Iridoid Glycosides From *Morinda Officinalis* F. C. How Alleviate Rheumatoid Arthritis and Its Associated Bone Deterioration and Protect Against Methotrexate-induced Toxicity in Collagen-induced Arthritic Rats

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Abstract

Background: *Morinda officinalis* F.C.How (MO), also known as “Ba-Ji-Tian” in Chinese, has long been used to treat osteoporosis and rheumatoid arthritis (RA) in China, knowing that iridoid glycosides (IG) isolated from this plant possess anti-inflammatory, anti-osteoporotic and analgesic activities. The study was to evaluate the ameliorating effect of MOIG on joint swelling and bone destruction and the protective effect against MTX toxicities.

Methods: The anti-arthritic activity of MOIG was investigated by clinical arthritis scoring, paw swelling inspection, as well as histological analysis in CIA rats. The anti-bone loss activity of MOIG was evaluated by bone mineral density (BMD) and bone morphometric analysis assessed by Micro-CT and biochemical parameters in serum related with bone metabolism. The serum metabolomics and NF- κ B signaling pathway were used to explore and clarify the action mechanism.

Results: The results showed that MOIG was able to alleviate the paw swelling and arthritic severity, and reduce the synovial tissue proliferation and inflammatory cell infiltration in the CIA rats. In addition, MOIG decreased bone loss and improved the micro-structure of the bone trabecular in CIA rats through regulating bone formation and bone resorption. MOIG could also reduce effectively protect against MTX-induced damage to the liver, lung and stomach. The result of metabolomics analysis showed that MOIG was involved in the regulation of D-glutamine and D-glutamate metabolism, taurine and hypotaurine metabolism, valine, leucine and isoleucine biosynthesis, and alanine, aspartate and glutamate metabolism. Furthermore, MOIG inhibited OC formation and differentiation through participating in LPS-induced NF- κ B signaling.

Conclusion: MOIG could attenuate the paw swelling and bone loss through regulation of amino acid metabolism, reduce the side effects caused by MTX, and may serve as a novel therapeutic for the management of patients with rheumatoid arthritis (RA).

1 Introduction

Rheumatoid arthritis (RA) is the most prevalent rheumatic disease characterized by joint inflammation and hyperplasia of the synovial tissue, which provokes massive cell infiltration and overproduction of inflammatory mediators, eventually leading to cartilage and bone destruction and functional disability[1]. Bone loss has been recognized as a complication of RA for more than a century, and may result in an increased risk of osteoporotic fracture in RA patients[2]. Current therapies available for RA include non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and disease-modifying anti-rheumatic drugs (DMARDs)[3]. Skeletal complications of RA are declining with the utilization of DMARDs probably due to decrease in pro-inflammatory cytokines that drive chronic inflammation[4]. However, despite significant progress in the treatment of RA, some skeletal manifestations of disease including fracture and osteoporosis still occur at high frequencies[2, 5]. The medications for the treatment of osteoporosis, such as bisphosphonates, anti-RANKL (receptor activator of nuclear factor kappa B ligand) antibody,

denosumab, anabolic agent and teriparatide, have been shown to improve the bone mineral density (BMD) in RA patients, but their ability to improve generalized bone loss is limited[6–8]. Long-term use of anti-rheumatoid drugs often causes further bone loss. It is therefore necessary to develop new medications that can both ameliorate the syndrome of joint inflammation and reduce bone loss for the sake of improving the life quality of RA patients.

Fibroblast-like synoviocytes (FLSs) are known to play an essential role during the process of development and progression of RA. Activated FLSs not only manifest the properties of hyper-proliferation and insufficient apoptosis, but participate in inflammatory response through secretion of various pro-inflammatory cytokines including interleukin (IL)-1 β and IL-6[9]. In addition, bone erosion starts early following the onset of RA and progresses throughout the course of the disease. On the one hand, the proliferating synovium produces an elevated amount of proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF)- α , and matrixdegrading enzymes matrix metalloproteinases and cathepsins to exacerbate bone and cartilage destruction[10]. On the other hand, elevated levels of inflammatory cytokines activate the RANKL to increase osteoclast formation in the joints, thus promoting bone destruction in RA[11]. Osteoclasts (OCs) are derived from bone marrow-derived macrophages (BMMs). Their formation, differentiation and maturation are mainly regulated by macrophage colony-stimulating factor (M-CSF) and RANKL[12]. RANKL, TNF- α and IL-1 bind to RANK on the surface of OCs to recruit TRAF6[13, 14]. The accumulation of TRAF6 activates NF- κ B, and separates the NF- κ B subunit from I- κ B, which immediately enters the nucleus and binds to the target gene promoter[13]. Therefore, the activation of key transcription factors of OCs, such as the activated protein 1 (AP-1) family, C-Fos and NFATC1, promotes OC formation and bone resorption[15].

Methotrexate (MTX) has been considered an anchor drug and is used as the first-line therapeutic modality for RA. However, long-term MTX treatment may result in some adverse effects, including hepatotoxicity, ulcerative stomatitis, leukopenia, neurological damage, and memory loss, especially in RA patients with gastrointestinal and hepatobiliary disorders[16–19]. These adverse effects lead to discontinuation of the drug in 10–37% RA patients. It is therefore urgent to develop drugs that can both mitigate the symptom of RA and reduce the toxicity of MTX.

Bajitian, the dried root of *Morinda officinalis* F.C.How (MO), is one of the best-known traditional Chinese herbs that has been used as a *Yang* tonic agent to invigorate kidney function and improve sexual performance for more than 2000 years in China[20]. Currently, a number of chemical constituents have already been isolated from this plant, including anthraquinones, flavonoids, iridoid glycosides, polysaccharides and oligosaccharides[20]. Iridoid glycosides, such as monotropein (MON), possess a wide range of bioactivities, including anti-inflammatory, anti-osteoporotic, anti-apoptotic and anti-catabolic activities in chondrocytes[21]. The MO roots have been used to treat RA and osteoporosis, and iridoid glycosides from MO root (MOIG) exhibit anti-inflammatory and anti-osteoporotic activities. Therefore, it is hypothesized that MOIG may be able to reduce inflammation-induced bone loss and bone destruction. Indeed, our previous study found that MON could decrease bone loss induced by

ovariectomy and lipopolysaccharide (LPS) stimulation in mice, and also counteract the injury of LPS in osteoblasts (OBs)[22].

Metabolomics, which focuses on the dynamic changes of endogenous metabolites of biological organism or tissue cells, has been used to explore pharmacological mechanisms of traditional Chinese medicines according to the systematic biology theory and methods. The present study aims to investigate the ameliorating effects of MOIG on joint swelling and bone destruction and its protective effect against MTX toxicities, and explore the action mechanism of MOIG based on serum metabolomics in CIA rats and the inhibitory effect on FLSs and OCs through the NF- κ B signaling pathway, in an attempt to gain global insights into the action of MOIG on RA and its complicated bone erosion.

2 Materials And Methods

2.1 Animals and reagents

6–8 weeks old Wistar rats (160–180 g) (Sippur Will Kay Company, Shanghai, China, Certificate No. SCXK 2013-0016) were housed at the Experimental Animal Center of Zhejiang Chinese Medical University. The rats were acclimatized for a week on a 12 h light-dark cycle under a temperature of $(24 \pm 0.5) ^\circ\text{C}$ and humidity of $47.5\% \pm 2.5\%$ before drug administration. All animals were handled according to the National Institute of Health (NIH) guidelines on the ethical use of animals, and received humane care. This study was carried out in accordance with the recommendations of the Guideline for ethical review of animal welfare (GB/T 35892 – 2018), and was approved by the Bioethic Committee of Zhejiang Chinese Medical University.

The materials and reagents used in this study were monetropein (MON, 98% purity) and deacetyl asperulosidic acid (DA, 98% purity) (Yuanye Biological technology Co., Ltd., shanghai, China); tripterygium glycosides (TGs) (Fudan Fuhua Pharmaceutical Co., Ltd, shanghai, China); MTX (Xinyi Pharmaceutical Co., Ltd., Shanghai, China); alendronate (Alen) (MoShaDong Pharma (SINGAPORE) Pte. Ltd. Savio Industrial S.r.l.); Bovine type II collagen (CII); incomplete Freund's Adjuvant (IFA) (Chondrex Co., USA and Sigma Co., USA); ELISA assay kits for IL-6 and IL-1 β (Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China); the bio-markers for bone metabolism in serum, including osteocalcin (OCN), osteoprotegerin (OPG), C-Telopeptide of type I collagen (CTX-I), deoxypyridinoline (DPD), tartrate resistant acid phosphatase (TRAP) and RANKL (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All the other reagents were of analytical-grade purity, and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2 Preparation of iridoid glycosides from MO roots and HPLC analysis

The dried specimen and the roots of *Morinda officinalis* were collected by Yi Shen (the first author of this manuscript), from Zhangzhou, Fujian Province of China, in October 2017, and identified by Professor Qiao-yan Zhang of the College of Pharmaceutical Sciences, Zhejiang Chinese Medical University in

Hangzhou. The GPS information of the collection place is as follows: the longitude is 113°12'39.6", the latitude is 34°54'7.0", the altitude is 722 meters. *Morinda officinalis* was identified according to Flora of China, 1999, 71(2): 199. The details of the chemical composition analysis of the ethanolic extract of MO roots have been reported in our earlier publication[23]. Recently, our publication details the method of enriching the preparation of MOIG[24]. Extraction and preparation methods were as follows: the dry roots of MO (2.0 kg) were powdered and extracted under permeation with 32.0 L solution of ethanol-water (70:30, v/v) for 20 h. The extracts were concentrated to dryness using rotary evaporator (SENCO, Shanghai, China) under reduced pressure to obtain MO extracts which were dissolved in appropriate volume of distilled water to obtain 1.0 g crude drug/mL working solution. The XDA-1 macroporous adsorption resin was used to adsorb the MO extracts (1.0 g crude drugs/mL), and eluted with water and 10% ethanol. The elutes of 10% ethanol were collected and concentrated to obtain MOIG, and the dried MOIG were kept at 4°C. Finally, based on HPLC, the major peaks were identified as MON and DA by comparison with the standard compounds (Supplementary Figure S1). According to the chromatographic conditions, the content of MON and DA in MO extracts was 12.66 mg/g and 6.49 mg/g, respectively. The yield of MOIG was 2.43%, and the content of MON and DA in MOIG was 385.68 mg/g and 236.17 mg/g, respectively.

2.3 Induction of CIA model in rats and drug treatment

The CIA model was established in Wistar rats by reference to the previous method with minor modifications[25]. Briefly, bovine CII dissolved in 0.1 M acetic acid was emulsified in an equal volume of IFA at 4 °C to obtain a final CII concentration of 1 mg/mL. On the first day of the experiment, except the normal control group, the other rats were induced by injecting 0.2 mL of CII emulsion intradermally per rat at the base of the tail, as the primary immunization. Three weeks after the primary immunization (on day 21), the rats were boosted again by injecting the same volume of CII emulsion in the same way.

Seventy-two rats were equally randomized to 8 groups: normal and model group, where the rats were orally administered with distilled water containing 0.5% Carbosy Methyl Cellulose (CMC-Na) sodium (1 mL/100 g body weight); positive control groups, where the CIA rats were orally administered with TGs (6 mg/kg body weight, once every day), Alen (1 mg/kg body weight, once every day) and MTX (0.5 mg/kg body weight, once every 3 days); MOIG treatment groups, where the CIA rats were orally administered with different doses of MOIG (25, 50 and 100 mg/kg body weight, once every day). The rats were administered orally with vehicle, TGs, Alen, MTX and MOIG for 8 weeks from the day 28 of immunization. The time schedule is detailed in Fig. 1.

2.4 Measurement of spleen and thymus index

At the end of the experiment, all rats were sacrificed by cervical dislocation after collection of the serum samples. The spleen and thymus were immediately removed and weighed after dissection. The spleen index and thymus index refer to the ratio of the spleen weight and the thymus weight versus body weight (g/kg), respectively.

2.5 Measurement of paw swelling and arthritis score

From day 28 after the first immunization, the thickness of the hind paw swelling was measured every 7 days with a Vernier calliper (S-H13050655, Shanghai) by two independent investigators who were blinded to the experimental scheme. The paws were checked and graded for severity and swelling loci using a four-point scale: “0”, no change; “1”, erythema; “2”, signs involving the toe and ankle joints with mild swelling; “3”, severe swelling involving the entire hind or fore paws and unable to walk. The maximum arthritis score per rat was set at 12 (3 points × 2 fores and 2 hind paws)[26].

2.6 Histopathological study

For histological analysis of the knee joints, the left hind limbs of the rat were removed postmortem, fixed in 4% paraformaldehyde for more than 24 h, and finally decalcified in ethylene diamine tetraacetic acid (EDTA) for a month at 4°C. After decalcification, the tissues were dehydrated, processed, paraffin embedded, sliced into 4-μm sections, stained with hematoxylin and eosin (HE), and finally observed under a panoramic scanner (Pannoramic MIDI, Hungary).

2.7 Determination of bio-markers levels in serum

Rat blood was obtained and allowed to curdle 2 hours at room temperature. Serum was recovered and frozen at -80°C for biochemical marker assay. The levels of inflammatory mediators including IL-1β, IL-6, and bio-markers related to bone metabolism including OCN, OPG, CTX-I, DPD, RANKL and TRAP were measured with commercially ELISA kits following the standard kit procedures.

2.8 Micro-CT analysis

For morphometric analysis of the distal femur, the rat right hind limbs were removed postmortem and then fixed in 4% paraformaldehyde for more than 24 h. The static parameters of bone histomorphometry at the distal femur were analyzed with micro-CT (V2.1.2, eXplore Locus SP, USA) under the following conditions: tube current was 80 μA, peak voltage was 80 kV, scanning resolution ratio was 14 μm, exposure time 2,960 ms, rotation angle 360 degree, increment 0.4 deg, frame average 4, and image resolution ratio 29 μm. The obtained images were imported into the Micview V2.1.2 software for 3D reconstruction. Parameter correction was based on the specific scanning protocol. It was necessary to enter the corresponding parameters at precision reconstruction when the relevant parameters such as BMD and bone mass were measured. After 3D reconstruction of the bone images, the morphometric parameters were measured using Advanced Bone Analysis software: including BMD (mg/cc), bone volume fraction (BVf or BV/TV; %), bone surface to bone volume (BS/BV; 1/mm), trabecular thickness (Tb.Th; mm), trabecular number (Tb.N; 1/mm), and trabecular separation (Tb.Sp; mm). All parameters were three-dimensionally calculated.

2.9 Observation of MTX toxicity and protective effects of MOIG

The CIA rat model was established as described in Sect. 2.3. Seventy rats were equally randomized to 7 groups, including normal control group and model control group (orally administered with 0.5% CMC-Na, 1 mL/100 g body weight); MTX treatment group (orally administered with MTX, 0.5 mg/kg body weight, once every 3 days); MOIG treatment group (orally administered with MOIG, 50 mg/kg body weight, once every day); MTX plus MOIG treatment groups (orally administered with MOIG-50 mg/kg, once every day in combination with MTX 0.25, 0.50 and 0.75 mg/kg body weight, once every 3 days). The rats were administered orally with these drugs for 7 weeks from day 28 of immunization.

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercially available ELISA kits following the standard kit procedures. The liver, lung and stomach organs were collected for histological analysis staining. The tissue sections were HE stained, and observed under an Olympus microscope (BX61VX, Japan).

2.10 Metabolomics analysis

2.10.1 Serum sample preparation

Rat serum (10 μ L) with 190 μ L 67% acetonitrile (ACN) was added into a 1.5 mL eppendorf tube, and mixed using vortex for 30 s. The mixture was centrifuged (12,000 rpm) at 4 °C for 15 min. Then, 10 μ L supernatant was analyzed by UPLC-MS. Quality control (QC) samples were prepared by pooling equal volumes of each analyzed sample.

2.10.2 UPLC-MS analysis

UPLC-Q-TOF-MS (Agilent 1290 infinity LC system and Agilent 6538 UHD Accurate-mass Q-TOF spectrometer, Agilent technologies, Santa Clara, CA, USA) was used for metabolomics analysis. A Waters Acquity HSS T3 column (2.1 $\times$ 100 mm, 1.8 μ m; Waters, Milford, MA, USA) was used as the stationary phase and the column temperature was maintained at 25°C. The mobile phase (A) was ACN containing 0.1% formic acid, and mobile phase (B) was water containing 0.1% formic acid. The program of gradient elution was as follows: 5–5% A at 0–2 min, 5–15% A at 2–8 min, 15–30% A at 8–10 min, 30–95% A at 10–13 min, and 95%A at 13–15 min, with a flow rate of 0.4 mL/min. The main working parameters of the Q-TOF mass spectrometer are summarized as follows: capillary voltage of 4 kV for positive ion mode and 3.5 kV for negative ion, nebulizer 45 p.s.i., drying gas 11 L/min, gas temperature 350 °C and fragmentor voltage 120 V, skimmer voltage 60 V. The mass range was set at m/z 100–1100.

2.10.3 Data processing and multivariable data analysis

Massbank-data and Human Metabolome Database (HMDB) were used to perform the data deconvolution, alignment and data reduction, which can obtain a list of mass and retention time pairs with corresponding intensities for all the detected peaks from each data file in the data set. After area normalization of the data, the resultant data matrices were introduced to the WUKONG platform Online (<http://www.omicsolution.org/wu-kong-beta-linux/hca/>) for unsupervised principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA). Variable importance plot (VIP) is a

parameter which estimates the importance of each variable in a PLS-DA model. The importance of the metabolites was reflected by the VIP value and Student's t-test of the value. Metabolites with VIP > 1 and $P < 0.05$ were chosen as potential biomarkers.

2.10.4 Metabolic pathway analysis

The comprehensive metabolic network was mapped by integration of all potential biomarkers identified in the present research by means of Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.ad.jp/kegg/>), HMDB (<http://www.hmdb.ca/>), and MetaboAnalyst 4.0 (<http://www.metaboanalyst.ca/>) were used to identify the affected metabolic pathways and facilitate further biological interpretation.

2.11 The culture and assay of proliferation and inflammatory cytokines of fibroblast-like synoviocytes (FLSs)

Synovial tissue samples were obtained from the knees of five patients with active RA (as diagnosed according to the 2010 Rheumatoid arthritis classification criteria) during knee joint arthroscopic operations. The synovial tissue was cut into 1–2 mm³ pieces and digested with DMEM containing 4 mg/mL I collagenase at 37 °C for 2 h. The FLSs were collected by centrifuge at 1,500–2,000 rpm for 4 min, and cultured in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humid incubator containing 5% CO₂. Cell medium was changed every 3–4 days. The FLSs were grown in a monolayer, and cells between the third and sixth generations were used for all experiments.

Cell viability was detected using the CCK-8 kit (Dojindo, China) according to the provided instructions. Briefly, cultured RA-FLSs were plated in 96-well plates at a density of 4,500 cells/well in DMEM containing 10% FBS, and stimulated with or without 10 ng/mL TNF-α for 4 h, and then treated with MOIG (25, 50, and 100 µg/ml) for additional 24 h. After this incubation period, 8 µL CCK-8 solution was added to each well and cells were incubated for 3 h. The absorbance at 450 nm was then measured via a microplate reader.

3×10^4 RA-FLSs were incubated in 24-well plates for 24 h, stimulated with 10 ng/mL TNF-α for 4 h, and then treated with MOIG at a concentration of 25, 50 and 100 µg/mL for 24 h. Supernatants were then collected to determine IL-6 and IL-8 levels according to the instructions of the ELISA kit.

2.12 Osteoclast culture

C57BL/6 mice aged 6 weeks were used to isolate bone marrow macrophages from the femur. The extracted bone marrow cells were cultured in α-MEM containing with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 5 ng/mL M-CSF in a humidified atmosphere of 5% CO₂ for 24 h. Non-adherent cells, which were considered as BMMs, were collected and cultured in α-MEM medium containing 30 ng/mL M-CSF for 3 days, and then cultured in α-MEM medium containing 30 ng/mL M-CSF and 20 ng/mL RANKL for 48 h, followed treatment with 30 ng/mL M-CSF and 200 ng/mL LPS for 6 days to further induce cells to differentiate into OCs.

2.13 Viability and TRAP activity assay and TRAP staining of OCs

OCs induced from BMM cells in 96-well plates were treated with various concentrations of MOIG for 48 h. The viability of OCs was measured with a CCK-8 kit, and the TRAP activity was determined according to our previous methods. The TRAP activity was represented as nanomoles p-nitrophenol per minute per 100 OCs. For TRAP positive multinucleated cell staining, BMMs-derived OCs were fixed with 4% paraformaldehyde and stained for TRAP activity using an Acid Phosphatase Kit (Sigma-Aldrich, St. Louis, MO, USA). The number and area of OCs containing 3 or more nuclei were counted and photographed using an inverted microscope (Nikon Corporation, Tokyo, Japan).

2.14 Western blot

Cells were washed with PBS and lysed in RIPA assay buffer for 30 min at 4 °C. The cell protein was collected by centrifugation, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% BSA in tris-buffered saline with tween (TBST) buffer for 1 h and then incubated with primary antibodies overnight at 4 °C. Then the membranes were washed with TBST and incubated with HRP-conjugated second antibody for 1 h. Then, protein bands were detected using electrochemiluminescence (ECL) reagent (Tanon, Shanghai, China), and then imaged using E-Gel Imager (Tanon-5200 Multi, Shanghai, China).

2.15 Statistical analysis

All data analyses were completed using Graphpad Prism version 5.0 software and IBM SPSS statistic 21.0 software. Data are expressed as the mean $\pm$ SD. Data comparison was shown by using Student's t-test. *P* values lower than 0.05 were considered statistically significant.

3 Results

3.1 Effects of MOIG on body weight and spleen and thymus indexes in CIA rats

The CIA rats showed significantly lower body weight compared with normal rats (Fig. 2A). The body weight of rats administered with MOIG, TGs, Alen and MTX was increased significantly as compared with that of the CIA rats. The spleen index of the CIA rats was significantly increased, and administration of TGs significantly decreased the spleen index in the CIA rats but administration of MOIG, Alen and MTX had no significant effect on the spleen index of the CIA rats (Fig. 2B). The thymus index of the CIA rats was significantly decreased, and administration of MOIG at a dose of 100 mg/kg significantly increased the thymus index of the CIA rats (Fig. 2C).

3.2 MOIG mitigates paw swelling and arthritis score in CIA rats

The paw swelling and arthritis score were determined to evaluate the severity of arthritis. As expected, the hind paw volume was obviously increased after CIA immunization. However, TGs, Alen and MTX showed significant potency in inhibiting the paw swelling of the CIA rats. Interestingly, MOIG oral administration (25, 50 and 100 mg/kg) could dramatically reduce paw edema from day 28 compared to that of model control CIA rats. Similarly, the CIA rats treated with TGs, Alen and MTX exhibited decreased arthritis scores compared with CIA group from day 28 (Fig. 2D). As shown in Fig. 2E, the increase of the arthritis scores in the CIA rats was also notably weakened by 8-week MOIG treatment at doses of 25, 50 and 100 mg/kg. All these data were parallel to the degree of the paw swelling of the rats.

3.3 MOIG reverses the joint histopathological change of CIA rats

The therapeutic effect of MOIG on the CIA rats was further confirmed by histological analysis. Massive mononuclear cell infiltration of the synovial tissue and synovial hyperplasia, pannus formation, cartilage hyperplasia and erosion were observed in the ankle joint of the CIA rats (Fig. 3A), while these symptoms were reduced significantly in the CIA rats treated with TGs, Alen and MTX, as well as in the CIA rats administered with MOIG (25, 50 and 100 mg/kg) as compared with the model control CIA rats. These results indicate that MOIG at doses of 25, 50 and 100 mg/kg successfully alleviated the arthritic symptom induced by CIA.

3.4 MOIG inhibits the levels of pro-inflammatory cytokines in serum of CIA arthritic rats and FLS cells

Knowing that pro-inflammatory cytokines play a vital role in the maintenance of chronic inflammation and tissue damage during RA progression, we measured the serum levels of pro-inflammatory cytokines IL-1 β and IL-6 to assess the severity of arthritis using by ELISA kits. The results showed that the serum levels of IL-1 β (Fig. 3B) and IL-6 (Fig. 3C) in the CIA model rats were markedly augmented compared to those in the normal rats. However, the serum levels of these cytokines were significantly down-regulated after treatment with TGs, MTX and MOIG for 55 days when compared with the CIA model rats.

As shown in Fig. 3D, MOIG did not significantly proliferate in normal FLS cells. However, it inhibited the proliferation of TNF- α -stimulated FLSs (Fig. 3E). In addition, MOIG significantly inhibited IL-6 and IL-8 production in TNF- α -stimulated FLSs (Fig. 3F and 3G).

3.5 MOIG modulates the serum biochemical parameters related to bone metabolism in CIA arthritic rats

Bone metabolism-related biochemical parameters were detected using ELISA kits. As shown in Fig. 4A, serum OCN, an indicator of bone formation, was increased significantly in the CIA rats compared with the normal rats at the end of 8-week treatment, while treatment with TGs and MOIG significantly reduced the

OCN level in the CIA rats. Serum OPG was decreased significantly in the CIA rats compared with the normal group, and treatment with MOIG-100 mg/kg significantly increased the levels of OPG in the CIA rats (Fig. 4B). The serum levels of bio-markers related with bone resorption including CTX-I (Fig. 4C), DPD (Fig. 4D), RANKL (Fig. 4E) and TRACP (Fig. 4F) were significantly enhanced in the CIA rats compared with normal rats, and treatment with TGs, Alen, MTX and MOIG significantly decreased the levels of these indicators.

3.6 MOIG increases BMD and improves the micro-architecture of the femurs of the CIA arthritic rats

The CIA rats exhibited juxta-articular bone osteopenia as evidenced by micro-CT analysis. The micro-CT images showed a lower bone volume in the trabecular bone of the distal femur and greater spacing between the trabecular bones in the CIA rats (Fig. 4G). Also, there was destruction of the 3D trabecular bone structure in the distal femur (Fig. 4H). However, trabecular bone loss of the distal femur was inhibited in TGs, Alen and MTX and MOIG treated CIA-rats.

As shown in Fig. 4I, BMD was significantly decreased in the CIA rats as compared with the normal rats. However, the BMD value in TGs, Alen, MTX and MOIG treated rats was significantly higher than that in the model control CIA rats. Further analysis of the bone structural properties revealed that the trabecular bone micro-architecture in the CIA rats were significantly decreased in BVF (Fig. 4J), Tb.Th (Fig. 4M) and Tb.N (Fig. 4L), and had a significant increase in BS/BV (Fig. 4K) and Tb.Sp (Fig. 4N) compared with the normal rats. Trabecular BV/TV, Tb. N, and Tb. Th were increased, and Tb. Sp and BS/BV were decreased significantly after treatment with TGs, Alen, MTX and MOIG. These findings indicate that arthritis had an especially strong deteriorating effect on the peri-articular bone adjacent to the inflamed joints, and MOIG could reverse this detrimental effect.

3.7 MOIG prevents against the toxicities of MTX in CIA rats

As shown in Fig. 5A-B, the content of ALT and AST were significantly increased in the CIA rats compared with that in the normal rats ($P < 0.01$). The serum level of ALT and AST in MTX group was increased ($P > 0.05$). However, the level of ALT and AST was significantly decreased when MOIG and MOIG-MTX were given to the CIA rats ($P < 0.05$, $P < 0.01$), indicating that MOIG had a protective effect on the liver. Compared with the normal group, the liver tissue of the CIA model rats was mildly fibrotic with inflammatory cell infiltration, and after MTX treatment the inflammatory cell infiltration was significantly increased, the hepatocyte was expanded, and severe focal lobular central necrotic fibrosis was observed. As shown in Fig. 5C, MOIG alleviated severe hepatitis and fibrosis caused by MTX, indicating that MOIG could attenuate the adverse effects of MTX and protect the liver effectively.

As shown in Fig. 5D, mildly inflammatory cell infiltration appeared in the lung tissue of CIA model rats. After MTX administration, the inflammatory cell infiltration was significantly increased and fibrosis in lung become more severe. After MOIG treatment, the inflammatory cell infiltration in the lung was significantly decreased, indicating that MOIG could alleviate lung inflammation caused by MTX and

obviously inhibit the formation of pulmonary fibrosis. All these results indicate that MOIG could reduce the adverse effects of MTX and protect the lung effectively.

Compared with the normal group, mildly inflammatory cell infiltration appeared in the gastric mucosa of CIA model rats. As shown in Fig. 5E, inflammatory cell infiltration in the stomach was significantly increased after MTX administration, and decreased significantly after MOIG treatment, demonstrating that MOIG could reduce the gastrointestinal reaction of MTX and protect the gastric mucosa effectively.

3.8 Metabolomics insights

3.8.1 Multivariate data analysis and identification of potential biomarkers in serum of CIA rats

The QC sample was injected regularly (every four injections) through the entire running process to monitor the system stability. The results showed that the LC-MS analysis was stable and reliable.

Multivariate analysis was performed to find metabolites that mostly discriminated the study groups. PCA was performed using the features obtained from LC-MS data to investigate metabolic differences between normal control and CIA model control group samples. However, the two group samples were not fully distinguishable in the PCA score plot (Fig. 6A). To maximize separation, PLS-DA, a supervised pattern recognition method, was used to the same datasets. PLS-DA score plot indicated that the CIA model group samples were clearly separated from the normal control samples (Fig. 6B), with a modeling fit R^2X value of 0.498, a R^2Y value of 0.999, and a good prediction Q^2 value of 0.820. These results showed that metabolic changes indeed occurred in the serum of CIA rats. Based on the variables with $VIP > 1$ and $P < 0.05$, a total of 13 differential metabolites between the normal and model groups were selected as potential biomarkers (Table 1).

Table 1
Potential biomarkers and their metabolic pathway

No.	Metabolites Identified	Ion (m/z)	ESI	VIP	Normal vs. Model	MOIG vs. Model	Pathway
1	Glutamine	145.0618	[M-H]-	1.84	↓##	↑	D-Glutamine and D-glutamate metabolism
2	L-Glutamine	130.0497	[M + H] ⁺	1.77	↓##	↑	Alanine, aspartate and glutamate metabolism,D-Glutamine and D-glutamate metabolism
3	2-Oxobutyrate	101.0244	[M-H]-	1.76	↓##	↑	Unknown
4	Pantothenic acid	220.1176	[M + H] ⁺	1.79	↑#	↑	Pantothenate and CoA biosynthesis
5	Purine	119.0363	[M-H]-	1.67	↓#	↓	Unknown
6	Succinate	248.9601	[M-H]-	1.47	↓#	↑	Alanine, aspartate and glutamate metabolism,Citrate cycle (TCA cycle)
7	Palmitic acid	255.2321	[M-H]-	1.53	↓#	↑	Biosynthesis of unsaturated fatty acids
8	Mesaconic acid	129.0190	[M-H]-	1.40	↓#	↑	Unknown
9	Pyridoxamine 5'-phosphate	248.9984	[M + H] ⁺	1.27	↓#	↑	Vitamin B6 metabolism
10	Cytidine	244.0923	[M + H] ⁺	1.74	↓#	↑**	Pyrimidine metabolism
11	Piperidine	86.0965	[M + NH ₄] ⁺	1.53	↓#	↑**	Unknown
12	Xanthosine	285.0830	[M + H] ⁺	1.59	↓#	↑*	Purine metabolism
13	3-Phenyllactic acid	165.0550	[M-H]-	1.50	↑#	↓*	Unknown
14	Choline	104.1065	[M + H] ⁺	1.67	↑	↑*	Glycine, serine and threonine metabolism
15	Betaine	118.0862	[M + H] ⁺	1.35	↓	↑*	Glycine, serine and threonine metabolism

#*P* < 0.05, ##*P* < 0.01 Normal vs Model group. **P* < 0.05, ***P* < 0.01 MOIG vs Model group.

No.	Metabolites Identified	Ion (m/z)	ESI	VIP	Normal vs. Model	MOIG vs. Model	Pathway
16	L-Glutamate	148.0605	[M + H] ⁺	1.61	↓	↑**	D-Glutamine and D-glutamate metabolism, Alanine, aspartate and glutamate metabolism
17	Taurine	126.0214	[M + H] ⁺	1.36	↓	↑*	Taurine and hypotaurine metabolism
18	Isoleucine	132.1017	[M + H] ⁺	1.51	↓	↑*	Valine, leucine and isoleucine biosynthesis
19	Uracil	113.0301	[M + Na] ⁺	1.46	↑	↑*	Pantothenate and CoA biosynthesis
20	Arachidonyl dopamine	440.3128	[M + H] ⁺	1.49	↑	↑*	Unknown
21	Carnitine	162.1120	[M + H] ⁺	1.60	↑	↓**	Fatty acid metabolism
22	Adenosine	556.9290	[M + H] ⁺	1.67	↑	↓**	Purine metabolism
23	Kynurenine	209.0916	[M + H] ⁺	1.51	↑	↓*	Tryptophan metabolism
24	γ-Linolenic acid	277.2166	[M-H] ⁻	1.21	↓	↓*	Biosynthesis of unsaturated fatty acids
#P < 0.05, ##P < 0.01 Normal vs Model group. *P < 0.05, **P < 0.01 MOIG vs Model group.							

3.8.2 Metabolic pathway analysis of the CIA rats

Based on the identified potential biomarkers, the relevant metabolic pathways for RA were established by MetaboAnalyst online website. Pathways with an impact value more than 0.1 were screened out as the most potential target pathways involved in RA. The pathway analysis results indicated that the most important pathway related to RA was alanine, aspartate and glutamate metabolism (0.15) (Fig. 6C).

3.8.3 Metabolomic evaluation of MOIG treatment on CIA rats

To investigate changes in serum metabolites between the model and MOIG treatment groups, PCA and PLS-DA models were generated (Fig. 7A and B). PLS-DA score plot demonstrated a clear separation between the CIA rats and MOIG treatment rats ($R^2X = 0.425$, $R^2Y = 0.995$, and $Q^2 = 0.878$). The permutation test was repeated 200 times to validate the PLS-DA model. Finally, based on the variables with $VIP > 1$ and $P < 0.05$, a total of 24 significantly differentially expressed biomarkers were selected. As shown in Table 1, 24 biomarkers could be regulated by MOIG, and 9 out of them were not significantly different

with the model group. Then, the pathway was identified by MetaboAnalyst online website. The results showed that MOIG could regulate the amino acid metabolic pathway including D-glutamine and D-glutamate metabolism (1.00), taurine and hypotaurine metabolism (0.43), valine, leucine and isoleucine biosynthesis (0.33), alanine, aspartate and glutamate metabolism (0.26) (Fig. 7C). Interestingly, these differential metabolites regulated by MOIG were also involved into purine metabolism, pantothenate and CoA biosynthesis, and biosynthesis of unsaturated fatty acids. Glutamine, L-glutamine, 2-oxobutyrate, succinate, betaine, L-glutamate, taurine, isoleucine, choline and kynurenine, which are known to be related to amino acid metabolism, were all reversed by MOIG, suggesting that MOIG obviously had a reverse effect in regulating the dysfunction of amino acid metabolism in the CIA rats. Meanwhile, carnitine, gamma-linolenic acid and palmitic acid involved in lipid metabolism were significantly reversed by MOIG, demonstrating that MOIG could distinctly regulate the pathological changes of lipid metabolism in CIA rats. Xanthosine and adenosine involved in purine metabolism were also reversed by MOIG, indicating that MOIG could reverse the disturbance of purine metabolism in CIA rats. In summary, based on the differential metabolites and pathway analysis, the corresponding metabolic networks regulated by MOIG were depicted according to the KEGG (Fig. 7D).

3.9 MOIG inhibits the formation and differentiation of LPS-stimulated OCs through NF- κ B pathway

The effect of MOIG on OCs induced by LPS was investigated. As shown in Fig. 8A-F, MOIG had no significant effect on the viability of BMMs at the concentrations of 0.04, 0.4, and 4 μ g/mL, but significantly inhibited the TRAP activity of OCs, and decreased the number and the area of TRAP positive multinucleated OCs. In addition, MOIG could significantly inhibit the expression of NFATC1, C-Fos, CtsK and MMP-9 of OCs induced by LPS (Fig. 9A-E, Fig. S2-3), demonstrating that MOIG could inhibit the expression of related makers of OCs, which is necessary for the differentiation and function of OCs.

To explore the mechanism of MOIG in inhibiting the formation and differentiation of OCs, the regulatory effect of MOIG on the NF- κ B pathway of OCs was further investigated. As shown in Fig. 10A-D (Fig. S4-5), MOIG inhibited the accumulation of TRAF6, phosphorylation of p65 of the NF- κ B pathway of OCs stimulated by LPS, indicating that MOIG inhibited the activation of the NF- κ B pathway stimulated by LPS.

4 Discussion

The CIA rat is an established model for studying the pathology and treatment of RA. In the CIA model, arthritis is induced in rats by immunization with heterologous CII in adjuvant. The chief pathological features of CIA include a proliferative synovitis with infiltration of polymorpho-nuclear and mononuclear cells, pannus formation, cartilage degradation and erosion of bone[26]. It was found in our study that MOIG treatment decreased the paw swelling and the arthritis score, alleviated synovial hyperplasia and inflammatory cell infiltration in the CIA rats, indicating that MOIG possesses anti-arthritic activities.

Pro-inflammatory cytokines TNF, IL-1, IL-6, IL-8 and IL-17 produced by inflamed synovial tissues in RA are known to affect the differentiation of OCs and OBs with effects on the bone[27]. These cytokines induce RANKL expression of OBs and/or synovial fibroblasts and decrease OPG expression of OBs[28]. TNF- α is a key regulatory cytokine in the pathogenesis of RA synovitis and joint destruction. TNF- α promotes the proliferation of OC precursor cells and bone resorption activity, and also inhibits OB differentiation and bone formation[29]. Furthermore, IL-1 also impairs mineralizing nodule formation to inhibit OB maturation. The elevated levels of inflammatory cytokines and the ratio of RANKL/OPG lowered the bone formation capacity of OBs, and enhanced the OC bone resorption activity, finally leading to bone loss. MOIG reduced the serum level of IL-1 β and IL-6 in the CIA rats. What's more, MOIG can inhibit the proliferation of TNF- α -stimulated FLS cells and IL-6 and IL-8 production by TNF- α -stimulated FLS cells. In addition, MOIG can regulate the biochemical parameters related with bone formation, and decrease biochemical parameters of bone resorption, suggesting that MOIG is associated with the regulation of bone metabolism through its anti-inflammation effect.

In the present study, we employed 3D micro-CT imaging to analyze the trabecular bone micro-architecture, thus allowing for quantitative assessment of bone loss and the micro-architecture. To assess the effect of synovial inflammation on the joint structure, we specifically focused on the distal femur, knowing that it is consistently involved in the developmental process of osteoarthritis. Consistent with the improved clinical scoring and reduced inflammation, BMD and some other bone parameters were significantly improved after MOIG treatment. RA can induce local bone loss and generalized bone loss, but the present study was limited to local bone loss, and did not cover the generalized bone loss. Bone loss in the cortical tissue often occurs in RA, and this study also lacked the observation on alteration of cortical bone in the CIA rats. Thus, additional research is needed to clarify the treatment effect of MOIG on the cortical bone.

MTX is the first-line disease-modifying drug of choice in controlling active inflammation of synovial cells that characterizes RA, a chronic autoimmune inflammatory condition[30]. It was reported that MTX could cause varying degrees of liver fibrosis, and even cirrhosis, while the accumulation of MTX and continuous liver enzymes in the body are related to liver pathology[31]. Furthermore, oxidative stress is one of the main causes of lung injury induced by MTX. Long-term administration of MTX can induce severe interstitial pneumonia[32] and lung injury[33]. In the other way, it was reported that MTX could also cause severe gastrointestinal reaction. It was reported that the patient developed left cervical lymphadenopathy and an ulcerative lesion in the stomach after MTX treatment for RA, while the symptoms disappear after MTX was discontinued[34]. In addition, endoscopy revealed an increase in round lesions in the stomach, and biopsy specimen showed atypical lymphoid cell proliferation. The endoscopy after cessation of MTX demonstrated gradual regression of the elevated lesions[35]. The present study demonstrated that MOIG significantly alleviated the serious adverse reactions of hepatitis and fibrosis, pneumonia and pulmonary fibrosis and gastritis caused by MTX indicating that MOIG has a certain protective effect against MTX-induced injury to the liver, lung and stomach.

Metabolomics analysis of the CIA rats serum found several distinct metabolic pathways involved in the progress of RA. MOIG was mainly associated with the regulation of amino acids metabolism, lipid and

purine metabolism. Some differential metabolites were closely associated with inflammation in different metabolite pathways. It was reported that the alanine, aspartate and glutamate and choline metabolism pathways were closely correlated with TNF- α synovial expression[36]. Furthermore, taurine, which has anti-oxidant properties, can down-regulate the production of pro-inflammatory cytokines such as TNF- α , and also protect OBs against oxidative damage via Wnt/ β -catenin-mediated activation of the extracellular regulated protein kinase (ERK) signaling pathway[37]. Additionally, elevation of branched-chain amino acids (BCAA: leucine, isoleucine and valine) can cause inflammation and oxidative stress in endothelial cells, thereby facilitating inflammatory cell adhesion and endothelial dysfunction[38]. On the other hand, metabolism of some amino acids can also regulate bone metabolism. Glutamic acid could ameliorate estrogen deficiency-induced menopausal-like symptoms in ovariectomized mice and regulate the bone mass and architecture[39, 40]. Kynurenine can inhibit bone marrow derived mesenchymal stem cell (BMMSC) proliferation, alkaline phosphatase expression and activity and the expression of osteogenic markers (Osteocalcin and Runx2)[41]. Another study also reported that tryptophan and its metabolites like kynurenine could suppress BMMSCs and regulate bone metabolism[42]. It was found in this study that the level of Kynurenine was increased and the level of L-glutamate, glutamine, L-glutamine, isoleucine, taurine was decreased in the CIA rats, demonstrating that RA might disturb amino acid disorders. Dietary choline and betaine intake was found to be related to the concentration of inflammatory markers and decrease the level of TNF- α and IL-6[43]. What's more, betaine could suppress the IL-1 β production by inhibiting the activation of NLRP3 inflammasome via interaction of forkhead box O1 (FOXO1) and thioredoxin-interacting protein (TXNIP)[44]. In purine metabolism, the adenosine mediates exerted an effect on inflammatory cells by engaging one or more cell-surface receptors[45]. Xanthosine was significantly altered in the SJL mouse model of inflammation[46]. Furthermore, carnitine as a lipid metabolism intermediate associated with fatty acid β -oxidation, is essential for muscle energy metabolism. The increased level of carnitine detected in the serum of CIA rats indicated that RA is related to lipid metabolism dysfunction. It was found in our study that the level of choline and adenosine was decreased and the level of betaine and xanthosine was decreased in the CIA rats, suggesting that RA might also disturb choline and purine metabolic disorders. MOIG treatment significantly improved the abnormality of choline, lipid and purine metabolisms, suggesting that MOIG may mitigate arthritis and bone deterioration through its anti-inflammatory effect.

NF- κ B signaling pathway is known to be involved in OC activation induced by inflammation. TRAP is an acid phosphatase secreted by OCs and has the function of dissolving bone mineralization matrix. TRAP is also a specific marker enzyme for OC differentiation and maturation. In this study, LPS and M-CSF were used to induce BMM differentiation to OCs. The results showed that MOIG could significantly inhibit the TRAP activity of OCs induced by LPS. What's more, the result of CCK-8 proved that the inhibitory effect of MOIG on OCs was not related to its cytotoxicity. Furthermore, OC transcription factors NFATC1 and C-Fos could regulate the formation and differentiation of OCs[47]. The partial function of NFATC1 is dependent on the expression of C-Fos[48]. It was found in our study that MOIG markedly inhibited the activation of NF- κ B of OCs, decreased the expression of NFATC1 and C-Fos, curbed the expression of OC-related genes such as MMP9 and CtsK. In the process of OC differentiation and maturation, it can

accumulate TRAF6 and activate downstream signaling pathways like NF- κ B, JNK and p38[49]. Studies demonstrated that NF- κ B knockout mice developed osteopetrosis due to the decreased expression of OCs[50], suggesting that MOIG could inhibit the degradation of I κ B α and phosphorylation of P65. All these results suggest that MOIG may be involved in LPS-induced NF- κ B signaling pathway in OCs inhibiting the expression of C-Fos and NFATC1 in the process of OC formation and differentiation.

The present study demonstrated that MOIG could attenuate the paw swelling and bone loss through its anti-inflammatory effect and the regulatory effect on bone metabolism in the treatment of inflammatory arthritis. Furthermore, MOIG can also alleviate the adverse effect of MTX on the liver, lung and stomach. The results of metabolomics demonstrated that MOIG mainly regulated amino metabolism, which might be related to the NF- κ B signaling pathway. By studying the effect of MOIG in CIA rats, the new clues and understanding of anti-inflammatory and anti-bone loss mechanisms may not only facilitate further insights into the therapeutic mechanism of MOIG in RA, but also have the potential to discover other active constituents for treating RA.

Abbreviations

ACN: acetonitrile; Alen: alendronate; ANOVA: one-way analysis of variance; BCAA: branched-chain amino acids; BMD: bone mineral density; BMMSCs: bone marrow derived mesenchymal stem cell; BS/BV: bone surface to bone volume; BVF: bone volume fraction; CIA: collagen-induced arthritis; CII: type II collagen; CMC-Na: carbosy methyl cellulose; CTX: C-Telopeptide of type I collagen; DA: deacetyl asperulosidic acid; DMARDs: disease-modifying anti-rheumatic drugs; DPD: deoxypyridinoline; EDTA: ethylene diamine tetraacetic acid; ELISA: enzyme linked immunosorbent assay; ERK: extracellular regulated protein kinases; FOXO1: forkhead box O1; HMDB: Human Metabolome Database; IFA: Freund's adjuvant incomplete; IL-6: interleukin; IL-1 β : interleukin 1 beta; KEGG: Kyoto Encyclopedia of Genes and Genomes; LPS: lipopolysaccharides; MO: *Morinda officinalis* F.C.How; MOIG: *Morinda officinalis* iridoid glycosides; MON: monotropein; MTX: methotrexate; OCN: osteocalcin; OPG: osteoprotegerin; PCA: principal components analysis; PLS-DA: Partial least squares discrimination analysis; QC: Quality control; RA: rheumatoid arthritis; RANKL: receptor activator of nuclear factor kappa B ligand; Tb.N: trabecular number; Tb.Sp: trabecular separation; Tb.Th: trabecular thickness; TCM: traditional Chinese medicine; TGs: tripterygium glycoside; TNF- α : tumor necrosis factor- α ; TRAP: tartrate resistant acid phosphatase; TXNIP: thioredoxin-interacting protein; UHPLC-MS: ultra performance liquid chromatography-quadrupole-time of flight-mass spectrometry; VIP: variable importance plot;

Declarations

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Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author's contributions

YS, QYZ and LQi contributed to the study design. QLZ and YS contributed the reagents, materials and analysis tools. YS, YH, QZ, JZ, ML, KL and PS performed the experiments. YS YH analyzed and interpreted the data. YS and QYZ wrote and revised the manuscript. All authors have read and approved the final version of the manuscript.

Ethical approval and consent to participate

The Bio-ethic Committee of Zhejiang Chinese Medical University approved all experimental protocols associated with this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest.

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Figures

Fig. 1

Figure 1

Chart for experimental design.

Fig. 2

Figure 2

Effects of MOIG on body weight (A), spleen index (B), thymus index (C), paw swelling (D) and arthritis score (E) of CIA rats. The data were expressed as mean $\pm$ SD (n=9), #P < 0.05, ##P < 0.01 vs. normal group; *P < 0.05, **P < 0.01 vs. model group.

Fig. 3

Figure 3

Determination of inflammation index. (A) Histopathological changes of joints in CIA rats treated with MOIG. The blue arrow indicates cartilage hyperplasia and erosion; the green arrow indicates the synovia hyperplasia; the yellow arrow indicates inflammatory cell infiltration; the red arrow indicates pannus formation. Effects of MOIG on serum levels of IL-1 β (B) and IL-6 (C) of CIA rats (n=9). Effects of MOIG on proliferation in normal FLS cells (D) and TNF- α -stimulated FLS cells (E). Effects of MOIG on IL-6 (F) and IL-8 production (G) (n=3). The data are expressed as means $\pm$ SD. #P < 0.05, ##P < 0.01 vs. control group; *P < 0.05, **P < 0.01 vs. model group.

Fig. 4

Figure 4

Effects of MOIG on serum levels of OCN (A), OPG (B), CTX-I (C), DPD (D), RANKL (E), and TRACP (F) of CIA rats. Effects of MOIG on histo-morphological alteration and BMD of the distal femur in CIA rats. (G) flat scanning images; (H) 3D scanning images of the rat femur; (I) BMD; (J) BVF; (K) Tb.Th; (L) Tb.N; (M) BS/TV and (N) Tb.Sp were analyzed by Micro-CT analysis. The data are expressed as the mean $\pm$ SD (n=9), #P < 0.05, ##P < 0.01 vs. normal group; *P < 0.05, **P < 0.01 vs. model group.

Fig. 5

Figure 5

Effects of MOIG on serum levels of ALT (A) and AST (B). (C) Histological staining of the liver in CIA rats. (D) Histological staining of the lung in CIA rats. (E) Histological staining of the stomach in CIA rats. The data are expressed as means $\pm$ SD (n=10). [#]P < 0.05, ^{##}P < 0.01 vs. control group; ^{*}P < 0.05, ^{**}P < 0.01 vs. model group.

Fig. 6

Figure 6

PCA (A) and PLS-DA (B) score plots derived from LC-MS data of serum samples between normal control and CIA model control groups, suggesting metabolic changes by CII induction. (C) summary of ingenuity pathway analysis with MetaboAnalyst based on differentiated metabolites of normal group and CIA model groups.

Fig. 7

Figure 7

PCA (A) and PLS-DA (B) score plots derived from LC-MS data of serum samples between CIA model control and MOIG treatment groups, demonstrating metabolic differences induced by MOIG treatment. (C) Summary of ingenuity pathway analysis with MetaboAnalyst based on significantly differentiated metabolites of CIA model and MOIG treatment groups. The size and color of each circle were based on the pathway impact value and p-value, respectively. (D) Construction of the metabolic pathway based on differential metabolites of CIA model group and CIA treatment with MOIG group. Metabolites with red dashed area present significant increase and blue dashed area present significant decrease in MOIG group compared to model group. Metabolites with gray dashed area present no significant change in serum. PTK-1: type 1 pantothenate kinase; $\square$ glutaminase (GLS) $\square$ glutamate-ammonia ligase (GLUL); $\square$ glutamate dehydrogenase (GLUD1); $\square$ taurine-2-oxoglutarate transaminase; $\square$ glutamate decarboxylase 1 (GLAD1) or glutamate decarboxylase 2 (GLAD2) ; $\square$ branched-chain amino acid aminotransferase (BCAT1).

Fig. 8

Figure 8

MOIG inhibits OC formation and differentiation stimulated by LPS. The effect of MOIG on the proliferation (A) and TRAP activity (A) of OCs. (C-D) inhibitory effects of MOIG on OC formation in a dose-dependent way. BMMs were incubated with M-CSF (30 ng/mL), RANKL (50 ng/mL) and different concentration (0.04, 0.4 and 4 $\mu\text{g/mL}$) of MOIG for 6 days and then stained with Acid Phosphatase Kit.

The mean number (E) and area (mm2) (F) of TRAP + OC with ≥ 3 nuclei. The data are expressed as means $\pm$ SEM (n=8). *P < 0.05, **P < 0.01 vs. ctrl group.

Fig. 9

Figure 9

MOIG suppresses LPS-mediated induction of c-Fos and NFATc1, and other marker genes expression in osteoclastogenesis. (A-E) The effects of MOIG on the protein expression of NFATc1, c-Fos, MMP9, CtsK and BMMs were determined by pre-treatment with 4 μg/mL MOIG for 4 h and then incubation with LPS (200 ng/mL) for indicated times. The cell extracts were used to determine NFATc1, C-Fos, MMP9 and CtsK protein levels through Western blot analysis. The data are expressed as means $\pm$ SEM (n=3). *P < 0.05, **P < 0.01 vs. ctrl group.

Fig. 10

Figure 10

Effects of MOIG on NF-κB activity of OCs induced by LPS. (A-D) MOIG blocked the NF-κB signaling pathway of OCs stimulated by LPS. BMMs were pretreated with 4 μg/mL MOIG for 4 h and then incubated with LPS (200 ng/mL) for indicated times. The cell extracts were used to determine TRAF6, P-P65, P65, IκBα protein levels through Western blot analysis. The data are expressed as means ± SEM (n=3). *P < 0.05, **P < 0.01 vs. ctrl group.

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