



ARTICLE

Artesunate attenuates LPS-induced osteoclastogenesis by suppressing TLR4/TRAF6 and PLC γ 1-Ca²⁺-NFATc1 signaling pathway

Xiang-zhou Zeng^{1,2,3}, Yue-yang Zhang^{1,2}, Qin Yang^{1,2}, Song Wang⁴, Bin-hua Zou^{1,2}, Yan-hui Tan^{1,2}, Min Zou², Shu-wen Liu² and Xiao-juan Li^{1,2}

In chronic infectious diseases caused by gram-negative bacteria, such as osteomyelitis, septic arthritis, and periodontitis, osteoclastic activity is enhanced with elevated inflammation, which disturbs the bone homeostasis and results in osteolysis. Lipopolysaccharide (LPS), as a bacteria product, plays an important role in this process. Recent evidence shows that an antimalarial drug artesunate attenuates LPS-induced osteolysis independent of RANKL. In this study we evaluated the effects of artesunate on LPS-induced osteoclastogenesis in vitro and femur osteolysis in vivo, and explored the mechanisms underlying the effects of artesunate on LPS-induced osteoclast differentiation independent of RANKL. In preosteoclastic RAW264.7 cells, we found that artesunate (1.56–12.5 μ M) dose dependently inhibited LPS-induced osteoclast formation accompanied by suppressing LPS-stimulated osteoclast-related gene expression (Fra-2, TRAP, Cathepsin K, β 3-integrin, DC-STAMP, and Atp6v0d2). We showed that artesunate (3.125–12.5 μ M) inhibited LPS-stimulated nuclear factor of activated T cells c1 (NFATc1) but not NF- κ B transcriptional activity; artesunate (6.25, 12.5 μ M) significantly inhibited LPS-stimulated NFATc1 protein expression. Furthermore, artesunate treatment markedly suppressed LPS-induced Ca²⁺ influx, and decreased the expression of PP2B-A α (calcineurin) and pPLC γ 1 in the cells. In addition, artesunate treatment significantly decreased the expression of upstream signals TLR4 and TRAF6 during LPS-induced osteoclastogenesis. Administration of artesunate (10 mg/kg, ip) for 8 days effectively inhibited serum TNF- α levels and ameliorated LPS (5 mg/kg, ip)-induced inflammatory bone loss in vivo. Taken together, artesunate attenuates LPS-induced inflammatory osteoclastogenesis by inhibiting the expression of TLR4/TRAF6 and the downstream PLC γ 1-Ca²⁺-NFATc1 signaling pathway. Artesunate is a valuable choice to treat bone loss induced by gram-negative bacteria infection or inflammation in RANKL-independent pathway.

Keywords: artesunate; osteoclast; LPS; PP2B-A α ; Ca²⁺; NFATc1; TLR4; RAW264.7 cells; chronic infectious diseases

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INTRODUCTION

Osteoclasts are multinucleated cells derived from the monocyte-macrophage lineage. Osteoclasts and osteoblasts can mediate bone resorption and bone formation, respectively, to maintain bone homeostasis [1]. In chronic infectious diseases, such as osteomyelitis, septic arthritis, and periodontitis, that are caused by gram-negative bacteria, osteoclastic activity and inflammation increases, which disturbs bone homeostasis and results in osteolysis [2]. Efficacious means of treating these diseases are limited and mainly include surgical repair, antibiotics, and nonsurgical mechanical debridement [3].

Lipopolysaccharide (LPS) is an important component of the outer membrane of gram-negative bacteria and can induce inflammation [4]. In vivo, LPS enhances the activity of osteoclasts mainly by increasing the expression of receptor activator of NF- κ B ligand (RANKL) [5].

Interestingly, LPS directly causes osteoclast formation and bone resorption independent of RANKL in preosteoclastic RAW264.7 cells, and TNF- α but not RANKL antibody treatment blocks LPS-induced osteoclastogenesis in RAW264.7 cells [6, 7]. In the LPS-induced osteoclastogenesis signaling pathway in vitro, TLR4 (the receptor of LPS) expression increases in both osteoclast differentiation and survival; TRAF6 gene expression increases in osteoclast differentiation but not in survival [8, 9]; as an early event, NF- κ B, MAPK (pP-38, pJNK, pERK) expression levels are upregulated [10, 11]; elevated c-Jun, c-fos, fra-1, and fra-2 participate in the activation of the AP-1 pathway [8]; in addition, PLC γ 1 phosphorylation and Ca²⁺ influx also increases; finally, the level of the crucial classical osteoclastogenesis factor nuclear factor of activated T cells c1 (NFATc1) is increased in the nucleus, where it cooperates with other nuclear factors to induce the expression of osteoclast-specific genes, such as tartrate-resistant

¹Laboratory of Anti-inflammatory and Immunomodulatory Pharmacology, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China; ²Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China; ³Department of Pharmacology, Hainan Medical College, Haikou 571199, China and ⁴Department of Surgery, Guangdong Hospital of Traditional Chinese Medicine, Guangzhou 510120, China
Correspondence: Shu-wen Liu (liusw@smu.edu.cn) or Xiao-juan Li (lixiao@smu.edu.cn)

These authors contributed equally: Xiang-zhou Zeng, Yue-yang Zhang

Chemical compounds studied in this article: artesunate (PubChem CID: 16394563); cyclosporin A (CsA) (PubChem CID: 5284373); BAY11-7082 (PubChem CID: 5353431).

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acid phosphatase (TRAP), MMP-9, Atp6v0d2, cathepsin K, and β 3-integrin [11]. Furthermore, ROS, RANKL, and inflammatory factors, such as IL-1 β , IL-6, and COX-2, participate in LPS-induced osteoclastogenesis [10, 12].

Artesunate is a semisynthetic derivative of artemisinin, which is widely used in the clinical treatment of falciparum malaria with little toxicity [13]. Artesunate exhibits anti-inflammatory and immunosuppressive effects to ameliorate autoimmune diseases and inflammatory diseases [14]. Recently, artesunate was found to attenuate ACLT-induced osteoarthritis by suppressing osteoclastogenesis [15]. Artesunate was also found to decrease RANKL-induced osteoclastogenesis in vitro and ovariectomy-induced bone destruction or LPS-induced calvarial bone loss in vivo [16, 17]. However, the mechanisms of artesunate action in LPS-induced osteoclast differentiation independent of RANKL is still unknown.

Hence, we evaluated the effects of artesunate on LPS-induced osteoclastogenesis in vitro and femur osteolysis in vivo and explored the underlying mechanism of artesunate in LPS-induced osteoclast differentiation independent of RANKL. Our results suggest that artesunate can be used to treat RANKL signaling-independent inflammation-induced osteolysis.

MATERIALS AND METHODS

Chemicals and materials

Artesunate (purity > 98% by HPLC; Fig. 1a) was obtained from Tauto Biotech (Shanghai, China). NFAT-luc stably transfected RAW264.7 cells and NF- κ B-luc stably transfected RAW264.7 cells were as described previously [16]. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were obtained from Gibco (Rockville, MD, USA). LPS (serotype O55:B5), the Leukocyte Acid Phosphatase (TRAP) kit, BAY11-7082, and cyclosporin A (CsA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Primary antibodies to NFATc1 (D15F1), β -actin, and lamin A/C (4C11) were obtained from Cell Signaling Technology (Beverly, MA, USA). A primary antibody to TLR4 was obtained from Bioworld Technology (Saint Louis Park, MN, USA). Primary antibodies to PLC γ 1 (1249), pPLC γ 1 (Tyr 783), and PP2B-A α (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A primary antibody to TRAF6 was obtained from Epitomics (Burlingame, CA, USA). Mouse TNF- α ELISA kits were obtained from Dakewe Biotech (Beijing, China).

Mice

Eight-week-old female ICR mice were obtained from the Guangdong Medical Laboratory Animal Center. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

Osteoclastogenesis assay in vitro

A total of 1×10^3 RAW264.7 cells/well were seeded in a 96-well plate and treated with or without LPS at a concentration of 100 ng/mL alone or in combination with different artesunate at concentrations of 1.56, 3.125, 6.25, and 12.5 μ M. On the 5th day, the cells were fixed and stained with TRAP according to the manufacturer's instructions (Sigma-Aldrich, USA). TRAP-positive cells with three or more nuclei were counted as osteoclasts.

NFATc1 or NF- κ B luciferase reporter gene assays

To determine the effect of artesunate on the LPS-induced NFATc1 or NF- κ B luciferase reporter gene activity, we first used

artesunate, the NFAT inhibitor CsA or the NF- κ B inhibitor BAY11-7082 to pretreat RAW264.7 cells (1×10^4 cells/well) stably transfected with an NFATc1 or NF- κ B luciferase reporter construct in a 96-well plate for 30 min, followed by stimulation with LPS (1 μ g/mL). Six hours later, we measured the luciferase activity using a luciferase assay system (Promega, Madison, WI, USA).

Real-time polymerase chain reaction (PCR) analysis

A total of 2×10^6 RAW264.7 cells were seeded in each well of a six-well plate and then pretreated with or without artesunate at concentrations of 3.125 and 12.5 μ M for 30 min, followed by stimulation with LPS (100 ng/mL) for 24 h.

Total RNA was isolated from cultured RAW264.7 cells with TRIzol reagent (Invitrogen Carlsbad, CA, USA). cDNA was synthesized by reverse transcription with 1 μ g of total RNA using reverse transcriptase (TaKaRa Biotechnology, Otsu, Japan). Real-time PCRs were run on an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus) (TaKaRa Biotechnology), and the amplification parameters were as follows: 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s for a total of 40 cycles. The primer sequences for TRAP, cathepsin K, β 3-integrin, Atp6v0d2, NFATc1, and GAPDH were as previously described [16]. Other primer sequences were as follows: DC-STAMP (Forward: 5'-TCCTCCATGAACAAA CAGTTCCAA-3'; Reverse: 5'-AGACGTGGTTTAGGAATGCAGCTC-3'), TRAF6 (Forward: 5'-GCTCAAACGGACCATTCGGA-3'; Reverse: 5'-GGATTGTGGGTCGCTGAAA-3'), Fra-2 (Forward: 5'-ATCCACGCTCAC ATCCCTAC-3'; Reverse: 5'-GTTTCTCTCCCTCCGGATTC-3'), TLR4 (Forward: 5'-CCCTGCATAGAGGTAGTTC-3'; Reverse: 5'-AGCTCAGA TCTATGTTCTTGGT-3'). The $2^{-\Delta\Delta CT}$ method was used to assess relative gene expression, and all values were normalized to those of the housekeeping gene GAPDH.

Western blot analysis

RAW264.7 cells (2×10^6 cells/well) were seeded in a six-well plate and cultured overnight. After treatment with or without artesunate (3.125, 6.25, and 12.5 μ M) for 30 min, the cells were stimulated with 1 μ g/mL LPS for 30 min (to detect PLC γ 1) or 15 min (for TLR4 and TRAF6) or with 100 ng/mL LPS for 24 h (to detect NFATc1 and PP2B-A α). Whole cell proteins and cytoplasmic and nuclear proteins were extracted from cultured cells using RIPA buffer (Sigma-Aldrich, St Louis, MO, USA), and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) containing protease and phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₂VO₄, and 1 mmol/L NaF). Samples of whole cell proteins, cytoplasmic proteins, and nuclear proteins were treated as described previously [16].

LPS-induced bone loss in mouse femurs

Eight-week-old female ICR mice were randomly divided into three groups with five mice per group. LPS (5 mg/kg body weight) or PBS was injected intraperitoneally on days 0 and 4 [18]. Artesunate (10 mg/kg body weight) was injected intragastrically 1 day before LPS injection and continued for 8 days. At the end of the study, the serum and hind limbs of mice were collected; the left femurs were scanned as described previously [16], and the trabecular microarchitectures were evaluated by high-resolution micro-computed tomography (micro-CT) (ZKKS-MCT-Sharp, Guangzhou Zhongkekaisheng Medical Technology Co. Ltd, Guangzhou, China). The scanned images and the levels of bone volume fractionation (BV/TV), bone mineral density (BMD), trabecular number (Tb.N.), as well as trabecular separation (Tb.Sp.) were obtained. For HE staining, the tibiae were fixed in 10% buffered formalin, decalcified in 12% EDTA for 4 weeks, embedded in paraffin, and cut into 5 μ m slices. The serum concentration of TNF- α was determined using an ELISA kit according to the manufacturer's instructions.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical significance was analyzed using one-way analysis of variance as well as Dunnett's post hoc test. *P* values < 0.05 were considered statistically significant.

RESULTS

Artesunate suppresses LPS-induced osteoclastogenesis in vitro
To assess whether artesunate inhibits LPS-induced osteoclast differentiation induced, RAW264.7 cells were treated with 1.56–12.5 μM artesunate in the presence of LPS. We found via the TRAP assay that treatment with artesunate reduced LPS-treated osteoclast differentiation in a dose-dependent manner (Fig. 1b, c). Artesunate treatment at 1.56–12.5 μM showed no cytotoxicity in osteoclast precursor RAW264.7 cells [16].

Artesunate inhibits LPS-induced osteoclast-specific gene expression

To confirm the inhibitory function of artesunate on osteoclast differentiation, we explored the effect of artesunate on the mRNA expression of LPS-induced osteoclast-specific genes. The mRNA expression levels of Fra-2, TRAP, Atp6v0d2, β3-integrin, cathepsin K, and DC-STAMP were markedly enhanced after stimulation with LPS. This in expression levels was significantly decreased by treatment with artesunate in a dose-dependent manner (Fig. 2), which is consistent with the inhibitory effects of artesunate on LPS-induced osteoclast differentiation in the TRAP assay.

Artesunate has no effect on LPS-induced NF-κB-luc activity at low concentrations

We further examined the effect of artesunate on NF-κB activation by an NF-κB luciferase reporter assay. As shown in Fig. 3b,

artesunate treatment had no effect on LPS-induced NF-κB activation within the concentration range of 3.125–12.5 μM, which clearly inhibited LPS-induced osteoclast formation. At high concentrations of 25 and 50 μM, artesunate significantly reduced NF-κB activation during LPS-induced osteoclastogenesis.

Artesunate inhibits LPS-induced activation of NFATc1

NFATc1 is the master transcription factor required for osteoclast differentiation, and it regulates the expression of multiple osteoclast-specific marker genes [19]. As artesunate suppressed LPS-induced osteoclast differentiation as well as osteoclast-specific gene expression, we next examined whether artesunate inhibits LPS-induced NFATc1 activation by assessing the influence of artesunate on the transcriptional activity, mRNA expression, and protein expression of NFATc1. A luciferase reporter assay showed that 12.5 μM artesunate treatment suppressed the LPS-induced transcriptional activity of NFATc1 (Fig. 3a). By real-time PCR analysis, we found that 3.125 and 12.5 μM artesunate decreased LPS-induced increases in NFATc1 mRNA expression (Fig. 3c). We also found that artesunate at concentrations ranging from 6.25 to 12.5 μM efficiently inhibited LPS-induced nuclear NFATc1 protein levels as assessed by western blot assay (Fig. 3d). Taken together, these findings illustrate that artesunate inhibits osteoclastogenesis by attenuating LPS-induced activation of NFATc1.

Artesunate suppresses LPS-induced Ca²⁺ influx and calcineurin expression

The Ca²⁺ signal is a critical factor for the activation of NFATc1 via calcineurin in osteoclasts [20]. Therefore, we measured LPS-induced Ca²⁺ flux. The concentration of intracellular Ca²⁺ was significantly increased in LPS-stimulated cells. Treatment with artesunate at concentrations of 3.125 and 12.5 μM or CsA at 0.5 μM dramatically reduced the intracellular Ca²⁺ level (Fig. 4a, b). In addition, treatment with artesunate at concentrations of 6.25 and

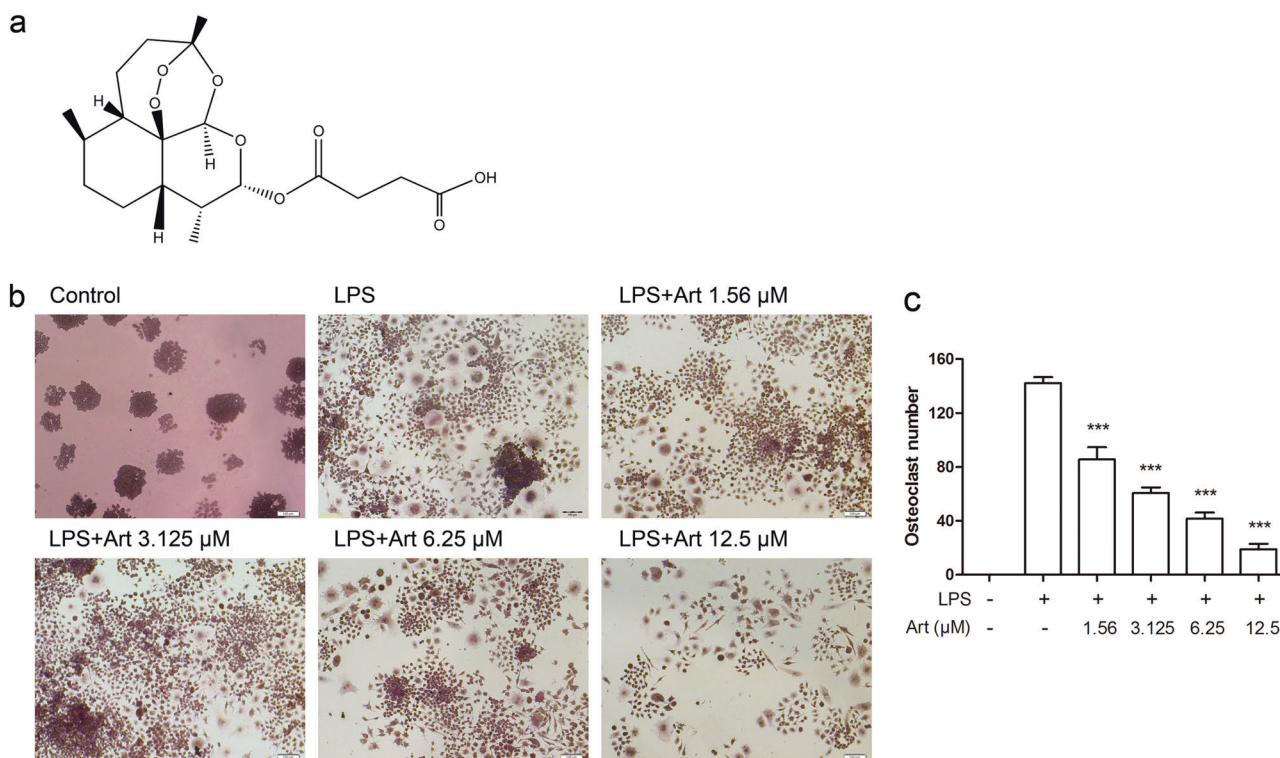


Fig. 1 Artesunate inhibits LPS-induced osteoclastogenesis. **a** The chemical structure of artesunate. **b** Artesunate inhibits LPS-induced osteoclast formation as assessed by a TRAP assay in preosteoclastic RAW264.7 cells. **c** The osteoclast number was counted per well of each group. Bars show the mean ± SEM from three independent experiments. ****P* < 0.001, compared with the LPS-treated group

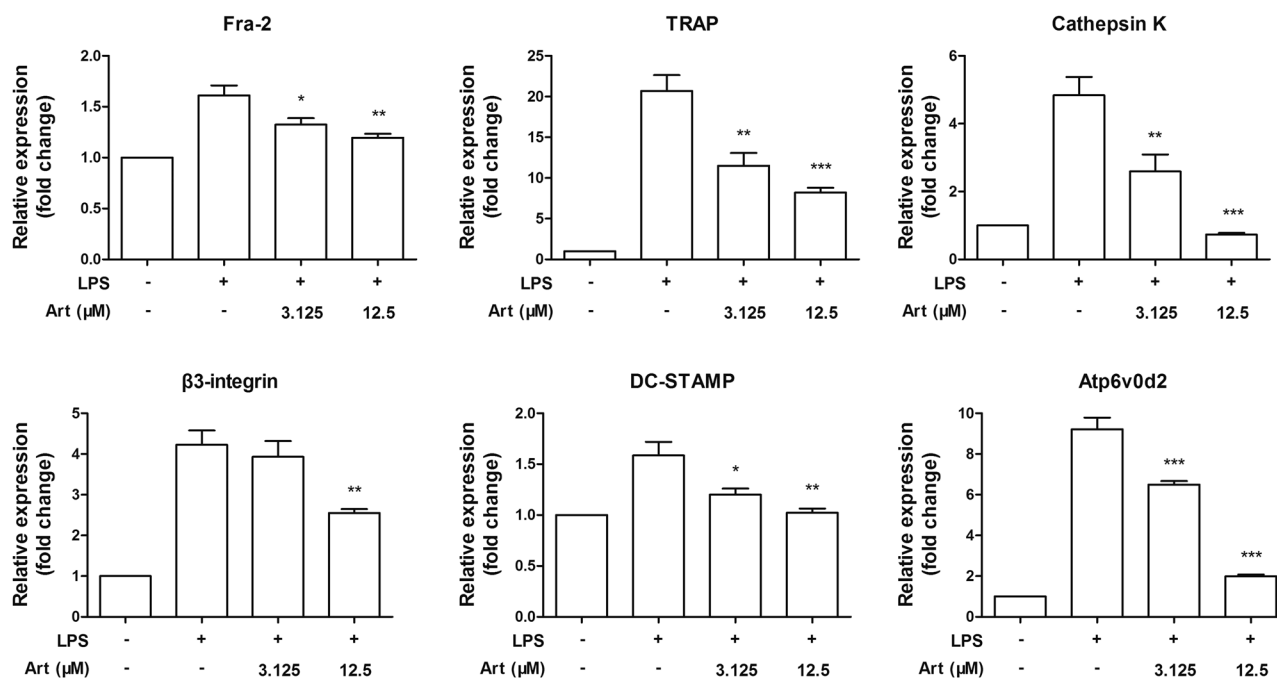


Fig. 2 Artesunate suppresses LPS-induced osteoclast-related gene expression. The expression of osteoclast-specific genes was measured by real-time PCR in preosteoclastic RAW264.7 cells after artesunate and LPS administration. Bars represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the LPS-treated group

12.5 μ M inhibited the LPS-induced expression of PP2B-A α protein, which is a catalytic subunit of calcineurin (Fig. 4c).

Artesunate inhibits LPS-induced PLC γ 1 phosphorylation during osteoclastogenesis
Since PLC γ 1 increases intracellular Ca²⁺ [21], we evaluated PLC γ 1 activation using an anti-phospho-PLC γ 1 antibody by western blot. Treatment with 3.125–12.5 μ M artesunate reduced the phosphorylation of PLC γ 1 in LPS-induced osteoclastogenesis in RAW264.7 cells (Fig. 4d), indicating that artesunate inhibits LPS-induced Ca²⁺ influx, which may be mediated by the decreased phosphorylation of PLC γ 1.

Artesunate decreases TLR4 and TRAF6 expression in LPS-induced osteoclastogenesis
TLR4 is an LPS receptor. After binding to TLR4, LPS recruits TRAF6 to induce proinflammatory signaling [22]. Here, we observed by real-time PCR and western blot analysis that the expression of both TLR4 and TRAF6 was upregulated in RAW264.7 cells after LPS injection. This result is consistent with previous reports [8]. Here, artesunate treatment reduced both TLR4 and TRAF6 mRNA levels at a concentration of 12.5 μ M in LPS-induced osteoclast differentiation (Fig. 5a). In addition, artesunate treatment at concentrations ranging from 3.125 to 12.5 μ M decreased the expression of both TLR4 and TRAF6 proteins after LPS stimulation in RAW264.7 cells (Fig. 5b), which suggests that artesunate-mediated suppression of upstream signals after LPS stimulation is involved in the inhibition of osteoclastogenesis.

Artesunate decreases TNF- α and LPS-induced bone loss in vivo
Osteoclasts are a unique type of cell that absorbs bone after activation. Intraperitoneal injection of LPS for 7 days induced extensive bone loss in mouse femurs (Fig. 6a). Intra-gastric administration of artesunate (10 mg/kg) reduced LPS-induced bone erosion as evaluated by a micro-CT assay in vivo (Fig. 6a, b). The quantitative analysis of bone parameters revealed that artesunate significantly increased BMD, BV/TV, and Tb.N, which

decreased in LPS-induced osteolysis, and decreased Tb.Sp, which was upregulated in LPS-induced osteolysis in vivo (Fig. 6b). Moreover, histological examination confirmed the protective effects of artesunate on LPS-induced bone erosion (Fig. 6c). LPS-induced osteoclast formation depends on TNF- α rather than RANKL [6]. Thus, the TNF- α level in peripheral blood was also assessed by ELISA to elucidate the efficacy of artesunate in vivo. The TNF- α level was dramatically increased after LPS injection but suppressed by artesunate treatment (Fig. 6d). Taken together, these data show that artesunate reduces TNF- α production and prevents LPS-induced bone loss in vivo.

DISCUSSION

Inhibiting the elevated formation or activity of osteoclasts is a valuable strategy for treating pathological osteolysis [23]. Gram-negative bacterial infections result in bone loss diseases, including osteomyelitis, periodontitis, and septic arthritis [24]. LPS is a primary component of the outer membrane of gram-negative bacteria [4]. LPS can induce osteoclast formation independent of RANKL [6]. Therefore, searching for drugs to inhibit LPS-induced osteolysis is a promising strategy for the prevention of osteolysis in infective bone diseases [24]. In this study, we found that artesunate, a semisynthetic antimalarial drug, suppresses LPS-induced osteoclastogenesis independent of RANKL in vitro, and further described the underlying mechanisms.

Our results indicate that artesunate significantly inhibited LPS-stimulated osteoclastogenesis in vitro at concentrations of 1.56–12.5 μ M without toxicity. Artesunate also had protective effects on LPS-induced femur bone loss in vivo, which is consistent with a recent report that artesunate inhibits LPS-induced calvarial osteolysis [17]. TNF- α is believed to be the main factor that mediates LPS-induced osteoclast formation [6, 25]. Artesunate treatment also reduced the serum TNF- α level accompanied by increased bone mass in LPS-induced osteolysis in vivo. These results illustrate that artesunate attenuates

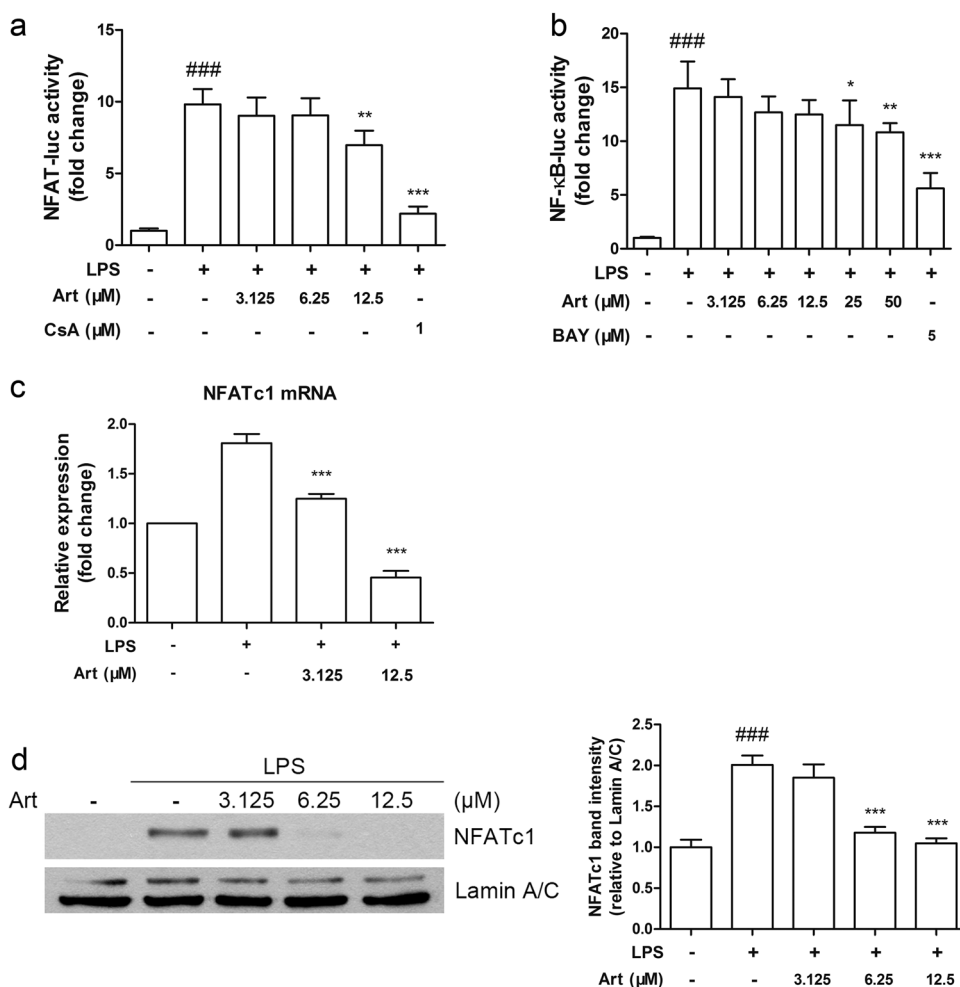


Fig. 3 Artesunate inhibits LPS-induced activation of NFATc1. **a** The NFAT luciferase activity of NFAT-luc-RAW264.7 cells and **b** the NF-κB luciferase activity of NF-κB-luc-RAW264.7 cells were measured. **c** mRNA levels of NFATc1 were assessed by real-time PCR. **d** Protein levels of NFATc1 were detected by Western blot (left panel). The relative density of nuclear NFATc1 was calculated (right panel). Bars represent the mean ± SEM from three independent experiments. ###*P* < 0.001, compared with the untreated group; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the LPS-treated group

LPS-induced osteoclast differentiation in vitro and bone destruction in vivo.

NFATc1, activated during osteoclastogenesis, is a master transcription factor that is required for the terminal differentiation of osteoclasts [19]. NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts, while osteoclast precursor cells with high NFATc1 expression levels efficiently differentiate into osteoclasts, even in the absence of RANKL [26]. Therefore, NFATc1 plays a particularly important role in osteoclast differentiation. In the present study, we found that artesunate treatment at concentrations of 3.125–12.5 μM inhibited LPS-stimulated NFATc1 but not NF-κB transcriptional activity. Meanwhile, artesunate treatment at high concentrations of 25 and 50 μM inhibited NF-κB-luc transcriptional activity without toxicity, which is consistent with the effects of artesunate in RANKL-induced osteoclastogenesis that we recently reported [16]. This effect is also consistent with the effects of another derivative of artemisinin, dihydroartemisinin, in RANKL-induced osteoclastogenesis [27].

Then, we focused on the effects of artesunate on NFATc1 activation. We observed that artesunate treatment strongly inhibited LPS-induced NFATc1 mRNA levels as well as nuclear NFATc1 protein expression upregulation. NFATc1 is a calcium-dependent factor [26]. In classical osteoclastogenesis activated by

RANKL, PLCγ binds to the IP₃ receptor (IP₃R), induces calcium release from the endoplasmic reticulum, and then increases calcium levels to activate calcineurin, which is followed by the dephosphorylation and nuclear translocation of NFATc1 [28]. In LPS-induced osteoclastogenesis, PLCγ1, Ca²⁺, and calcineurin levels are also reportedly increased with elevated NFATc1 [20]. Here, we observed that LPS upregulated the phosphorylation of PLCγ1, calcineurin (PP2B-Aα) protein expression, and Ca²⁺-influx during osteoclastogenesis in RAW264.7 cells. Artesunate dose dependently suppressed LPS-induced PLCγ1 activation. In addition, artesunate treatment reduced intracellular Ca²⁺ levels, which was similar to the effects of the NFAT inhibitor CsA. We also observed that artesunate reduced the protein expression of calcineurin (PP2B-Aα). As a result, we conclude that artesunate inhibits LPS-induced osteoclastogenesis, which might be related to the suppression of PLCγ1-Ca²⁺-calcineurin and NFATc1 activation.

In the nucleus, NFATc1 binds to promoter regions to modulate the expression of osteoclast-specific genes, including TRAP, Fra-2, cathepsin K, β3-integrin, DC-STAMP, and Atp6v0d2, which participate in osteoclast formation and osteoclastic bone resorption. Here, we found that artesunate treatment inhibited the expression of these genes, which contributed to the inhibitory effects of artesunate on osteoclastogenesis.

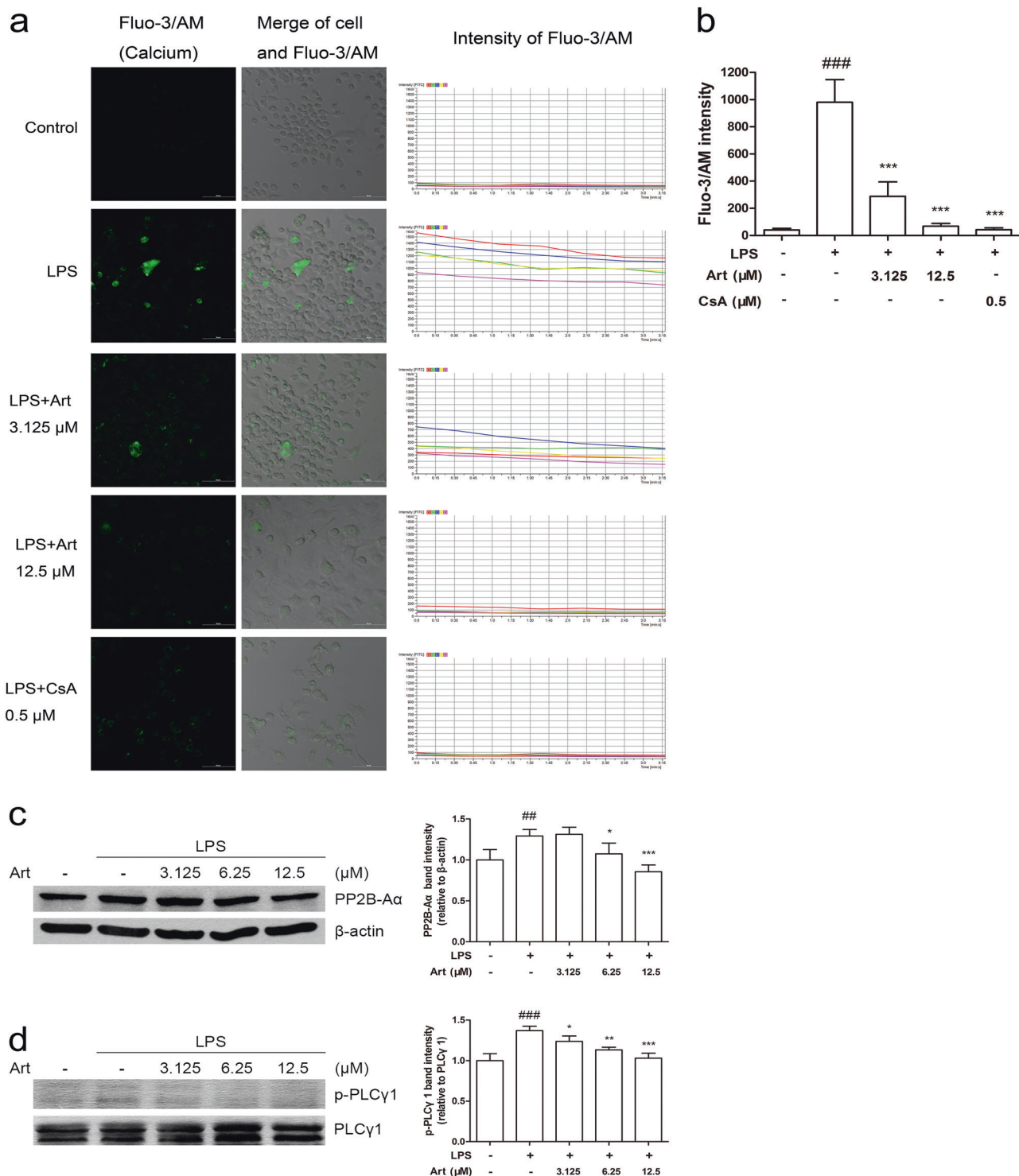


Fig. 4 Artesunate suppresses LPS-increased Ca^{2+} levels and calcineurin expression by decreasing the phosphorylation of PLC γ 1. **a** LPS-induced Ca^{2+} influx was determined by confocal microscopy. **b** The fluorescence intensity in 200 s was analyzed to indicate intracellular $[\text{Ca}^{2+}]$. Protein levels of **c** PP2B- α and **d** phosphorylated PLC γ 1 were analyzed by Western blot. Bars show the mean \pm SEM from three independent experiments. ## $p < 0.01$, ### $p < 0.001$, compared with the untreated group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the LPS-treated group

TLR4 and TRAF6 are important upstream signals in LPS-induced osteoclastogenesis [8]. LPS binds to its ligand TLR4 to activate inflammation and osteoclastogenesis [9]. Other groups and we have found that the expression of TLR4 and TRAF6 is upregulated during LPS-induced osteoclastogenesis [8, 20]. Previous reports indicated that artesunate improves acute

pancreatitis by inhibiting TLR4 [29]. Similarly, we found that artesunate treatment suppressed TLR4 mRNA and protein expression during LPS-induced osteoclast differentiation. Artesunate treatment also decreased TRAF6 expression after LPS activation in osteoclastogenesis. Consequently, artesunate-mediated suppression of TLR4/TRAF6 might be followed by a

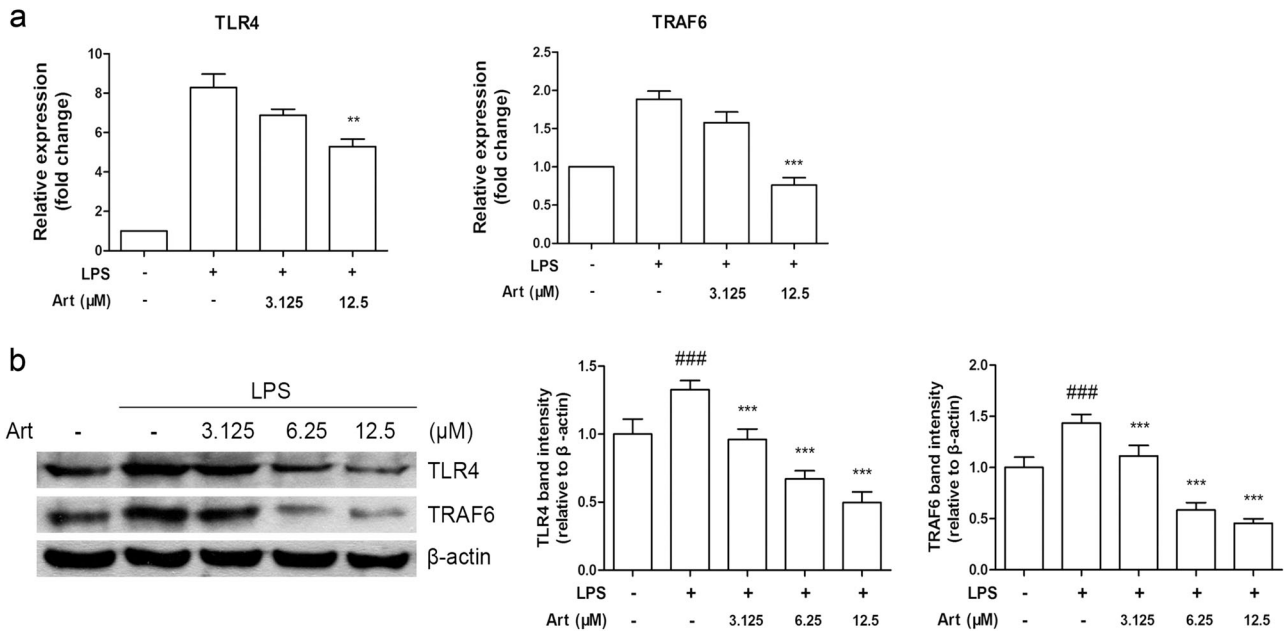


Fig. 5 Artesunate suppresses LPS-induced expression of TLR4 and TRAF6. **a** mRNA levels of TLR4 and TRAF6 were determined by real-time PCR. **b** Protein levels of TLR4 and TRAF6 were detected by Western blot (left panel). The relative TLR4 and TRAF6 densities were calculated (right panel). Bars show the mean \pm SEM from three independent experiments. ### $P < 0.001$, compared with the untreated group; ** $P < 0.01$, *** $P < 0.001$, compared with the LPS-treated group

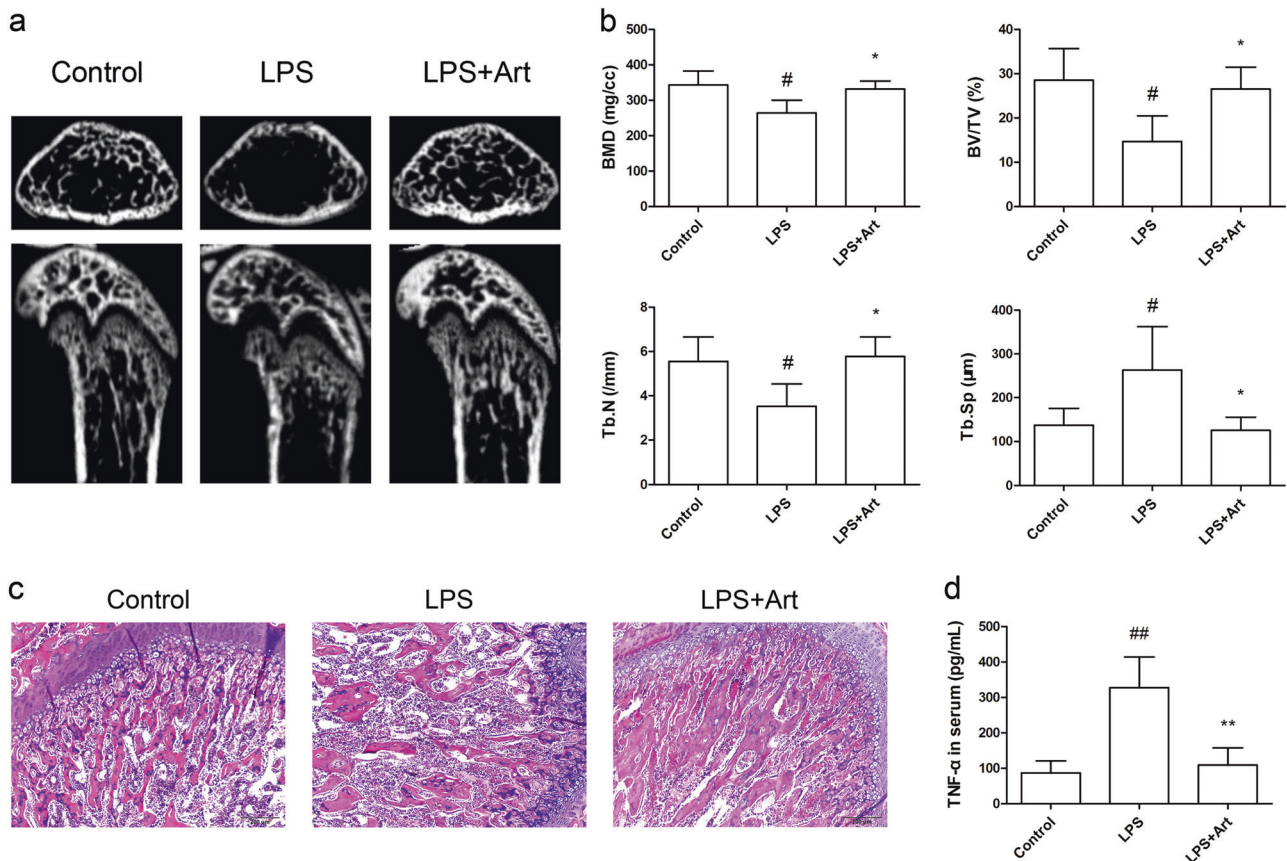


Fig. 6 Artesunate prevents LPS-induced osteolysis in vivo. **a** Distal femur images of micro-CT analysis. **b** Graphical depiction of trabecular bone parameters, including BMD, BV/TV, and Tb.N. and Tb.Sp. by micro-CT. **c** Histological changes of tibial sections were assessed by H&E staining ($\times 100$). **d** Serum levels of TNF- α were measured using an ELISA kit. Data are representative of two independent experiments, and bars show the mean \pm SEM ($n = 5$). # $P < 0.05$, ## $P < 0.01$, compared with the control group; * $P < 0.05$, ** $P < 0.01$, compared with the LPS-injected group

reduction in PLC γ 1-Ca²⁺-calcineurin and NFATc1, which contribute to osteoclastogenesis.

In conclusion, our results demonstrate that artesunate suppresses LPS-induced osteoclastogenesis in vitro and prevents LPS-induced femur bone loss in vivo. Moreover, artesunate exhibited inhibitory effects on LPS-induced osteoclastogenesis by reducing TLR4/TRAF6 and PLC γ 1-Ca²⁺-calcineurin-NFATc1. Our findings provide a mechanistic insight into the application of artesunate for LPS-mediated bone loss independent of RANKL. Artesunate might be a valuable therapeutic agent to treat bone loss diseases, including osteomyelitis, septic arthritis, and periodontitis induced by gram-negative bacterial infections. Artesunate has been clinically used to treat rheumatoid arthritis in China recently [30]. Patients with rheumatoid arthritis have elevated expression levels of both RANKL and inflammatory factors, including TNF- α [31]. Artesunate has been reported to inhibit RANKL-induced osteoclastogenesis [16], and it might exhibit a potential therapeutic effect on bone erosion in rheumatoid arthritis by inhibiting both LPS- and RANKL-related signaling pathways to reduce the pathological overactivation of osteoclasts.

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AUTHOR CONTRIBUTIONS

XJL, XZZ and SWL designed the research; XZZ, YYZ, QY, SW, BHZ and YHT performed the research; XZZ, SW and MZ analyzed the data; XZZ, QY, XJL and SWL wrote the paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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