Antcin K Inhibits TNF-α, IL-1β and IL-8 Expression in Synovial Fibroblasts and Ameliorates Cartilage Degradation: Implications for the Treatment of Rheumatoid Arthritis

David Achudhan1, Shan-Chi Liu2, Yen-You Lin3, Chien-Chung Huang1,4, Chun-Hao Tsai5,6, Chih-Yuan Ko6, I-Ping Chiang7, Yueh-Hsiung Kuo8,9,10* and Chih-Hsin Tang1,3,9,10*

1 Graduate Institute of Biomedical Science, College of Medicine, China Medical University, Taichung, Taiwan, 2 Department of Medical Education and Research, China Medical University Beigang Hospital, Yunlin, Taiwan, 3 Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan, 4 Division of Immunology and Rheumatology, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan, 5 Department of Sports Medicine, College of Health Care, China Medical University, Taichung, Taiwan, 6 Department of Orthopedic Surgery, China Medical University Hospital, Taichung, Taiwan, 7 Department of Pathology, China Medical University Hospital, Taichung, Taiwan, 8 Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan, 9 Department of Biotechnology, Asia University, Taichung, Taiwan, 10 Chinese Medicine Research Center, China Medical University, Taichung, Taiwan

Extracts from Taiwan’s traditional medicinal mushroom, Antrodia cinnamomea, exhibit anti-inflammatory activities in cellular and preclinical studies. However, this paper is the first to report that Antcin K, a triterpenoid isolated from A. cinnamomea, inhibits proinflammatory cytokine production in human rheumatoid synovial fibroblasts (RASFs), which are major players in rheumatoid arthritis (RA) disease. In our analysis of the mechanism of action, Antcin K inhibited the expression of the three cytokines (tumor necrosis factor alpha [TNF-α], interleukin 1 beta [IL-1β] and IL-8) in human RASFs; cytokines that are crucial to RA synovial inflammation. Notably, incubation of RASFs with Antcin K reduced the phosphorylation of the focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT) and nuclear factor-κB (NF-κB) signaling cascades, all of which promote cytokine production in RA. Intraperitoneal injections of Antcin K (10 mg/kg or 30 mg/kg) attenuated paw swelling, cartilage degradation and bone erosion, and decreased serum levels of TNF-α, IL-1β, IL-8 in collagen-induced arthritis (CIA) mice; in further experiments, IL-6 levels were similarly reduced. The inhibitory effects of Antcin K upon TNF-α, IL-1β and IL-8 expression in human RASFs was achieved through the downregulation of the FAK, PI3K, AKT and NF-κB signaling cascades. Our data support clinical investigations using Antcin K in RA disease.

Keywords: rheumatoid arthritis, tumor necrosis factor alpha, interleukin 1 beta, interleukin 8, antcin K, collagen-induced arthritis, rheumatoid arthritis synovial fibroblast, Antrodia cinnamomea
INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease with a complex pathogenesis that is not fully understood, but is known to involve the infiltration of inflammatory cells into the joints and subsequent swelling, synoviocyte proliferation, cartilage damage and bone erosion (1, 2). The production of proinflammatory cytokines from activated RA synovial fibroblasts (RASFs) disrupts the microenvironment that mediates bone homeostasis (3, 4). RASFs are considered to be the main catabolic factor in cartilage bone degradation, as they stimulate the production of proinflammatory cytokines typically associated with RA disease, including tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), IL-8 and IL-6 (5).

Symptomatic pain and inflammation associated with RA disease is generally prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatories (corticoids) in addition to existing RA treatment, while synthetic or biologic drugs (NSAIDs) and steroidal anti-inflammatories (corticoids) are commonly used in RA management. However, their long-term safety is yet to be confirmed, as a recent Cochrane Review of randomized clinical trial evidence on the efficacy and safety of these anti-inflammatories in the long-term treatment of RA concluded that the quality of the NSAID evidence was very low overall and meta-analysis evidence was lacking for corticoids (6).

Importantly, long-term pharmacotherapy in RA disease is often associated with significant side effects, such as cytopenia, poor tolerability, rash, and occasionally liver damage occurring with traditional DMARDs (7), while data from observational studies indicate higher risks of cardiovascular disease, infections, diabetes mellitus and mortality with glucocorticoids (8), and side effects relating to biologic treatment with tumor necrosis factor (TNF) inhibitors can include severe infection, sepsis, tuberculosis, lymphoma or demyelinating disorders (9).

Novel therapeutic alternatives with good tolerability are needed to halt the progression of RA disease (10). Traditional Chinese medicinal plants contain functional compounds that can cure various disease conditions (11) and are an important source of drug development. In particular, the traditional Chinese medicine (TCM) remedy Lianhua Qingwen markedly downregulates messenger RNA (mRNA) expression of cytokines TNF-α and IL-6 and of the chemokine monocyte chemoattractant protein-1 [(MCP)-1], also referred to as chemokine (CC-motif) ligand 2 (CCL2)] and C-X-C motif chemokine ligand 10 [(CXCL10], also known as interferon gamma-induced protein 10 (IP-10)] in human hepatocellular carcinoma (Huh-7) cells infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (12).

Antrodia cinnamomea is a species of mushroom endemic to Taiwan that has been used for hundreds of years in TCM and A. cinnamomea extracts are well known for their hepatoprotective, antioxidative, antihypertensive, antihyperlipidemic, immunomodulatory, anticancer and anti-inflammatory activities (13–15). In this study, we demonstrate that a triterpenoid isolated from A. cinnamomea, Antcin K, exhibits anti-inflammatory effects in cellular and preclinical experiments investigating RA disease. Interestingly, we found that Antcin K downregulated the production of proinflammatory cytokines including TNF-α, IL-1β and IL-8 in RASFs via the FAK, PI3K, AKT and NF-κB signaling cascades. Furthermore, Antcin K ameliorated cartilage degradation in mice with collagen-induced arthritis (CIA). Our report is the first to explain how Antcin K inhibits cartilage degradation in vitro and also the production of proinflammatory cytokines in vivo. According to our evidence, Antcin K appears to have therapeutic potential in RA disease.

MATERIALS AND METHODS

Materials

Antibodies against TNF-α (A11534), IL-1β (SC-7884), IL-8 (ab18672), p-FAK (Thr202/Tyr204; SC-7383), FAK (SC-5298), p-85 (SC-1637), p-AKT (Thr308; SC-16646-R), AKT (SC-5298), p-p65 (Ser536; SC-101752), p-65 (SC-8008), and β-actin (SC-58673) were purchased from Santa Cruz (Santa Cruz, CA, USA). Activators Angiotensin II (a FAK activator), 740-YP (a PI3K activator), SC-79 (an AKT activator) and prostratin (an NF-κB activator) were all purchased from Santa Cruz (Santa Cruz, CA, USA). Cell culture supplements were purchased from Invitrogen (Carlsbad, CA, USA). A Human Inflammation Antibody Array kit was supplied by RayBiotech, Inc. Human TNF-α, IL-1β and IL-8 ELISA kits were purchased from R&D Systems (MN, USA).

Cell Culture

Immortalized RASFs (MH7A cells) were bought from the Riken Cell Bank (Ibaraki, Japan) and cultured as a single culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin 100 U/mL. Cell incubation was performed in a humidified atmosphere of 37°C, 5% CO₂. The cells were passaged when they had grown to 80% confluence.

MTT Assay

RASFs were seeded at a density of 5 x 10^3 cells/well into 96-well plates and incubated with Antcin K (0, 0.3, 1, 3, or 10 μM) for 24 h, then incubated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 2 h. Dimethyl sulfoxide (DMSO) was added to the solution and absorbance at 550 nm was measured with a microplate reader (BioTek, Winooski, VT, USA).

Quantitative Real-Time PCR for mRNA

After incubating the RASFs with Antcin K (0, 0.3, 1, 3, or 10 μM) for 24 h, total RNA was extracted from the RASFs using...
TRizol™ reagent. The qPCR analysis was performed as per an established protocol (16–18). RNA concentration was measured using a NanoVue Plus™ Spectrophotometer (Biochrom Ltd., Cambridge, UK). 1 µg of total RNA was reverse-transcribed to complementary DNA (cDNA), which was then synthesized by the MMLV reverse transcription system (Invitrogen, Carlsbad, CA, USA) and mixed with Fast SYBR® Green Mix. Gene expression was examined by the StepOnePlus™ Real-Time PCR System. GAPDH served as the internal control and the primers used in the qPCR assays are listed in Table 1.

**Western Blot Analysis**
The RASFs were incubated with Antcin K (0, 0.3, 1, 3, or 10 µM) for 24 h, then washed with PBS and protein was extracted using RIPA buffer containing protease inhibitors. The Western blot analysis was performed as per an established protocol (19–22). The total proteins were quantified by the Thermo Scientific Pierce™ BCA Protein Assay Kit. Cell lysates were separated by SDS-PAGE electrophoresis then transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 4% BSA, then incubated with primary antibodies TNF-α, IL-1β, IL-8, p-FAK, FAK, p-85, p-AKT, AKT, p-p65, p-65 and β-actin overnight before being subjected to three consecutive washes in a mixture of tris-buffered saline and polysorbate 20 (TBST) and then the blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies at room temperature for 1 h. Enhanced chemiluminescent imaging of the blots was visualized with the UVP Biospectrum system (UVP, Upland, CA, USA).

**Enzyme-Linked Immunosorbent Assay (ELISA)**
The RASFs were plated in 6-well dishes and grown to confluence. The culture medium was then exchanged with serum-free RPMI 1640 medium. Cells were pretreated for 30 min with pharmacological activators Angiotensin II (a FAK activator), 740-YP (a PI3K activator), SC-79 (an AKT activator) and prostratin (an NF-κB activator) to stimulate the RSAFs, before 24 h of Antcin K (0, 0.3, 1, 3, or 10 µM) treatment. The conditioned medium was collected and stored at 4°C until use. Secreted expression of TNF-α, IL-1β and IL-8 was examined using ELISA kits for TNF-α, IL-1β and IL-8 (R&D Systems, MN, USA), according to the manufacturer’s protocols.

**CIA Mouse Model**
Forty C57BL/6J mice (aged 8–10 weeks) were obtained from the National Laboratory Animal Centre (Taipei, Taiwan) and randomly allocated to the four study groups (n=10 mice in each group). CIA was induced according to our previous report (23, 24), and the study protocol was approved by the Institutional Animal Care and Use Committee of China Medical University. After receiving two immunizations, 95% of the CIA group developed severe arthritis. Intraperitoneal injections of Antcin K (10 or 30 mg/kg; see the study timeline in (Figure 7A)) were administered on alternative days for a total of 4 weeks. Arthritis severity was assessed in each knee by plethysmometer measurements. The mice were sacrificed after 42 days of treatment. The phalanges and ankle joints were removed and fixed in 4% paraformaldehyde for micro-computed tomography (micro-CT) analysis.

**Human Synovial Tissue and Synovial Fluid Samples**
Human RA synovial tissues and synovial fluid samples (N=6) was obtained from patients with RA during knee replacement surgery and non-arthritis synovial tissue was obtained at arthroscopy after trauma joint derangement. The study protocol was approved by the Institutional Review Board of China Medical University Hospital (CMUH108-REC3-039). All patients completed written informed consent before study enrollment.

**Histopathological Analysis**
Histopathological changes were examined by hematoxylin and eosin (H&E) and Safranin-O staining using a light microscope. For immunohistochemical (IHC) staining, 5-µm sections were prepared from paraffin-embedded tissue, deparaffinized in xylene and rehydrated with ethanol or citrate buffer (pH 6.0) for 20 min at 95°C. The specimens were stained with specific primary antibodies anti-TNF-α (A11534), anti-IL-1β (SC-7884), or anti-IL-8 (ab18672). All specimens were separately scored by two researchers in a blinded manner and the inter-rater reliability was

**TABLE 1** | Sequences of RT-PCR primers.

| Gene     | Forward | Reverse |
|----------|---------|---------|
| GAPDH    | AAT GGACAAACTGGTCGTGGGAC | CCCTCCAGGGGATCTGTTTG |
| TNF-α    | CTTCTCTTCTATACGGCCCTCTG | GAGGACCTGGGAGTAGATGAG |
| IL-1β    | ATCATGCGCTTATACGGCCCTCTG | GTCGGGAGATTGGAGTAGGAG |
| IL-8     | CGGCGCCACACAGAATTATTTGA | CTGCGCCACACAGAATTATTTGA |

Serum levels of TNF-α, IL-1β, IL-8 and IL-6 in CIA mice were examined using ELISA kits for TNF-α and IL-1β (R&D Systems, MN, USA), IL-8 (MyBioSource, Inc, San Diego, USA) and IL-6 (Max™ Set Deluxe Kits, BioLegend, USA) according to the manufacturers’ protocols.
assessed. **Supplementary Table 1** presents the results from microscopic evaluation of inflammation scores and gives a detailed analysis of pathology findings in ankle joint synovium and adjacent tissue from healthy control mice and CIA mice (untreated or treated with Antcin K) in slides stained with H&E, according to previous research (25, 26). All of the slides were independently evaluated by two pathologists blinded to treatment.

**Statistical Analysis**

All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software). All results are represented as the mean ± standard deviation (SD) of at least six independent experiments. The Student’s *t*-test compared the means between experimental groups. The statistical difference was considered to be significant if the *p*-value was < 0.05.

**RESULTS**

**Upregulation of Proinflammatory Cytokines in Human RA Tissue and Plasma**

In RA, proinflammatory cytokines are the principal pathological factors underlying the destruction of bone architecture and tissue damage at the site of inflammation (27). We observed aggressive proinflammatory cytokine activities in RA synovial tissue and fluid samples compared with normal healthy samples. IHC staining revealed higher levels of TNF-α, IL-1β and IL-8 in human RA tissue compared with samples from healthy controls (*Figures 1A, B*). Similarly, ELISA assay data revealed significantly higher expression of these proinflammatory cytokines in human RA plasma compared with control plasma (*Figure 1C*).

**Antcin K Lowers the Production of Proinflammatory Cytokines in RASFs**

RASFs are critical in the initiation of proinflammatory cytokine secretion and their stimulation by proinflammatory cytokines influences cartilage and bone degradation in the RA microenvironment (28). In this study, we employed an immortalized RASF cell line (MH7A) that exhibits similar characteristics to human RASFs (29) and produces TNF-α (30–32), IL-1β (33–35) and IL-8 (35–38). According to MTT data, incubation of human RASFs for 24 h with Antcin K (0.3, 1, 3, or 10 μM) did not affect cell viability (*Figures 2A, B*), so we used all doses for investigating dose-dependent effects and only the highest concentration (10 μM) was used for the protein array and pathway activator analyses (*Figures 2C–E*). Findings from

![Figure 1: The identification of cytokines that interfere with RA severity. *(A, B)* RA and normal synovial tissue specimens were analyzed by IHC. *(C)* Cytokine plasma levels were upregulated in RA patients. ELISA assessments of blood samples (N=6) from RA patients and healthy volunteers determined levels of TNF-α, IL-1β and IL-8. Results are expressed as the mean ± SD. *P* < 0.05 compared with controls.](image-url)
FIGURE 2 | Antcin K inhibits TNF-α, IL-1β and IL-8 expression in RASFs. (A) Chemical structure of compound Antcin K. (B) Cells were incubated with Antcin K (0.3, 1, 3 or 10 μM) for 24 h and cell viability was examined by the MTT assay. (C–E) Cells were incubated with Antcin K (10 μM) for 24 h and the expression of 40 inflammatory factors was quantified by a human inflammation antibody array. (F) Inhibitory effects of Antcin K on TNF-α, IL-1β, IL-6, IL-8 and protein secretion were evaluated by Western blot. (G–I) Cells were incubated with Antcin K (0.3, 1, 3 or 10 μM) for 24 h, TNF-α, IL-1β and IL-8 mRNA expression and protein secretion were evaluated by qPCR, Western blot, and ELISA. Results are expressed as the mean ± SD. *P < 0.05 compared with controls.
the human inflammation protein array showed that TNF-α, IL-1β and IL-8 expression was significantly inhibited in human RASFs after 24 h of incubation with Antcin K (10 μM) compared with vehicle (Figures 2C–E), which was also the case with our Western blot results (Figure 2F). In further experiments, IL-6 expression was similarly significantly inhibited after RASFs were incubated with Antcin K (10 μM) for 24 h (Figures 2C–F). Antcin K was associated with significant dose-dependent (0.3, 1, 3, or 10 μM) reductions in levels of TNF-α, IL-1β and IL-8 mRNA, as well as protein expression in RASFs (Figures 2G–I). Thus, Antcin K appears to dose-dependently inhibit the production of proinflammatory cytokines in human RASFs.

The FAK/PI3K and AKT Signaling Cascades Are Implicated in Antcin K-Mediated Proinflammatory Cytokine Inhibition

The FAK/PI3K and AKT intracellular signaling pathways regulate cell growth and proliferation, and promote cytokine production (39). We examined whether FAK/PI3K and AKT signaling cascades interfere with the effects of Antcin K. Incubation of human RASFs with Antcin K (0.3, 1, 3, or 10 μM) for 24 h reduced the phosphorylation of FAK, PI3K and AKT (Figures 3A, 4A, 5A). Stimulating the RASFs with the FAK activator angiotensin, the PI3K activator 740-YP, and the AKT activator SC-79 (10 μM for each activator) significantly antagonized Antcin K-induced reductions of TNF-α, IL-1β and IL-8 production (Figures 3, 4, 5B, C). We then collected the cell culture medium from these treatments and we analyzed the levels of TNF-α, IL-1β and IL-8. Compared with untreated RASFs, levels of all three proinflammatory cytokines were significantly reduced in the RASFs treated with Antcin K (Figures 3–5). These findings suggest that Antcin K inhibits the production of these proinflammatory cytokines in RASFs via the FAK/PI3K and AKT signaling cascades.

The NF-κB Pathway Is Implicated in Antcin K-Mediated Proinflammatory Cytokine Inhibition

The NF-κB transcription factor is a critical player in inflammatory diseases (40) and is capable of activating several major proinflammatory cytokines including TNF-α, IL-1β and IL-8 (41, 42). We therefore examined whether Antcin K interferes with NF-κB phosphorylation. Treating RASFs with

**FIGURE 3** | The FAK pathway is involved in Antcin K-induced inhibition of TNF-α, IL-1β and IL-8 production in RASFs. (A) Cells were treated with Antcin K (0.3, 1, 3 or 10 μM) for 24 h and FAK phosphorylation was examined by Western blot. (B–D) Cells were pretreated with the FAK activator Angiotensin II for 30 min, then treated with Antcin K (10 μM) for 24 h. TNF-α, IL-1β and IL-8 mRNA expression and protein secretion were examined by qPCR, Western blot and ELISA. Results are expressed as the mean ± SD. *P < 0.05 compared with controls; #P < 0.05 compared with the Antcin K-treated group.
Antcin K (0.3, 1, 3, or 10 μM) for 24 h dose-dependently reduced NF-κB phosphorylation (Figure 6A). Prostratin rescued Antcin K-induced inhibition of TNF-α, IL-1β and IL-8 expression (Figures 6B–D). Transfecting the RASFs with the NF-κB luciferase plasmid after incubation with Antcin K (0.3, 1, 3, or 10 μM) for 24 h dose-dependently reduced NF-κB luciferase reporter activity (Figure 6E). Antcin K appears to inhibit proinflammatory cytokines by inhibiting the NF-κB signaling cascades.

Antcin K Attenuates Signs and Symptoms of RA Disease in CIA Mice

We next examined the anti-arthritic effects of Antcin K in CIA mice. Antcin K 10 mg/kg and 30 mg/kg significantly reduced the extents of paw swelling and bone degradation in CIA mice compared with controls (Figures 7A–C). Micro-CT imaging of the hind paws showed how Antcin K ameliorated bone erosion and enhanced bone mineral density (Figures 7D, E). Serum levels of TNF-α, IL-1β and IL-8 were significantly reduced in Antcin K-treated CIA mice (Figures 7F–H); notably, IL-6 levels were also significantly reduced in further experiments (Figure 7I).

IHC staining of synovium tissue revealed significantly higher TNF-α, IL-1β and IL-8 expression in CIA mice compared with controls; both concentrations of Antcin K (10 mg/kg and 30 mg/kg) significantly reduced the levels of all three cytokines (Figures 8A–D). Inflammatory cell infiltration into the synovial cell layers was significantly and dose-dependently reduced by Antcin K compared with untreated CIA mice (Figure 8E and Supplementary Table 1); synovium inflammation was also significantly and dose-dependently reduced by Antcin K compared with no treatment (Figure 8F and Supplementary Table 1). Thus, Antcin K appears to inhibit RA disease activity in CIA mice.

DISCUSSION

RA is associated with synovial inflammation and joint destruction (43). Onset of the disease is characterized by pannus formation and a hyperplastic synovium due to the deposition of synovial fibroblasts (10, 44). Compared with healthy fibroblasts, RASFs exhibit aggressive pathological characteristics in terms of structure and gene expression (45, 46). Moreover, activated RASFs exhibit a loss of contact inhibition (47) and accelerate the levels of adhesion molecules and proinflammatory cytokines including TNF-α, IL-1β and IL-8, causing synovial inflammation and cartilage damage (44).
Anti-TNF therapy has already proven effective in the treatment of RA (48), while IL-1β is also a key proinflammatory cytokine in inflammation-related diseases and cell injury, with disrupted IL-1β signaling accelerating the pathogenesis of inflammatory disease (49). As for IL-8, its abundant expression during inflammation promotes the formation of new blood vessels (50). Our study findings demonstrate high levels of TNF-α, IL-1β and IL-8 expression in human RA synovial tissue and plasma. As revealed by our CIA mouse model, the levels of these proinflammatory cytokines were closely associated with RA disease activity, with higher expression related to increasingly severe cartilage and bone degradation.

Several pharmacological inhibitors block these proinflammatory cytokines, including infliximab, etanercept, adalimumab and golimumab (51). However, loss of treatment responsiveness is common and side effects can develop that only subside after treatment discontinuation (52). Indigenous or traditional medicines involving herbal extracts are often considered to be 'natural' and causing fewer side effects than synthetic pharmacotherapies. The unique fungus A. cinnamomea has been used for hundreds of years in traditional medicines of Taiwan to treat liver diseases, food and drug intoxication, hypertension, cancers and inflammatory disorders, amongst other conditions (15, 53). Evidence has shown that extracts from A. cinnamomea have anti-inflammatory properties, such as the active compound ergostatrien-3β-ol (ST1), which significantly decreases mRNA levels of pro-inflammatory genes including IL-6 and inducible nitric oxide synthase (iNOS) and levels of TNF-α, NF-κB and IL-6 protein expression, reducing inflammatory reactions after skin flap surgery (54). Moreover, when cultured in the solid state and in wood, A. cinnamomea protects the lung against hyperoxia-induced acute lung injury in mice by reducing hyperoxia-induced generation of reactive oxygen species (ROS) and suppresses IL-6, TNF-α, IL-1β, IL-8, IKKα/β and iNOS expression (55). Amongst the functional compounds derived from the fruiting bodies of A. cinnamomea (56), the findings of this suggest that the triterpenoid Antcin K mediates anti-inflammatory activity in RA disease with no apparent signs of cytotoxicity.

The FAK/PI3K and AKT signaling cascades initiate proinflammatory cytokine production (57, 58) and are important in inflammation to regulate multicellular functions at the site of synovial proliferation and promote cytokine production from RASFs (59, 60). Antcin K treatment inhibits the migration and invasion of human hepatoma cells by suppressing the phosphorylation of FAK, PI3K and AKT.

![FIGURE 5](image-url)
Our study demonstrates that Antcin K downregulates FAK/PI3K and AKT phosphorylation. When we subsequently used specific activators to upregulate TNF-α, IL-1β and IL-8 expression, Antcin K treatment restored the levels to normal. These findings suggest that FAK/PI3K and AKT intracellular signaling cascades are pivotal in Antcin K-mediated proinflammatory cytokine production in RASFs.

NF-κB is a transcription factor that is not only vital in inflammatory responses, but also is a key molecule in the link between chronic inflammation and cancer (41, 62). We therefore examined whether Antcin K interferes with the NF-κB pathway. Interestingly, we found that 24 h of Antcin K treatment inhibited NF-κB phosphorylation and this activity was rescued by the NF-κB activator prostratin. Moreover, when we transfected RASFs with the NF-κB luciferase plasmid, we found that Antcin K dose-dependently inhibited NF-κB luciferase activity. These findings suggest that Antcin K has potential for blocking proinflammatory cytokine production via the inhibition of the FAK/PI3K, AKT and NF-κB signaling cascades.

Structural cartilage and bone degradation are the hallmarks of RA, with circulating RASFs promoting this damage to unaffected joints (63), contributing to the production of cytokines and proteolytic enzymes that degrade the extracellular matrix (45). Our experimental findings reveal that CIA mice administered intraperitoneal injections of Antcin K (10 mg/kg or 30 mg/kg) exhibit reductions in paw swelling, bone erosion and cartilage bone degradation, as well as lower serum levels of proinflammatory cytokines TNF-α, IL-1β, IL-8 and IL-6 compared with normal controls.

In conclusion, our observations indicate that the inhibition of TNF-α, IL-1β and IL-8 production in RASFs and in serum and synovial tissue from CIA mice attenuates signs and symptoms of RA (Figure 9) and that Antcin K significantly inhibits these proinflammatory cytokines via the FAK/PI3K, AKT and NF-κB signaling cascades. Derivatives of A. cinnamomea have exhibited varying pharmacological activity in experimental models of tumors, inflammation, immunomodulation, hepatotoxicity, diabetes and hyperlipidemia (53). In one study, triterpenoids isolated from A. cinnamomea mycelia were protective against acute alcohol-induced liver injury in mice (64) and in another study, A. cinnamomea polysaccharides inhibited vascular endothelial growth factor receptor (VEGFR) signaling in bovine aortic endothelial cells (65). This study has shown for the first time that the A. cinnamomea extract Antcin K inhibits RA disease activity in human RASFs and in a mouse model of RA. These findings offer new opportunities for RA disease management.
FIGURE 7 | Antcin K ameliorated paw swelling and cartilage degradation in CIA mice. (A) Workflow of CIA induction and Antcin K injections. (B, C) Hind paw swelling was photographed and measured with a digital plethysmometer in healthy controls, untreated CIA mice, and in CIA mice administered Antcin K 10 mg/kg or 30 mg/kg for 48 weeks. Representative micro-CT images of the hind paws were recorded on Day 48. (D, E) Quantifications of bone volume, bone surface, and bone density. (F–I) Serum levels of TNF-α, IL-1β, IL-6 and IL-8 were analyzed by ELISA. Results are expressed as the mean ± SD. *P < 0.05 compared with controls; #P < 0.05 compared with the untreated CIA mice.
FIGURE 8 | Effects of Antcin K quantified by histopathological changes in ankle joints from CIA mice. (A) Representative magnified images of histologic sections taken from ankle joints that were stained with H&E and Safranin-O, and IHC staining for TNF-α, IL-1β, and IL-8 antibodies from healthy controls, untreated CIA mice, and CIA mice administered Antcin K 10 mg/kg or 30 mg/kg (N=6 per group). (B–D) Quantification of TNF-α, IL-1β, and IL-8 expression by IHC score. (E, F) Quantification of inflammation scores and pathology findings in ankle joint synovium and adjacent tissue (details are provided in Supplementary Table 1). Results are expressed as the mean ± SD. *P < 0.05 compared with controls; #P < 0.05 compared with the untreated CIA mice.
INSTITUTIONAL REVIEW BOARD STATEMENT

The animal study and protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC Approval No: 2016-295).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The study protocol was approved by the Institutional Review Board of China Medical University Hospital (CMUH108-REC3-039). Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin. The animal study and protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC Approval No: 2016-295). Written informed consent was obtained from the owners for the participation of their animals in this study.

FUNDING

This work was supported by grants from the Ministry of Science and Technology in Taiwan (MOST 110-2320-B-039-022-MY3; MOST 110-2314-B-039-008; MOST 110-2314-B-039-012), and...
The authors thank Iona J. MacDonald from China Medical University (Taichung, Taiwan) for her editing of this manuscript. The authors thank Wang Chao-Qun (Department of Pathology, Affiliated Dongyang Hospital of Wenzhou Medical University, Dongyang, Zhejiang, People’s Republic of China) for assisting with the histological analysis and interpretation of the study results.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.790925/full#supplementary-material
