigate the effect of SCU on RA, the serum and tissue levels of RANKL and OPG were examined by ELISA. As shown in Figs. 5A–5D, the serum and tissues of RA mice documented increased RANKL and decreased OPG levels ($p < 0.001$). SCU or leflunomide treatment significantly downregulated RANKL levels in serum and tissues of RA mice while it significantly up-regulated OPG ($p < 0.01$). Further, there was no statistically significant difference in the effects of SCU and leflunomide on levels of RANKL and OPG.

SCU induced the Keap1/Nrf2/HO-1 signaling pathway in RA mice

Maintaining high levels of HO-1 is a promising strategy to protect against inflammation and arthritis (Poulet and Beier, 2016). Hence, we explored whether SCU regulates the Keap1/Nrf2/HO-1 signaling pathways to exert an anti-oxidation function in RA mice. As shown in Figs. 6A and 6B, significantly upregulated Keap1 level and downregulated Nrf2 and HO-1 levels were observed in the model group ($p < 0.001$). Further, SCU or leflunomide treatment significantly inhibited the mRNA and protein expression of Keap1 in synovium tissues of RA mice ($p < 0.01$). Compared with the leflunomide treatment, SCU exhibited more significant inhibition on the expression of Keap1 ($p < 0.05$). As Keap1 is an inhibitor of Nrf2, our observations showed that SCU or leflunomide treatment induced the expression of Nrf2 and HO-1 compared to the RA model group ($p < 0.05$; Figs. 6A and 6B). Further, SCU exerted a better efficiency than leflunomide in increasing the HO-1 expression ($p < 0.05$).

Discussion

SCU is a major effective constituent of the Chinese medicine *Erigeron breviscapus* and has been verified to inhibit collagen-induced arthritis (Zhang *et al.*, 2017). In the present study, we evaluated the therapeutic potential of SCU in a CFA-induced RA mouse model and also explored the underlying mechanism of SCU on RA. We found that SCU could alleviate the arthritis score and inflammatory response in CFA-induced RA mice. Furthermore, this protective effect was related to the regulation of the Th1/Th2 ratio and the Keap1/Nrf2/HO-1 pathway.

CFA is used in most procedures for the experimental induction of RA as it induces changes similar to RA in humans (Youssef *et al.*, 2022). The induction of arthritis causes some pathological alterations, such as inflammatory synovitis with enhanced infiltration of inflammatory cells, followed by cartilage degradation and joint deformation (Robinson *et al.*, 2016). In our study, there were obvious

histopathological changes in the RA model mice inclusive of the degenerated cartilage surface and abnormal hyperplastic synovium, accompanied by abundant inflammatory cell infiltration in the joint cavity. Further, increased apoptosis of chondrocytes and destroyed dynamic balance between cell proliferation and death rates were reported in RA patients (Faramarzi *et al.*, 2022). The chondrocytes are decreased substantially, accompanied by gradual degradation of extracellular matrix (ECM), eventually leading to degradation of articular cartilage. In the present study, we found abundant apoptotic chondrocytes in the synovium of RA mice. These results indicate the successful construction of the RA mouse model. Several studies have reported the protective effect of SCU on arthritis (Yang *et al.*, 2022; Zhang *et al.*, 2017). Our results revealed that SCU treatment alleviated inflammatory cell infiltration and synovium hyperplasia and reduced chondrocyte apoptosis in RA mice, which indicates that SCU has a therapeutic effect on RA.

Inflammatory responses play a major role in the physiopathology of RA, and high levels of immunoglobulins (IgA, IgE, IgD, and IgG) and pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-17, IL-22, and IL-6) are observed in the synovial fluid and the serum (Achudhan *et al.*, 2021; Shu *et al.*, 2022; Gioud-Paquet *et al.*, 1987). This increase in immunoglobulins and cytokines is partly correlated with the severity of cartilage destruction. CFA treatment can also increase pro-inflammatory cytokine levels in the serum and synovial fluid. Zhang *et al.* (2017) demonstrated that SCU could inhibit inflammatory cytokines levels in collagen-induced arthritis mice. Similarly, our findings also revealed that SCU treatment significantly reduced CFA-induced IgG, IgE, TNF-α, IL-1β, and IL-6 levels in RA mice, indicating that administration of SCU could inhibit the inflammatory response in RA mice.

Studies have shown that Th1 cells and their secreted cytokines are involved in joint inflammation, whereas Th2 cells and their secreted cytokine IL-4 help alleviate inflammatory responses (Cutolo *et al.*, 2022; Xie *et al.*, 2022). The Th1/Th2 cell ratio is implicated to be important in the pathogenesis of RA (Bao *et al.*, 2022). Further, an imbalance of Th1 and Th2 cells is commonly observed in RA patients (Li *et al.*, 2021). IFN-γ is an important pro-inflammatory cytokine secreted by Th1 cells in RA, which promotes the inflammatory response in RA by activating macrophages. Hence, for evaluating the anti-inflammatory effect of SCU, we analyzed percentages of Th1 and Th2 cells and the Th1/Th2 ratio in the mice spleens by flow cytometry. The treatment with SCU significantly decreased the percentage of IFN-γ+ Th1 cells and the Th1/Th2 ratio. These results suggest that SCU regulated the balance of Th1/Th2 cells in RA mice. The above data reveals that SCU ameliorates RA by inhibiting the inflammatory response in the mice models.

A precise balance between bone formation and resorption is required for joint functioning. The protein RANKL can induce the differentiation of progenitor cells into mature osteoclasts while OPG can inhibit RANKL function conversely (Tan *et al.*, 2017; Wang *et al.*, 2017). Our data showed that the administration of SCU increased RANKL expression and decreased OPG expression, which revealed the regulating effect of SCU on bone metabolism.

HO-1 is a member of the heat shock protein family and is an important anti-inflammatory, antioxidative, and cytoprotective enzyme regulated by the activation of Nrf2 (Zeng *et al.*, 2022). A previous study demonstrated that the HO-1/Nrf-2 signaling pathway was involved in the inflammatory response of RA (Zeng *et al.*, 2022). An important finding of the present study relates to the SCU-mediated upregulation of HO-1 expression that was associated with a reduced *in vivo* inflammatory response. The results were consistent with a previous report that HO-1 is upregulated by the transcriptional factor Nrf2 (Na and Surh, 2014). Puppala *et al.* (2022) revealed that perillyl alcohol alleviated RA by regulating the Keap1/Nrf2 signaling pathway. Our data showed that SCU significantly promoted the degradation of Keap1, an endogenous inhibitor of Nrf2. Considering that HO-1 is a regulator of the inflammatory response, it is conceivable that the upregulation of HO-1 by SCU was involved in regulating the inflammatory response in RA through the activation of the Keap1/Nrf2/HO-1 pathway.

Conclusions

Our results indicated the therapeutic effects of SCU on RA evidenced by the improvement of clinical responses and histological damage and reduced chondrocyte apoptosis in RA model mice treated with SCU. SCU also ameliorated the inflammatory response and decreased the Th1/Th2 ratio in RA mice. Additionally, this study demonstrated that the mechanism of SCU inhibiting the CFA-induced RA may involve the regulation of the Keap1/Nrf2/HO-1 pathway. Nevertheless, more experiments are needed to validate the relationship between SCU and the Keap1/Nrf2/HO-1 pathway in RA. In addition, the regulating effects of SCU on the balance between osteoblast and osteoclast need further investigation. Our results revealed that SCU treatment may help in the clinical treatment of RA, while more clinical studies need to be conducted.

Funding Statement: The authors received no specific funding for this study.

Author Contributions: The authors confirm their contribution to the paper as follows: Study conception and design: JL, QW, and XZ; data collection: JL and QW; analysis and interpretation of results: JL, QW, and XZ; draft manuscript preparation: JL. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval: All animal protocols were approved by the Animal Experimental Ethics Committee of The First People's Hospital of Xiaoshan District, Xiaoshan Affiliated Hospital of Wenzhou Medical University. The animal use license number is SYXK (Jiangsu) 2018-0027.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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