

# $\beta$ -Ecdysterone Prevents LPS-Induced Osteoclastogenesis by Regulating NF- $\kappa$ B Pathway in Vitro

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## Research article

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# Abstract

## Background

Lipopolysaccharide (LPS), a bacteria product, plays an important role in orthopedic diseases. Drugs that inhibit LPS-induced osteoclastogenesis are urgently needed for the prevention of bone destruction.

## Methods

In this study, we evaluated the effect of  $\beta$ -ecdysterone ( $\beta$ -Ecd), a major component of Chinese herbal medicines derived from the root of *Achyranthes bidentata* BI on LPS-induced osteoclastogenesis *in vitro* and explored the mechanism underlying the effects of  $\beta$ -Ecd on this.

## Results

We showed that  $\beta$ -Ecd inhibited LPS-induced osteoclast formation from osteoclast precursor RAW264.7 cells. The inhibition occurred through suppressing the production of osteoclast activating TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2, which led to down-regulating expression of osteoclast-related genes including RANK, TRAF6, MMP-9, CK and CA $\square$ . Besides,  $\beta$ -Ecd treatment can inhibit LPS-induced activation of NF- $\kappa$ B signaling pathway in RAW264.7 cells. Meanwhile, inhibition of NF- $\kappa$ B signaling pathway decreased the formation of osteoclasts and expression of pro-inflammatory cytokines which LPS-induced. Collectively,  $\beta$ -Ecd can prevent LPS-induced osteoclast formation *in vitro* by regulating NF- $\kappa$ B signaling pathway.

## Conclusions

These findings provide evidences that  $\beta$ -Ecd might be beneficial as a valuable choice for the prevention and treatment of bacteria-induced bone destruction disease, and give new insights for understanding its possible mechanism.

# Introduction

The osteoclast is a large, multinucleated cell from the monocyte-macrophage lineage [1]. It can degrade bone tissue by secreting H<sup>+</sup>, Cl<sup>-</sup>, cathepsin K (CtsK) and matrix metalloproteinase (MMPs) in the resorption area [2] and plays a key physiological role in bone remodeling and a pathological role in bone destruction diseases including osteopetrosis, osteoporosis, rheumatoid arthritis, inflammatory osteolysis [3], Paget's disease, osteopetrosis and pycnodysostosis [4]. Inheritance of osteoclast differentiation from hematopoietic progenitors requires macrophage colony stimulating factor (M-CSF) and receptor activated nuclear factor kappa-B (NF- $\kappa$ B) ligand (RANKL) to monitor differentiation [5-7]. These factors are the key determinants in stimulating the differentiation and activation of osteoclast, and are very crucial in bone remodeling [8].

The lipopolysaccharide (LPS) is a central component of the outer membrane in gram-negative bacteria that plays a key role in host-pathogen interactions with the innate immune system [9]. LPS is a potent

endotoxin and is highly resistant to degradation by mammalian enzymes thus providing a persistent inflammatory stimulus [10]. It can cause inflammatory bone loss, leading to severe morbidity and large medical expenses and it is also considered to be a key pathogen of inflammatory osteolytic diseases such as osteomyelitis, septic arthritis, periodontitis, and infection of orthopedic implants [11]. LPS-induced pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , prostaglandin E2 (PGE2) and IL-6, can directly stimulate osteoclast differentiation and ultimately lead to the destructive bone loss by means of increasing the expression of receptor activator for nuclear factor- $\kappa$ B ligand (RANKL) [12-14]. Furthermore, osteoclasts arise in the bone marrow from the fusion of haematopoietic cells of a monocyte/macrophage lineage after stimulation by M-CSF and RANKL [15]. LPS is considered to be an important factor in osteogenesis and osteoclast metabolism disorder in infectious bone diseases, but there are still no relevant reports on how to effectively regulate the local bone growth metabolism disorder caused by LPS from the molecular mechanism, and there is also no effective response in the clinic. So, exploring the molecular mechanism on how to intervene and regulate the impact of LPS on bone metabolism disorders is of great significance for understanding and treating infectious bone diseases.

$\beta$ -ecdysterone ( $\beta$ -Ecd), a major component of Chinese herbal medicines derived from the root of *Achyranthes bidentata* BI [16, 17].  $\beta$ -Ecd has been reported to possess antiapoptotic and anti-inflammatory pharmacological effects [16]. Besides, it has been demonstrated to exhibit a number of functions, including anabolic and hepatoprotective effects [18] and some studies have indicated that  $\beta$ -ecdysterone may increase the synthesis of collagen protein and inhibit cell apoptosis by regulation of autophagy [18-20]. Related studies have shown that multiple signaling pathways involved MAPK [21, 22], Smad/BMP2 [23, 24], Wnt/ $\beta$ -catenin [25, 26], NF- $\kappa$ B [27] and other signaling pathways are involved in the regulation of bone tissue metabolism. Among these pathways, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway is particularly closely related to the regulation of bone metabolism under inflammatory conditions, and it plays an important role in regulating osteoclast differentiation, activation and osteoblast activity [28].

Previous studies found that  $\beta$ -Ecd can inhibit LPS-mediated osteoclast differentiation and fusion, but the specific signaling pathway involved is unclear. So we hypothesize that  $\beta$ -Ecd may inhibit LPS-induced osteoclastogenesis by regulating NF- $\kappa$ B pathway *in vitro*. Indeed, the findings presented in this study perfectly support the hypothesis. This study provides a reference and direction for the correct evaluation of the pharmacological effects of these natural plant drugs on bone tissue under inflammatory conditions and the application of natural plant drugs in the regulation of bone metabolism in infectious bone diseases.

## Materials And Methods

### Cell culture and treatment

Osteoclast precursor RAW264.7 cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL penicillin, and 100 µg/mL streptomycin maintained at 37 °C with a 5 % CO<sub>2</sub> atmosphere. Cultured RAW264.7 cells were pretreated for 1 h with β-Ecd at various concentrations followed by exposure to LPS (100 ng/mL) at indicated times. RAW264.7 cells were used to assess if β-Ecd had toxic effect on them. For inhibitor studies, NF-κB pathway-specific inhibitor was added to cell culture 30 min before treatment with β-Ecd and maintained throughout the experiment.

### **Assessment of cell viability by CCK-8 assay**

The assay cell counting kit-8 (CCK-8) was used to measure whether the β-Ecd had toxic effects on the RAW264.7 cells. RAW264.7 cells were cultured to the logarithmic growth phase, and after digestion, 1×10<sup>5</sup>/mL cell suspension was prepared with the pre-warmed medium and inoculated into a 96-well cell culture plate with a volume of 100 µl per well. After the cells were adhered, the original medium was aspirated, and RAW264.7 cells were added at concentrations of 0 µM, 5 µM, 10 µM, 20 µM and 40 µM β-Ecd, PBS as a control. The next day, the CCK-8 solution was added 10 µl per well, and after another 4 hours' culture, the absorbance of each well was measured at 450 nm. This experiment was in quadruplicate.

### **Osteoclastogenesis assay *in vitro***

To assess whether β-ecdysterone inhibits LPS-induced osteoclast formation, a total of 1×10<sup>3</sup> 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 β-Ecd at concentrations of 5, 10, 20 and 40 µM. That is to say, the assay included six groups: PBS, LPS, LPS+5 µM β-Ecd, LPS+10 µM β-Ecd, LPS+20 µM β-Ecd and LPS+40 µM β-Ecd. Besides, to assess the effect of inhibiting NF-κB pathways on osteoclast formation *in vitro*, the assay was changed to four groups containing PBS, LPS, LPS+40 µM β-Ecd and LPS+sulfasalazine (NF-κB inhibitor). Cells were washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (pH 7.4) at room temperature (r.t.) for 15 min and stained for TRAP using leukocyte Acid Phosphatase Kit 387-A according to the manufacturer's protocol. TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast under a IX70 microscope (Olympus, Japan).

### **Western blot analysis**

RAW264.7 cells were cultured overnight. After treatment with or without β-Ecd (5, 10, 20 and 40 µM), the cells were stimulated with LPS to detect receptor activator of nuclear factor kappa B (RANK), TNF receptor associated factor 6 (TRAF6), matrix metalloproteinase 9 (MMP-9), cathepsin K (CK), carbonic anhydrase II (CA<sup>II</sup>) inhibitor of nuclear factor kappa B α (IκBα) or phosphorylation-IκBα (p-IκBα). The cellular lysates were prepared. The above proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 4% bovine serum albumin (BSA) for 1 h at r.t.

and then probed with rabbit anti-mouse antibodies against RANK, TRAF6, MMP-9, CK, CA $\alpha$ , I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$  and  $\beta$ -actin at 4°C overnight. After washing three times, the blots were subsequently incubated with a goat horseradish peroxidase-conjugated secondary antibody for 2 h at r.t. After four times washing for 10 min in Tris-buffered saline with Tween-20 (TBST), the membranes were detected using a chemiluminescence detection system. The intensity of the bands was quantified by ImageJ software.  $\beta$ -actin as a control.

### **Enzyme-linked immunosorbent assay(ELISA)**

ELISA was to detect the pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), prostaglandin E2 (PGE2) and cyclooxygenase-2 (COX-2) quantitatively. Similarly, PBS, LPS, LPS+5  $\mu$ M  $\beta$ -Ecd, LPS+10  $\mu$ M  $\beta$ -Ecd, LPS+20  $\mu$ M  $\beta$ -Ecd and LPS+40  $\mu$ M  $\beta$ -Ecd were performed. ELISA plates were coated with purified TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2 antibodies in carbonate-bicarbonate buffer at 4°C overnight. The plates were blocked with 200 $\mu$ l blocking buffer BSA at 37°C for 1 h. Then, the wells were added samples to be tested and incubated at 37°C for 2 h and washed four times carefully. Horseradish peroxidase (HRP)-conjugated TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2 antibodies were added to detect the specific proteins. After reaction at 37 °C for 1 h, the plates were washed four times. Next, the wells were incubated with the substrate for approximately 10-15 min, and the enzyme reaction was terminated with stop solution. The OD value was measured at 450 nm. The assay tested each sample in triplicate. The results are expressed as pg/ml.

### **Statistical analysis**

All the experiments were performed at least three times. Results are expressed as the mean  $\pm$  standard deviation (S.D.). Statistical comparisons between groups were carried out by one-way ANOVA with post hoc tests to compare differences between experimental and control group. Statistical analyses were performed using GraphPad Prism 7 software. Statistical significance was defined at  $p < 0.05$ .

## **Results**

### **$\beta$ -Ecd inhibited LPS-induced osteoclast formation**

To eliminate the inhibitory effects of  $\beta$ -Ecd on osteoclast formation could have been due to its cytotoxicity on RAW264.7 cells, CCK-8 assay were performed. The data showed that the cell viability of RAW264.7 cells was not distinctly reduced by  $\beta$ -Ecd with concentrations of 5, 10, 20 and 40 $\mu$ M, indicating that  $\beta$ -Ecd under 40 $\mu$ M was not nontoxic to RAW264.7 cells (Fig.1).

To observe the effect of  $\beta$ -Ecd on LPS-induced osteoclastogenesis, RAW264.7 cells were treated with different concentrations of  $\beta$ -Ecd as mentioned above for 72 h in the presence or absence of LPS, and then stained with TRAP, an osteoclast-specific protein. Data showed that LPS can exactly stimulate RAW264.7 cells differentiation into osteoclast.  $\beta$ -Ecd treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group (Fig.2A), and indeed inhibited LPS-induced osteoclast formation in

a dose-dependently manner (Fig.2B). Notably, 40  $\mu\text{M}$  of  $\beta\text{-Ecd}$  nearly inhibited LPS-induced osteoclast formation, the osteoclast number was counted with three different microscope fields and the average number of osteoclasts was only 12.5 % of the LPS-treated group. Besides,  $\beta\text{-Ecd}$  at 5  $\mu\text{M}$  inhibited osteoclastogenesis less severely than the other  $\beta\text{-Ecd}$ -treated groups.

### **$\beta\text{-Ecd}$ inhibits LPS-induced osteoclast-related gene expression**

To further understand the inhibitory function of  $\beta\text{-Ecd}$  on osteoclast formation, RAW264.7 cells were treated with different concentrations of  $\beta\text{-Ecd}$  in the presence or absence of LPS, and expression of osteoclast differentiation-related genes including RANK, TRAF6, MMP-9, CK and CA $\square$  were analyzed quantitatively using WB. The WB bands containing all the former proteins were shown in Fig.3A. Notably, the data indicated that LPS at 100 ng/mL significantly up-regulated proteins expression of RANK (Fig.3B), TRAF6 (Fig.3C), MMP-9 (Fig.3D), CK (Fig.3E) and CA $\square$  (Fig.3F) during osteoclast formation.  $\beta\text{-Ecd}$  treatment groups obviously inhibited expression of these relevant genes, and the inhibitory effects were also in a dose-dependent manner. Particularly, 40  $\mu\text{M}$  of  $\beta\text{-Ecd}$  exhibited the inhibition on these genes stronger than those of the other three concentrations.

### **$\beta\text{-Ecd}$ suppressed the release of pro-inflammatory cytokines**

It is reported that pro-inflammatory cytokines are considered as mediators of bone-loss [29]. Common inflammatory markers include TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and cyclooxygenase-2 (COX-2). These cytokines enhance osteoclast formation and migration in bone destructive diseases such as rheumatoid arthritis and periodontitis. Consistent with this, RAW264.7 cells incubated with LPS for 24 h resulted in insignificant increases in TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2 (Fig.4), and their levels were increased to 1.4-, 1.2-, 1.5- and 1.4-fold of untreated control group, respectively. Clearly,  $\beta\text{-Ecd}$  treatment for 24 h suppressed LPS-induced the secretion of these cytokines with a dose-dependent manner. Importantly,  $\beta\text{-Ecd}$  at 40  $\mu\text{M}$  caused a more obvious reduction of IL- $\beta$ , PGE2 and COX-2 compared with the remaining concentrations (Fig.4).

### **$\beta\text{-Ecd}$ inhibited LPS-triggered activation of NF- $\kappa\text{B}$ Pathway**

NF- $\kappa\text{B}$  signaling pathway plays an important role in activating inflammatory response and inducing apoptosis of chondrocytes, which is mediated by the degradation of I $\kappa\text{B}\alpha$  [16, 30].  $\beta\text{-Ecd}$  suppressed LPS-induced osteoclast formation as well as the expression of osteoclast-related genes. Therefore, to illuminate the possible molecular mechanisms underlying the inhibitory effect of  $\beta\text{-Ecd}$  on LPS-induced osteoclast formation, we explored whether  $\beta\text{-Ecd}$  affects the NF- $\kappa\text{B}$  pathway which LPS-induced. Given this, the proteins expression of I $\kappa\text{B}\alpha$  and p-I $\kappa\text{B}\alpha$  were analysed by WB. The WB bands were presented in Fig.5A. Stimulation of RAW264.7 cells with LPS can up-regulate the ratio of p-I $\kappa\text{B}\alpha$ /I $\kappa\text{B}\alpha$ , which presented the activation of NF- $\kappa\text{B}$  pathway (Fig.5B). When treated with different concentrations of  $\beta\text{-Ecd}$ , it can efficiently inhibited LPS-increased NF- $\kappa\text{B}$ -related protein levels as assessed by western blot assay. Notably, the level of p-I $\kappa\text{B}\alpha$ /I $\kappa\text{B}\alpha$  was decreased as the concentration increased. Taken together, these

findings illustrated that  $\beta$ -Ecd inhibited LPS-induced osteoclastogenesis through suppressing I $\kappa$ B $\alpha$  phosphorylated activation in NF- $\kappa$ B pathway.

### **Inhibition NF- $\kappa$ B pathway restrain LPS-induced osteoclast formation and inflammation**

The above results preliminary indicated that  $\beta$ -Ecd inhibited the osteoclast formation and inflammation. Next, the effects of inhibition NF- $\kappa$ B pathway on LPS-induced osteoclast formation and inflammation need to be elucidated. For this purpose, TRAP-staining and ELISA assays were performed as previous description. In TRAP-staining assay, RAW264.7 cells were treated with 40 $\mu$ M  $\beta$ -Ecd or sulfasalazine (NF- $\kappa$ B inhibitor) in the presence of LPS. LPS and control group were involved. After interaction for 72 h, RAW264.7 cells were stained with TRAP. As shown in Fig.6A,  $\beta$ -Ecd and sulfasalazine treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group and indeed inhibited LPS-induced osteoclast formation (Fig.6B). Notably, 40  $\mu$ M  $\beta$ -Ecd can inhibit LPS-induced osteoclast formation, and the number of osteoclast was 64% of the LPS-treated group. Besides, osteoclastogenesis was also inhibited in sulfasalazine group compared with LPS-treated group.

Next, the contents of TNF- $\alpha$ , IL-1 $\beta$ , PGE2, and COX-2 in the culture supernatants were measured by ELISA. RAW264.7 cells incubated with 40  $\mu$ M  $\beta$ -Ecd or sulfasalazine in the presence of LPS for 24 h. LPS-treated group caused the release of these pro-inflammatory cytokines. When treated with 40  $\mu$ M  $\beta$ -Ecd and sulfasalazine, the expressions of LPS-induced TNF- $\alpha$  (Fig.6C), IL-1 $\beta$  (Fig.6D), PGE2 (Fig.6E), and COX-2 (Fig.6F) represented a downward trend. Importantly, there was no significant difference in the levels of these cytokines between  $\beta$ -Ecd and sulfasalazine group. These results indicated that suppression of the NF- $\kappa$ B pathway indeed decreased the LPS-induced osteoclast formation and inflammation.

## **Discussion**

Infectious bone diseases caused by osteomyelitis, trauma, or post-operative infection of implants may cause persistent inflammatory responses in the body, while the affected sites may be secondary to local bone metabolic disorders, severe bone destruction, and bone death [31, 32].  $\beta$ -Ecd is a plant-derived natural organic compound isolated and extracted from commelinaceae plant dew grass. It has a variety of activities, including stimulating protein synthesis, promoting carbohydrate and lipid metabolism, relieving hyperglycemia and hyperlipidemia, modulating immune system, protecting endothelial cells from apoptosis and inducing their proliferation. Considering that LPS can induce some inflammatory cytokines and  $\beta$ -Ecd can modulate immune system. Therefore, it is worthy to study whether  $\beta$ -Ecd could inhibit LPS-induced inflammation in osteoclasts. According to current results, we found that  $\beta$ -Ecd could inhibit LPS-induced osteoclastogenesis through suppressing NF- $\kappa$ B-mediated inflammatory response in RAW264.7 cells.

Related research confirmed that LPS can stimulate secretion of prostaglandins (PG), histamine and other inflammatory mediators, and activate the release of immune factors such as interleukin (IL), TNF- $\alpha$ , and these inflammatory mediators can be produced by LPS stimulation through different mechanisms to promote the formation and bone resorption of osteoclast activity. Studies have demonstrated that LPS

can promote osteoclast differentiation by enhancing COX-2 expression and activating RANK, JNK and ERK1/2 pathways [33]. Besides, LPS can also accelerate osteoclast differentiation by activating NF- $\kappa$ B-NFATc1 signal pathway [34]. In this study, we found that LPS can increase the expressions of TRAP-positive cells, osteoclasts-related genes and pro-inflammatory cytokines in RAW264.7 cells. However, these events were inhibited by  $\beta$ -Ecd, which indicated that  $\beta$ -Ecd may be a potential candidate for treating and/or preventing osteoclast-associated diseases, including osteoporosis.

Several studies reported that some plant-derived natural organic compounds, such as isoflavones (coumestrol, daidzein and genistein) showed a direct inhibitory effect on cytokine-induced osteoclast differentiation [35, 36]. Another study illustrated that treatment with puerarin (10-50  $\mu$ mol/L) effectively inhibited the production of the inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , PGE2) induced by LPS [37]. Besides, it is reported that artesunate reduced TNF- $\alpha$  production and prevented LPS-induced bone loss *in vivo* [1]. Desoxyrhapontigenin inhibited the differentiation of bone marrow macrophages into mature osteoclasts by suppressing the RANKL-induced activator protein-1 and NFATc1 signaling pathways [38]. Punicalagin played an important role in the attenuation of LPS-induced inflammatory responses in RAW264.7 macrophages and that the mechanisms involved downregulation of the FoxO3a/autophagy signaling pathway [39]. In accordance with their results, we found that treatment with  $\beta$ -Ecd effectively inhibited the production of TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2 induced by LPS, which were consistent with the inhibitory effect of  $\beta$ -Ecd on osteoclast formation. Therefore,  $\beta$ -Ecd can inhibit the production of these pro-inflammatory cytokines which LPS-induced, and prevent later stages of osteoclast differentiation in the infective bone destruction.

NF- $\kappa$ B is the key transcription factor associated with the regulation of COX-2 and mPGES1 after LPS stimulation [40]. Abnormal activation of NF- $\kappa$ B signaling induces osteoclast formation by increasing expression of NFATc1 [41]. Islam et al. reported that LPS, as a potent bone resorbing factor, could induce osteoclastic cell differentiation in RAW264.7 cells [42]. However, the molecular mechanisms underlying the LPS-induced RAW264.7-osteoclasts formation still remains unclear. One speculation is that LPS might mimic RANKL-induced osteoclast formation via activation of NF- $\kappa$ B and SAPK/JNK [43]. Previous studies [44, 45] have indicated that NF- $\kappa$ B pathway serves as a central role in the regulation of osteoclast differentiation and survival. Therefore, NF- $\kappa$ B pathway can be served as a therapeutic target in inhibition of osteoclastogenesis.

Importantly, we found that  $\beta$ -Ecd treatment could markedly decrease the osteoclast numbers and down-regulate the expression of pro-inflammatory cytokines through inhibiting LPS-induced activation of NF- $\kappa$ B, which are critical for osteoclast differentiation. A primary level of control for NF- $\kappa$ B is through interactions with an inhibitor protein called I $\kappa$ B. I $\kappa$ B retains NF- $\kappa$ B in the cytoplasm through masking of the nuclear localization sequences. Removal of I $\kappa$ B activates the NF- $\kappa$ B NLSs so that NF- $\kappa$ B rapidly translocate into the nucleus, binds to select gene promoters in a sequence-specific manner, and activates gene transcription [46]. Activation of NF- $\kappa$ B to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$ . Therefore, the up-regulated ration of p-I $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$  can present the activation of NF- $\kappa$ B pathway [47]. In this study, we found that  $\beta$ -Ecd can inhibit NF- $\kappa$ B

pathway by decreasing the ratio of p-I $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$  and down-regulate the levels of pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , PGE2 and COX-2. Encouragingly, the inhibitory effect of  $\beta$ -Ecd was similar to that of sulfasalazine (the inhibitor of NF- $\kappa$ B). These results indicated that  $\beta$ -Ecd could inhibit NF- $\kappa$ B activation in LPS-treated RAW264.7 cells through suppressing the phosphorylation inactivation of I $\kappa$ B $\alpha$  to increase the transcriptional activity of NF- $\kappa$ B. Considering the key role of NF- $\kappa$ B signaling pathway in LPS-induced osteoclast formation [44, 45], we concluded that suppression of NF- $\kappa$ B can decrease the formation of osteoclasts and  $\beta$ -Ecd may serve as an effective therapeutic agent to treat bone loss diseases.

## Conclusions

Herein, we demonstrated that  $\beta$ -Ecd suppressed LPS-induced osteoclastogenesis *in vitro*. Moreover,  $\beta$ -Ecd exhibited inhibitory effects on LPS-induced osteoclastogenesis by inhibiting NF- $\kappa$ B signaling pathway. Our findings introduce a novel therapeutic approach to control bacteria-induced bone destruction disease, and demonstrate NF- $\kappa$ B signaling pathway may play an important role in this regulating process. The effect of  $\beta$ -Ecd on LPS-induced bone loss *in vivo* still need to be proved in the near future. Nevertheless, our research may provide a reference on correctly evaluation about the pharmacological effect of these natural plant drugs on bone tissue in inflammatory state and direction for the application of natural plant drugs in the regulation of bone metabolism in infectious bone disease.

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### **Authors' contributions**

Yuling Li, Jing Zhang and Ke Jiang designed the experiments. Yuling Li, Jing Zhang, Caiping Yan, Qian Chen, Chao Xiang carried out the experiments and performed the statistical analysis. Qingyan Zhang, Xingkuan Wang, and Yuling Li drafted the manuscript. All authors read and approved the final manuscript.

### **Acknowledgements**

Not applicable

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## Figures

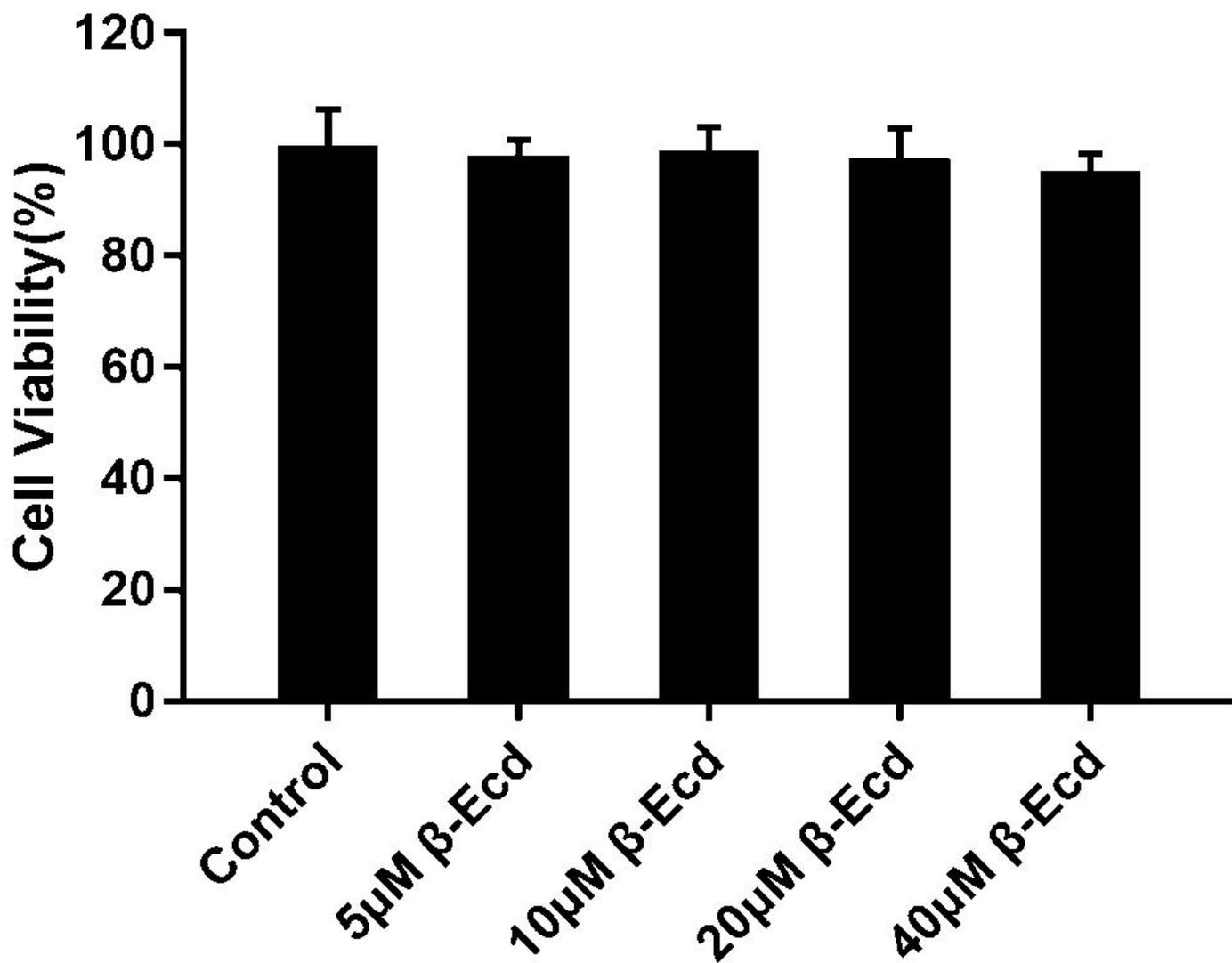


Figure 1

Effect of  $\beta$ -Ecd on cell viability of RAW264.7 cells. RAW264.7 cells were treated with or without  $\beta$ -Ecd (5, 10, 20 and 40  $\mu$ M) for 48 h. Cell viability was measured by CCK-8 assay. There was no significant difference between groups.

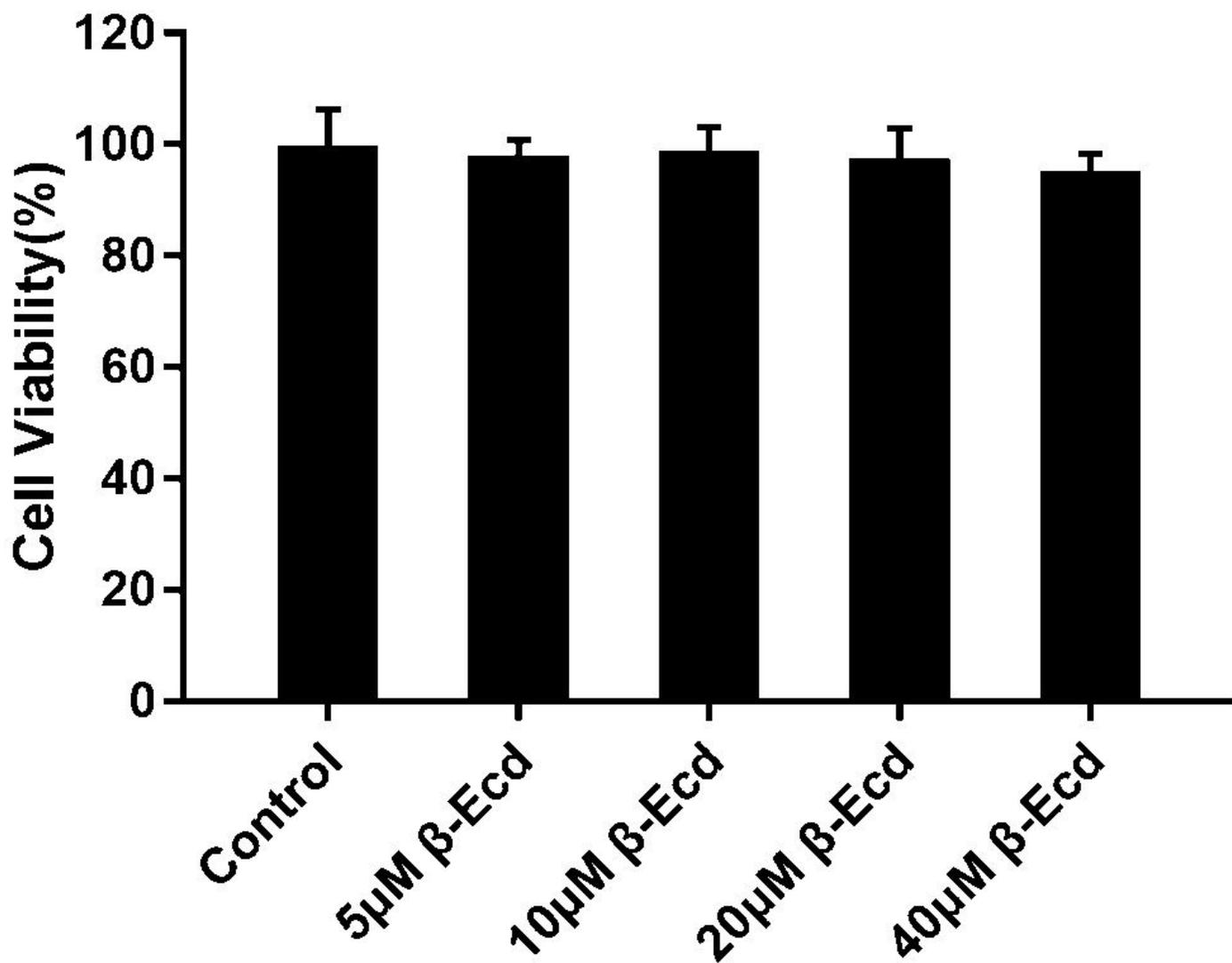


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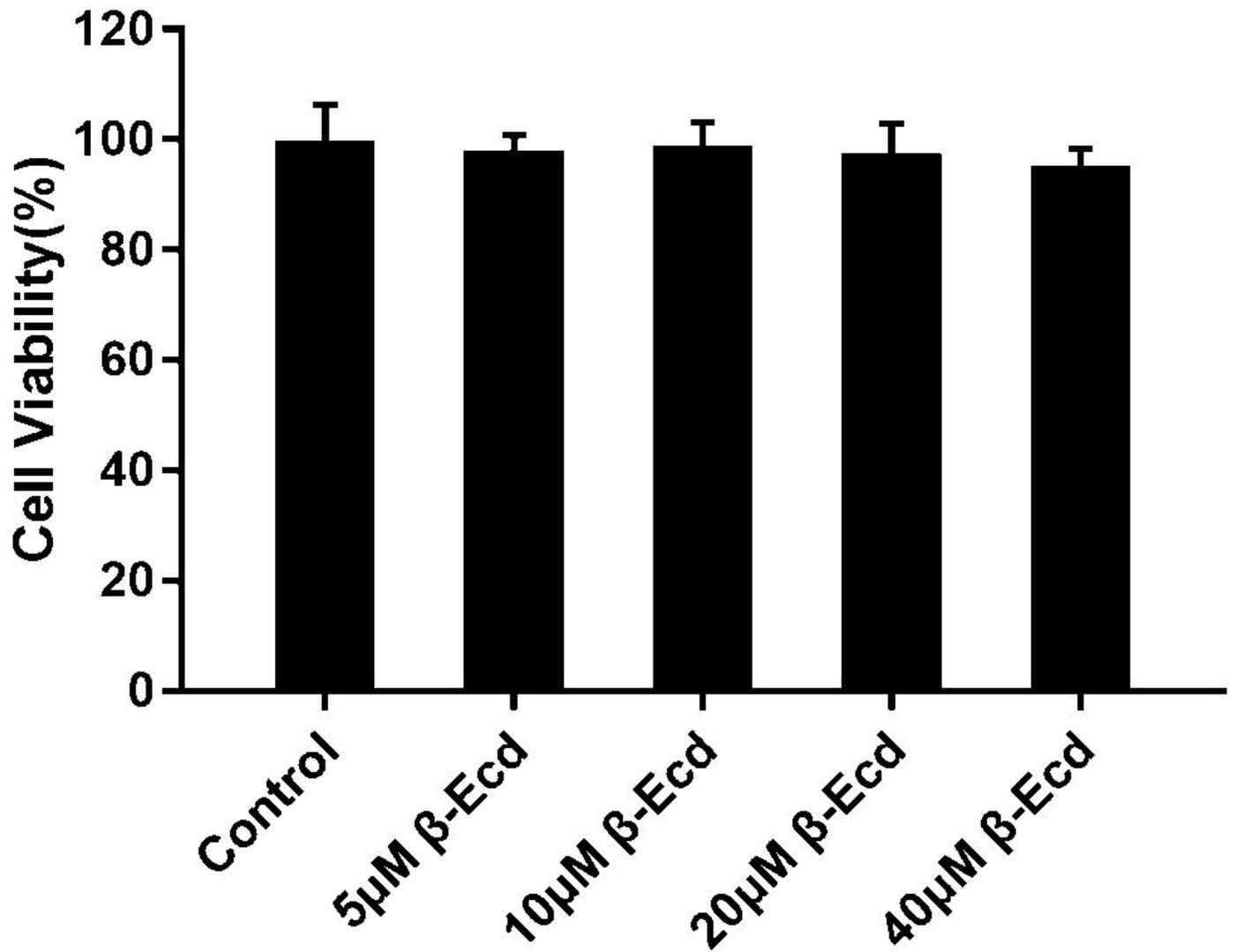
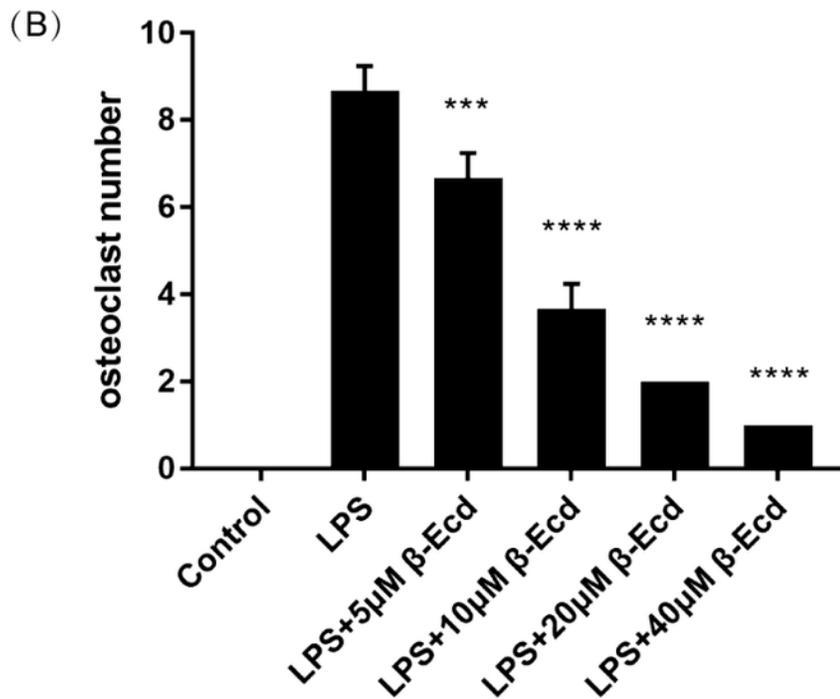
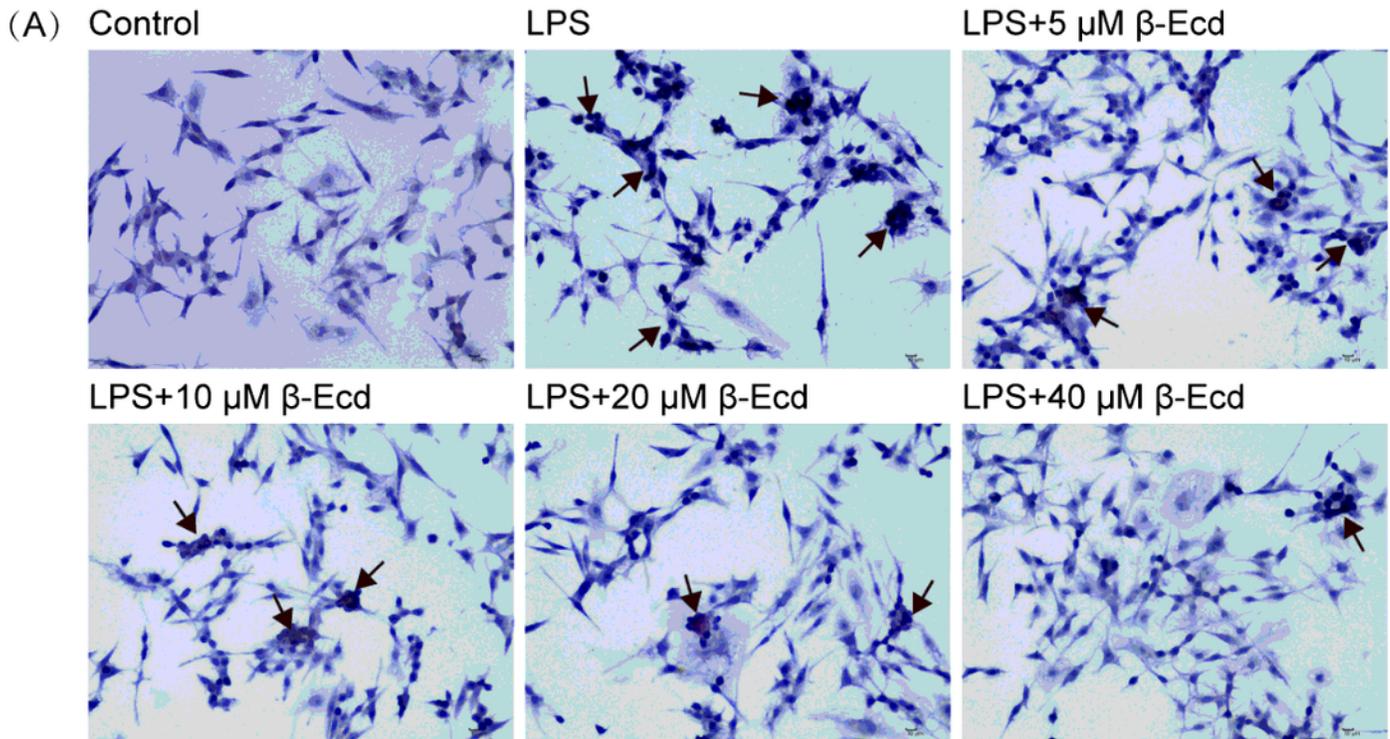


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**Figure 2**

$\beta$ -Ecd inhibited LPS-induced osteoclastogenesis. (A) After RAW264.7 cells were cultured for 24 h, cells were treated with  $\beta$ -Ecd (5, 10, 20 and 40  $\mu$ M) plus LPS (100 ng/mL) for 72 h. Cells were stained for TRAP staining and cells containing more than three nuclei were counted as osteoclast under IX70 microscope. (B) The osteoclast number was counted per field of microscope and the number of control group was

zero. Bars represent the mean  $\pm$  S.D. from three independent experiments. \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001 compared with LPS-treated group. Bars=10 $\mu$ m.

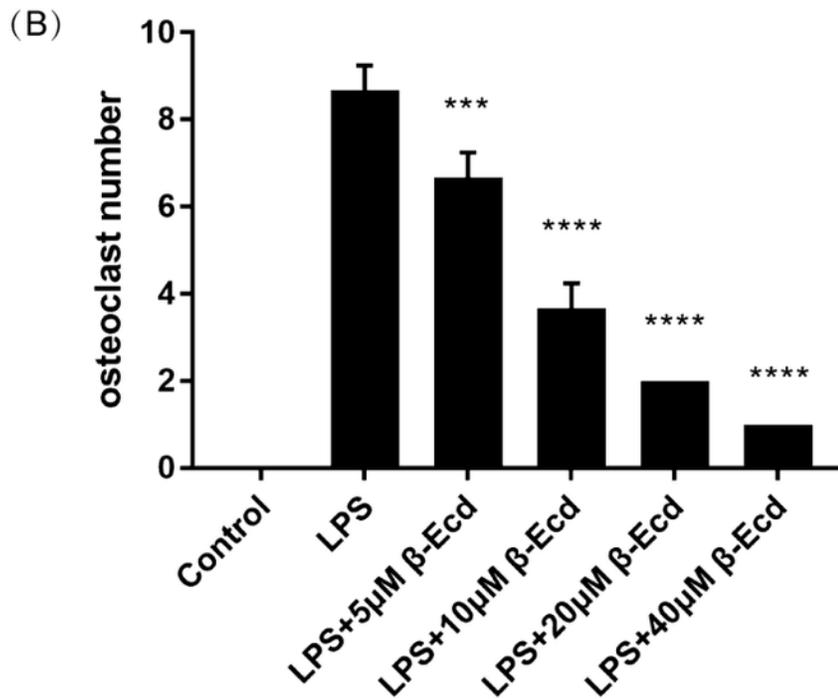
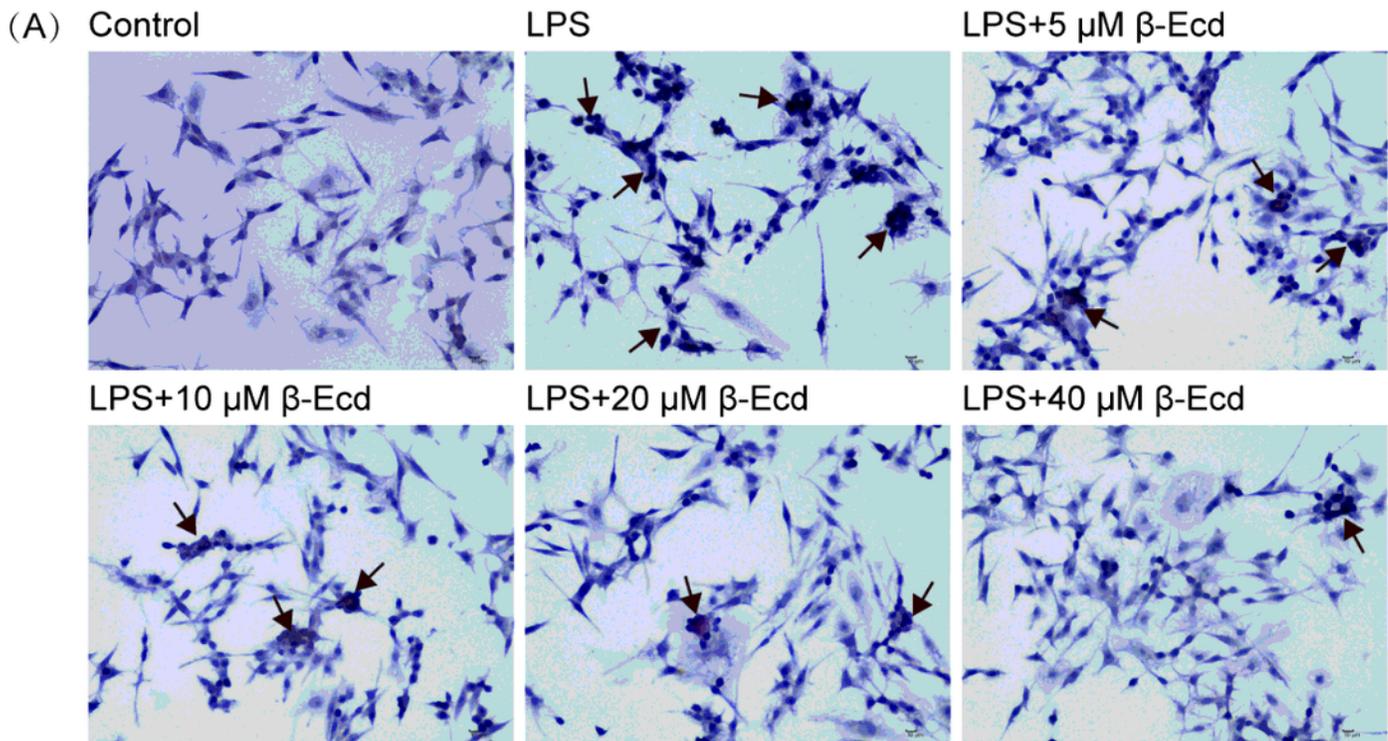


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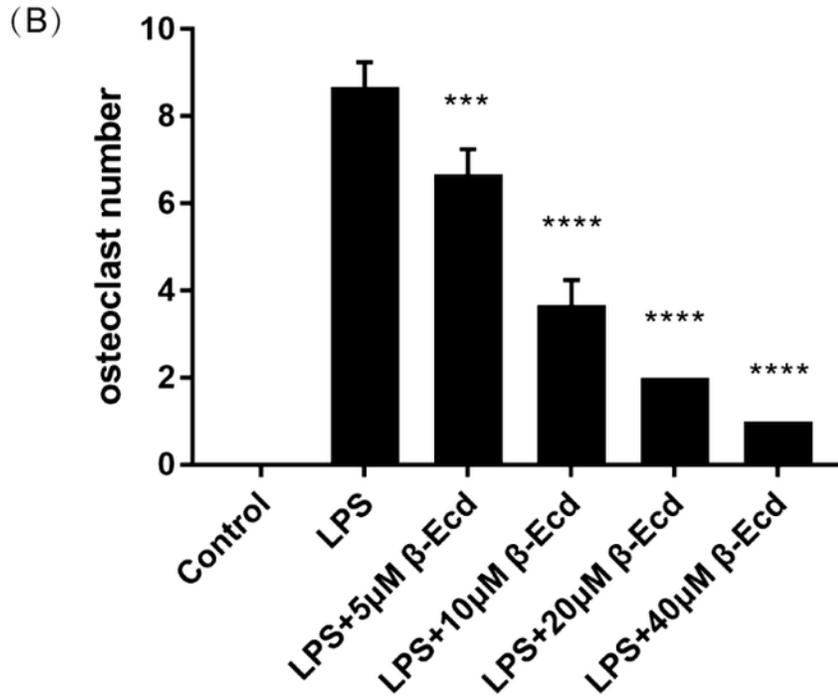
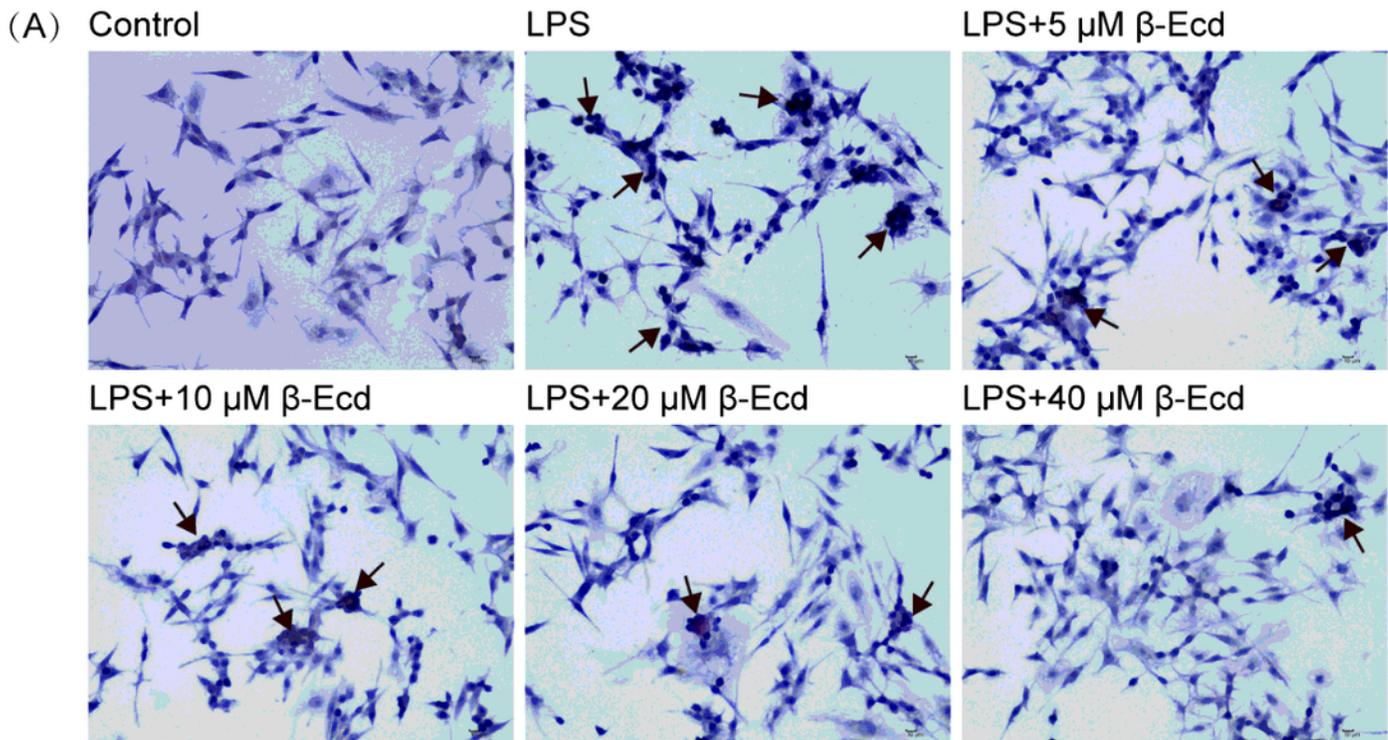
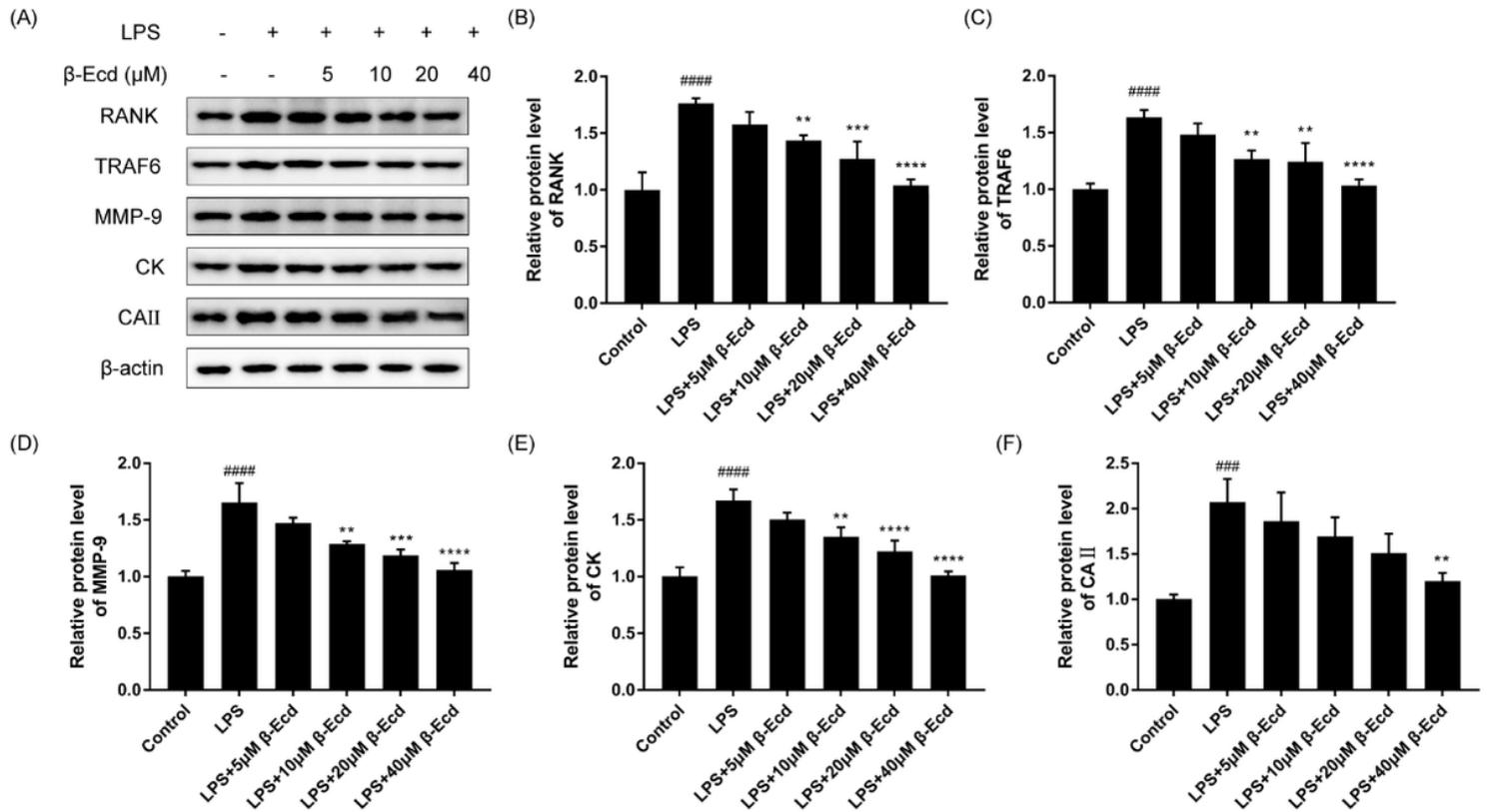


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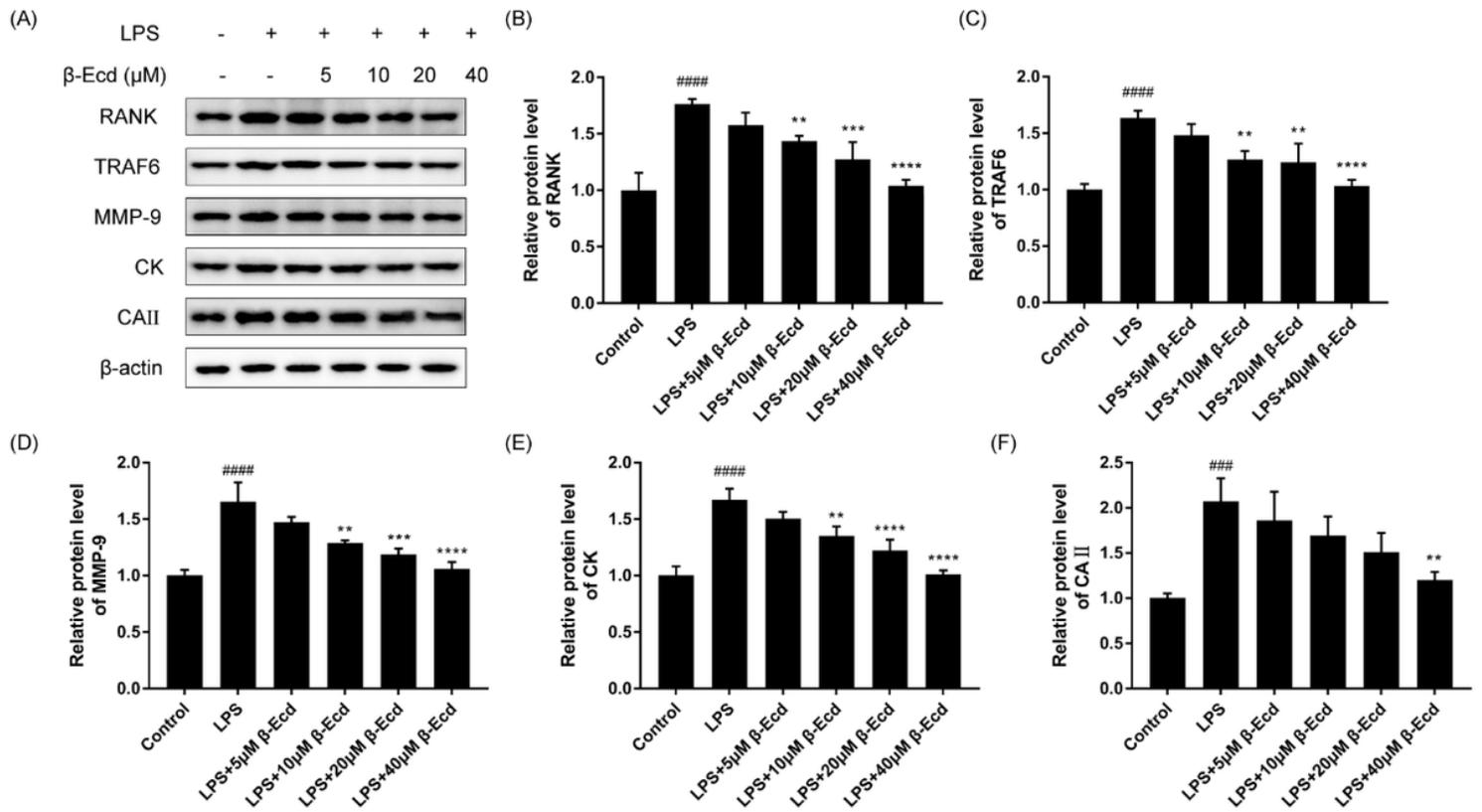
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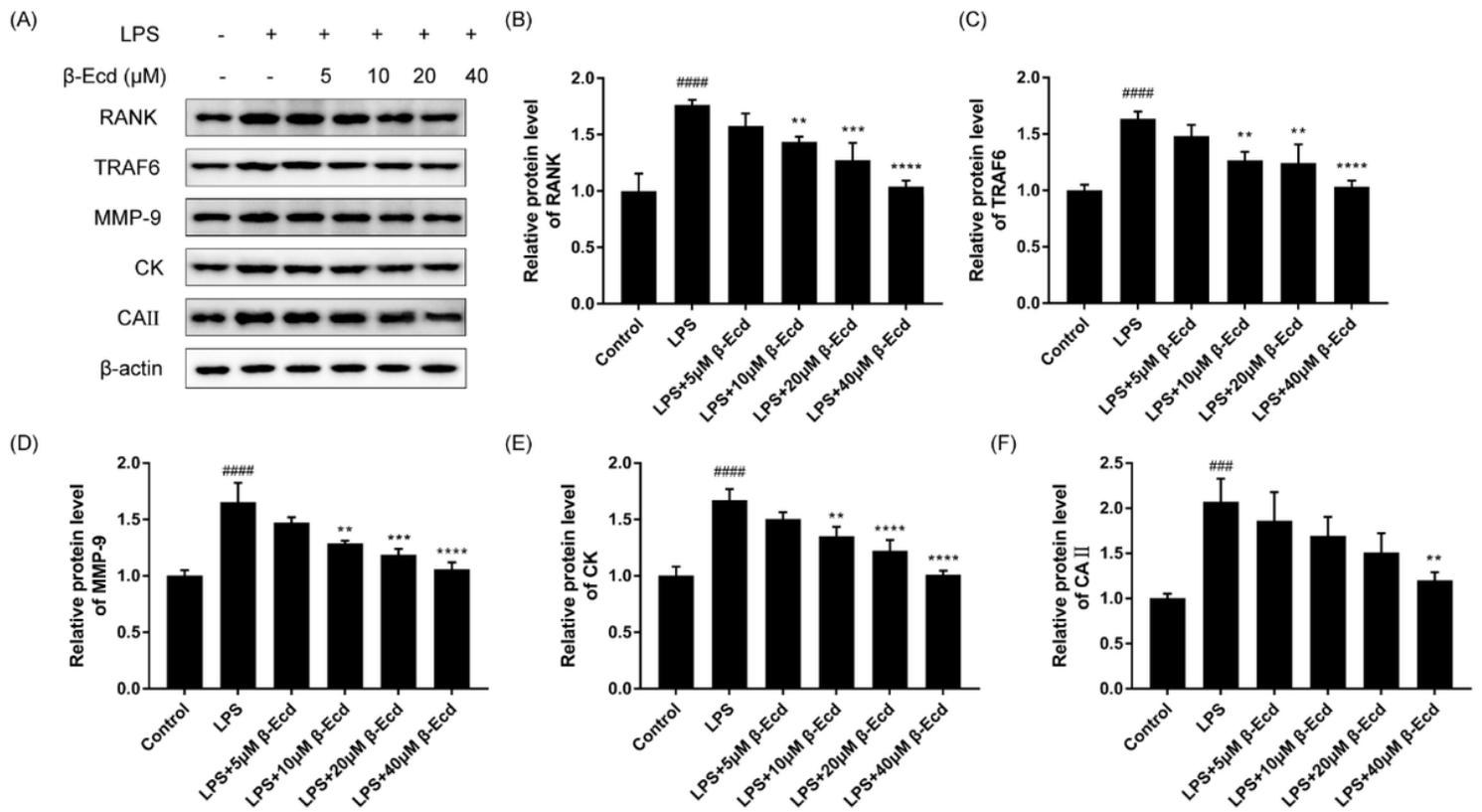
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$\beta$ -Ecd inhibits LPS-induced osteoclast-related protein expression. RAW264.7 cells were treated with 5, 10, 20 and 40  $\mu$ M  $\beta$ -Ecd for 24h with or without LPS. (A) Osteoclast-related genes were analyzed by western blotting using antibodies against RANK, TRAF6, MMP-9, CK and CAII.  $\beta$ -actin served as a loading control. The relative density of RANK (B), TRAF6 (C), MMP-9 (D), CK (E) and CAII (F) were quantified by western blotting analysis and normalized to control. Bars represent the mean  $\pm$  S.D. from three independent experiments. #### $P$ <0.0001 compared with control group, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $p$ <0.0001 compared with LPS-treated group.



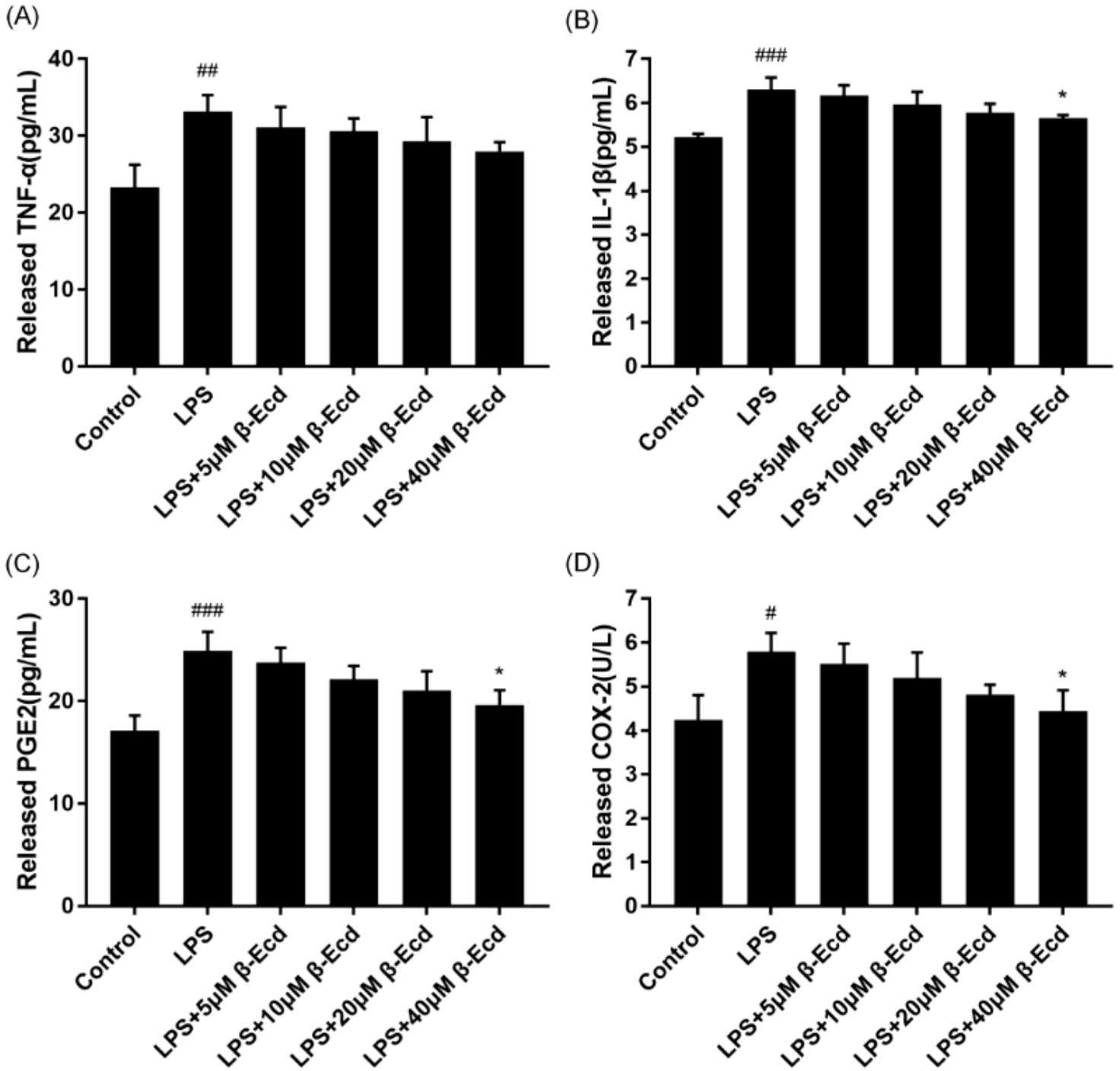
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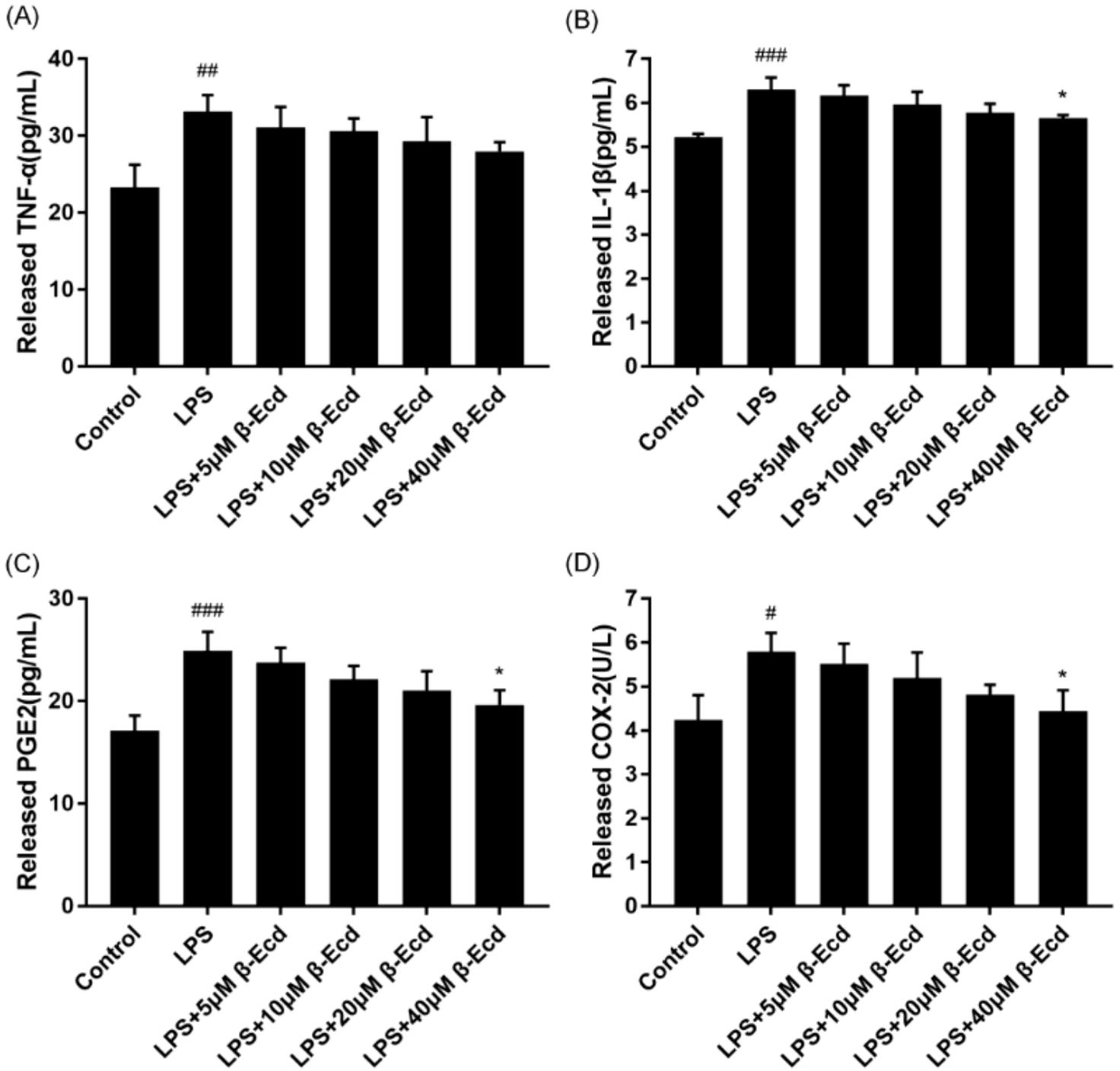
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**Figure 4**

$\beta$ -Ecd suppressed the release of pro-inflammatory cytokines, including TNF- $\alpha$  (A), IL- $\beta$  (B) and PGE2 (C), and COX-2 (D). RAW264.7 cells were treated with 5, 10, 20 and 40  $\mu$ M  $\beta$ -Ecd for 24 h with or without LPS. The expressions of pro-inflammatory cytokines were measured by ELISA. All values are given as means  $\pm$  S.D. from three independent experiments. # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.001 compared with control group, \* $P$  < 0.05 compared with LPS-treated group.



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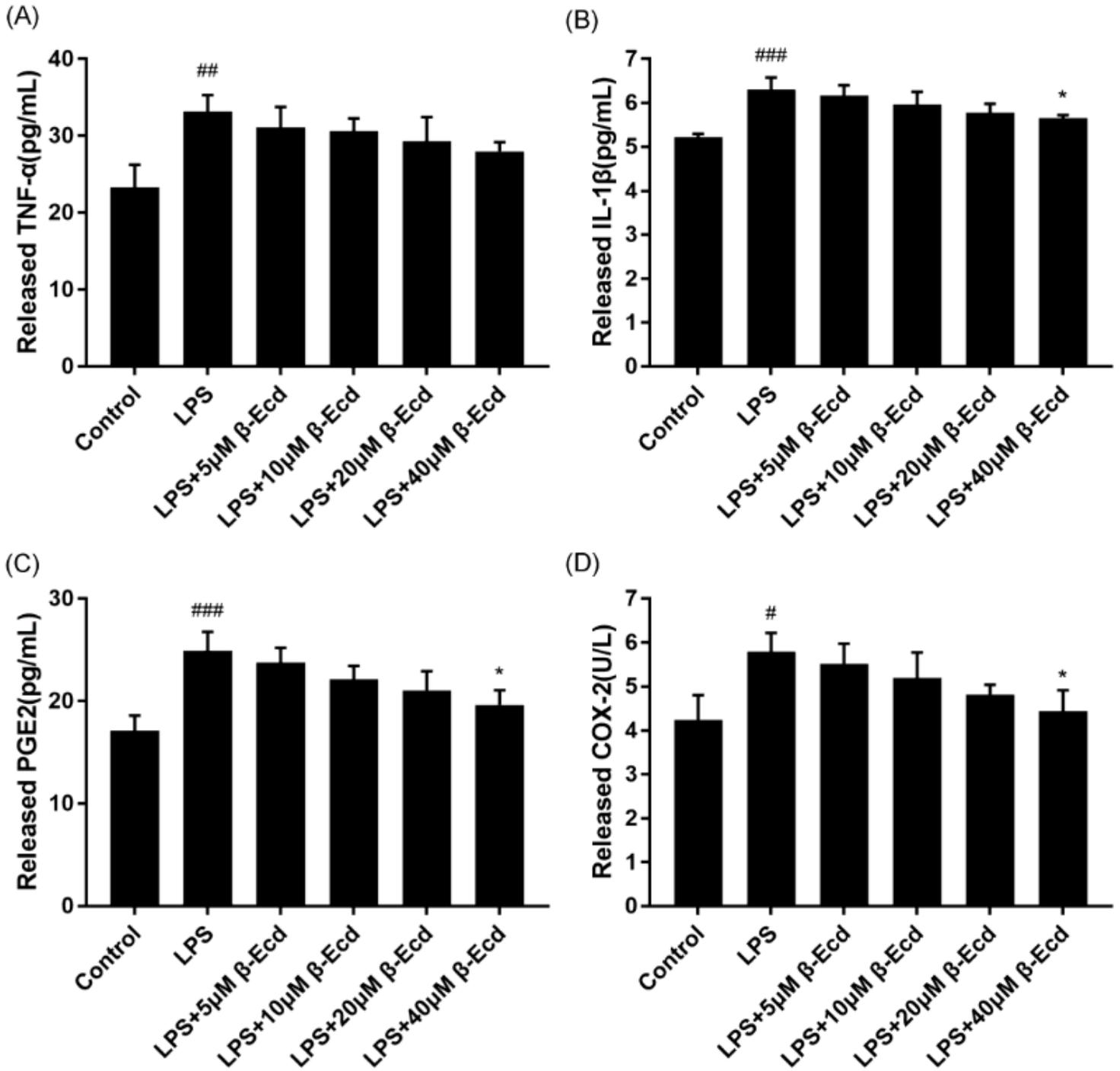
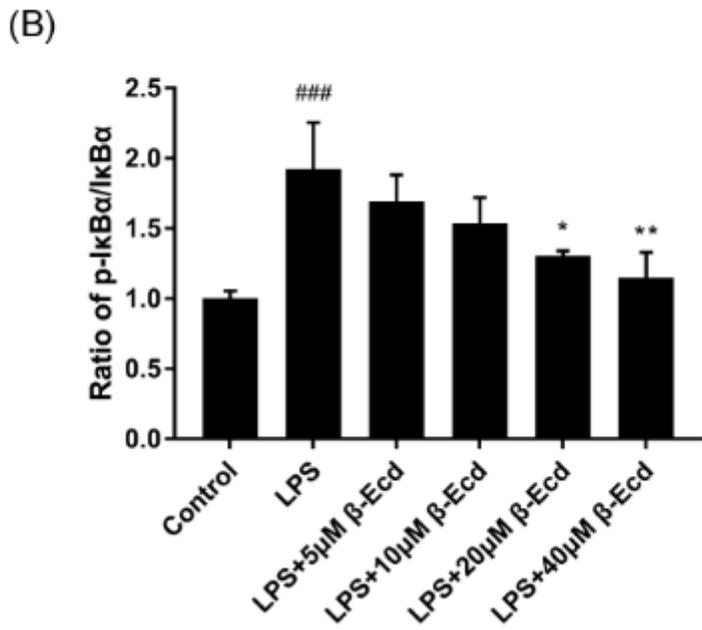
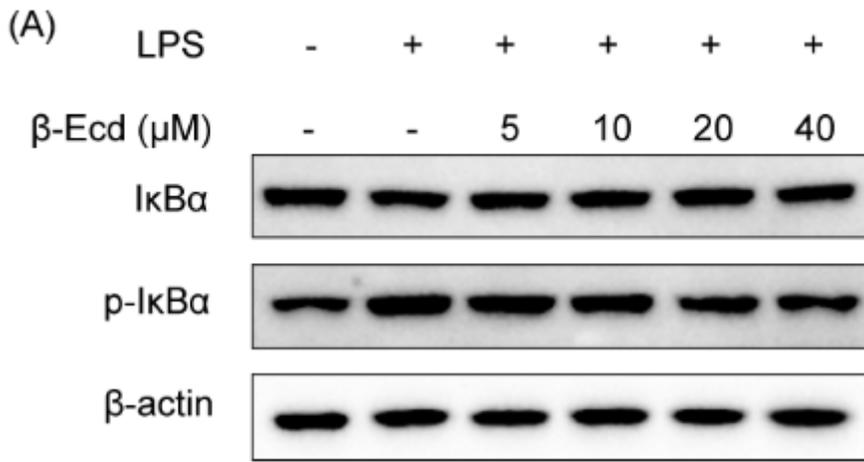


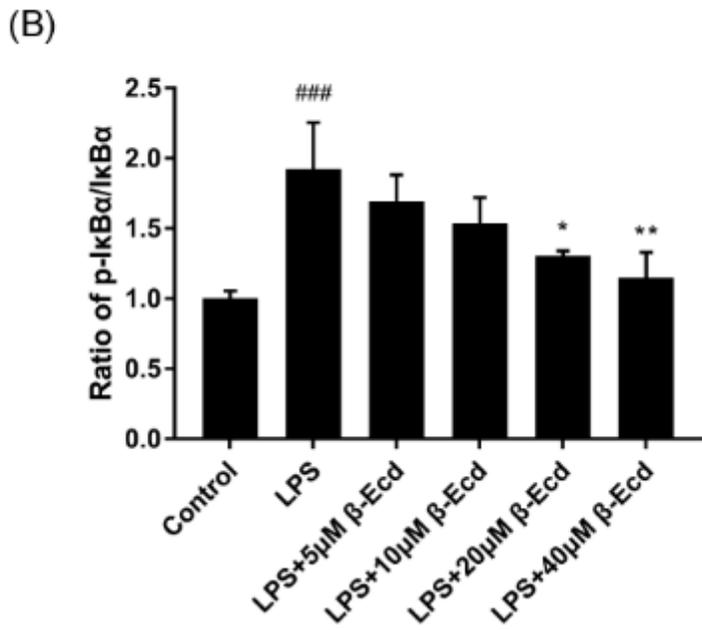
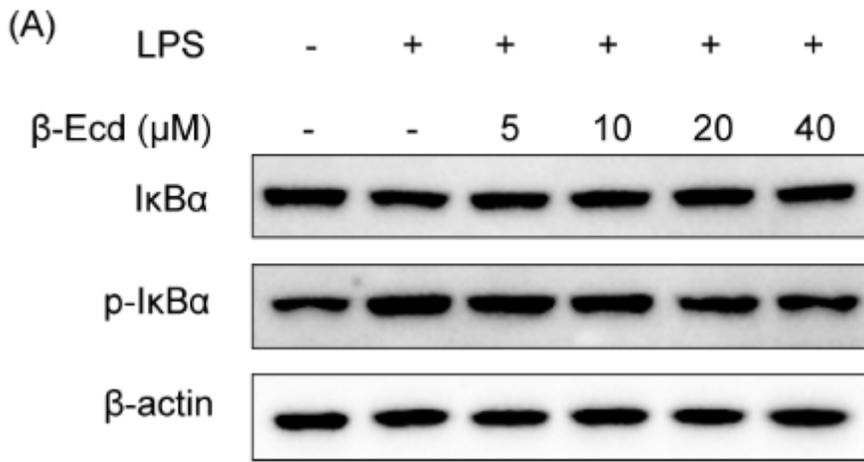
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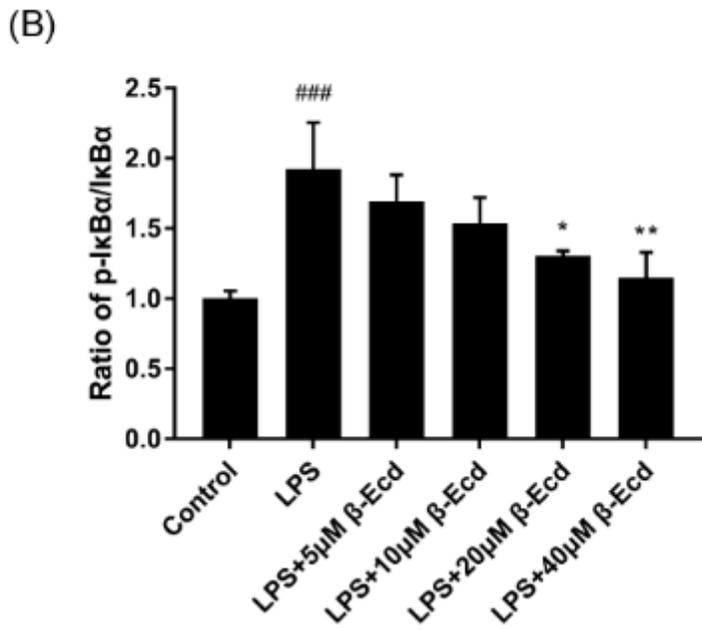
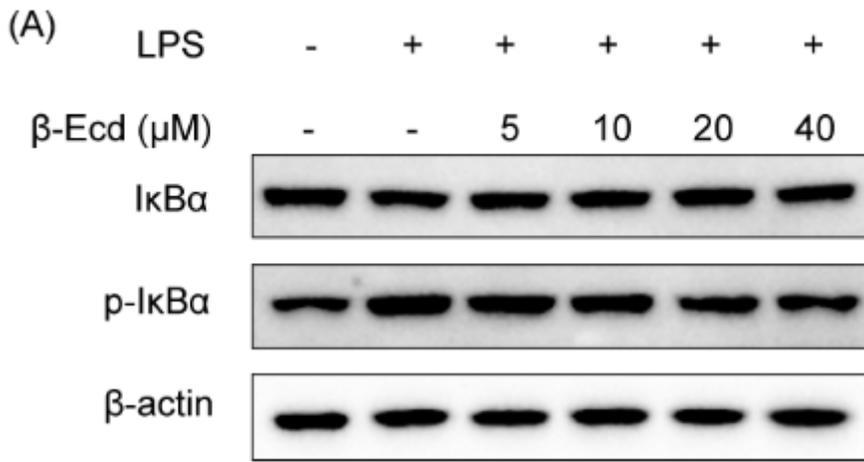
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$\beta$ -Ecd inhibited LPS-triggered activation of NF- $\kappa$ B pathway in RAW264.7 cells. (A) RAW264.7 cells were cultured for 24 h, and treated with 5, 10, 20, and 40  $\mu$ M  $\beta$ -Ecd for 48h with or without LPS. Cell lysates were prepared and analyzed by western blot with specific antibodies to detect the expression levels of I $\kappa$ B $\alpha$  and p-I $\kappa$ B $\alpha$ . (B) Densitometer was analyzed by image analyzing software, and data were expressed as the relative level of p-I $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$ . All values are given as means  $\pm$  S.D. from three independent experiments. ###P<0.001 compared with control group. \*p<0.05, \*\*P<0.01 compared with LPS-treated group.



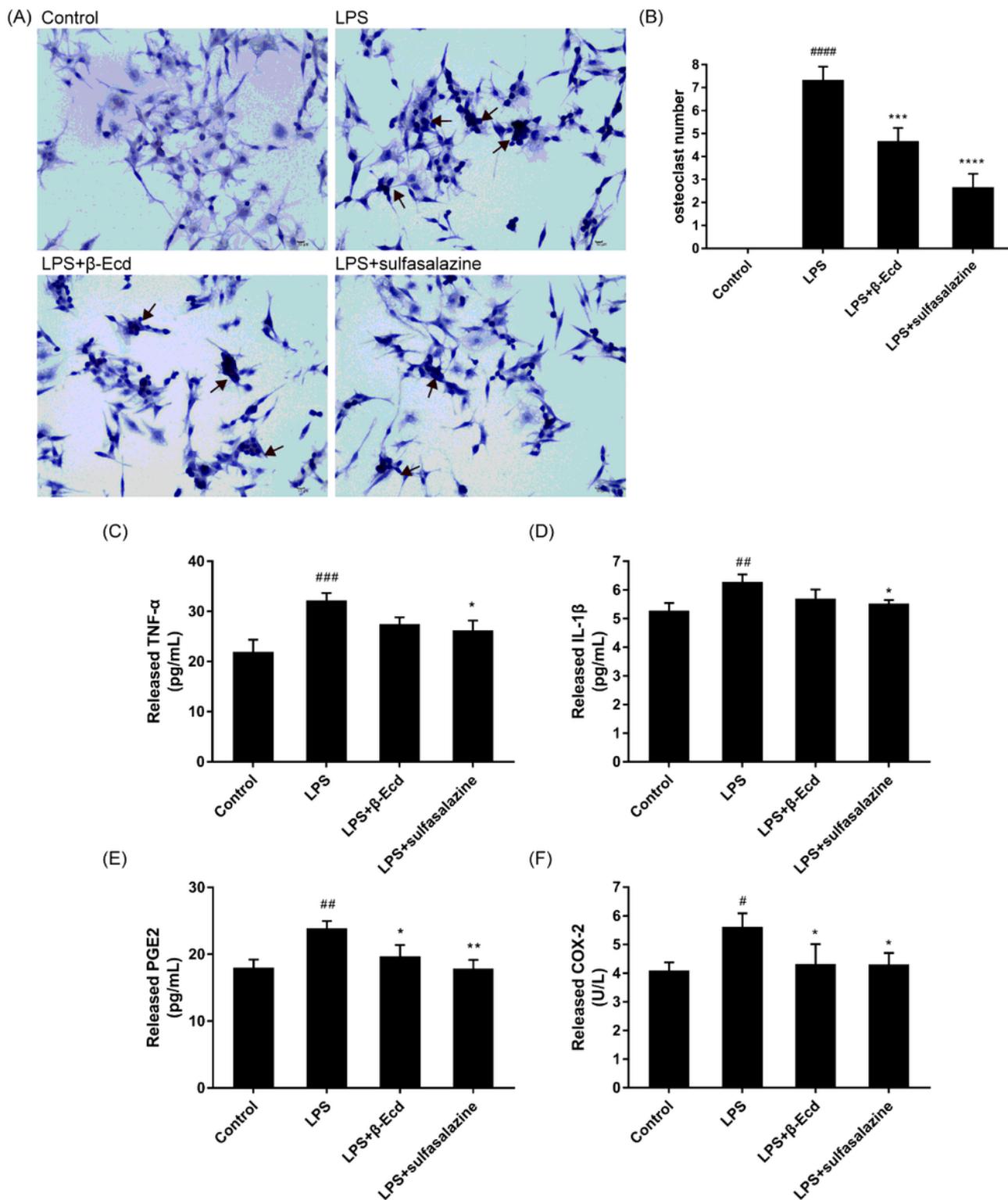
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**Figure 6**

Effect of inhibition NF-κB pathway on LPS-induced osteoclast formation and inflammation. (A) After RAW264.7 cells were cultured for 24 h, cells were treated with 40 μM β-Ecd plus LPS or sulfasalazine plus LPS for 72 h. Cells were stained for TRAP staining and cells containing more than three nuclei were counted as osteoclast under IX70 microscope. Bars=10μm. (B) The numbers of osteoclasts were counted per field of microscope and the number of control group was zero. The expressions of pro-inflammatory

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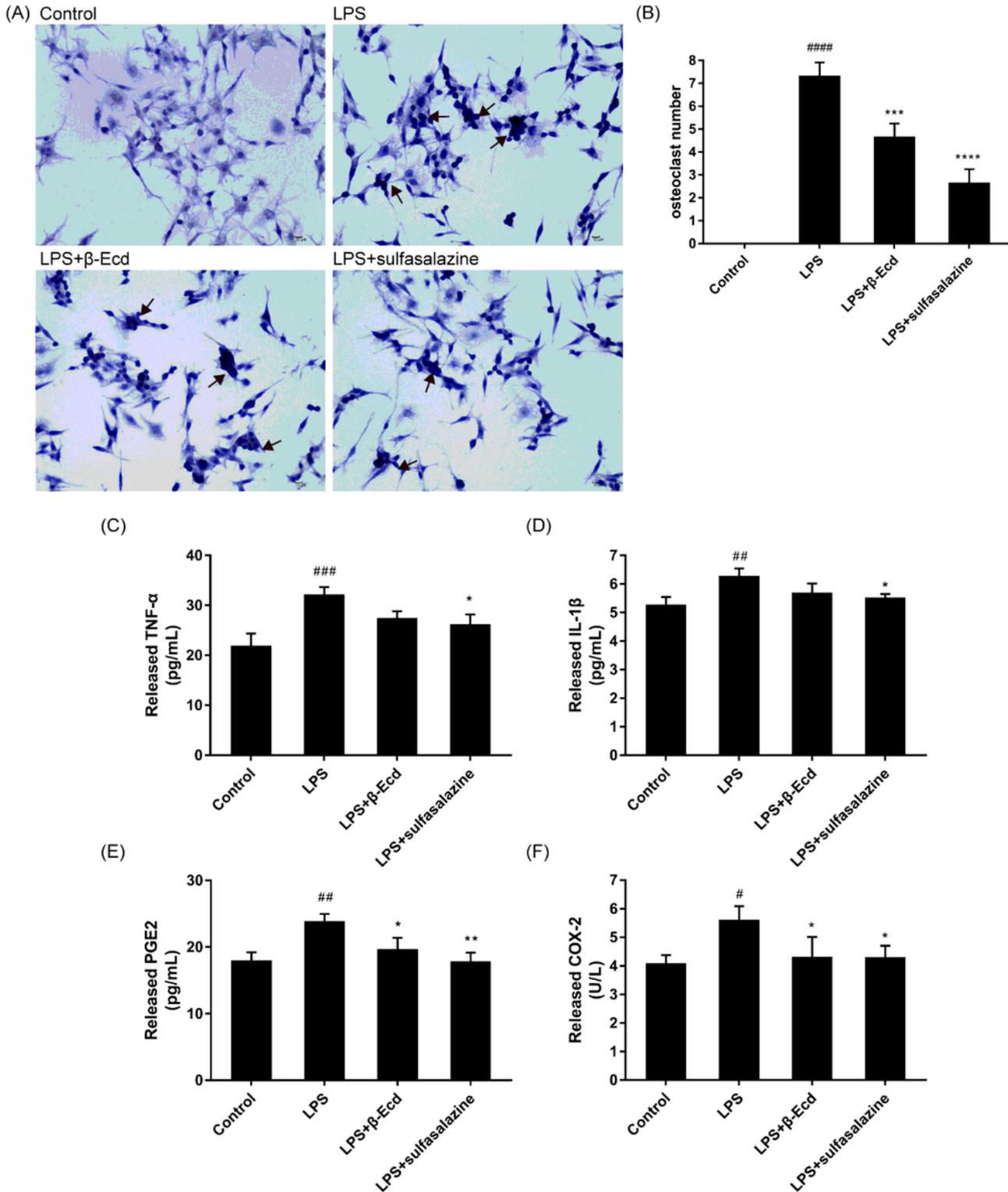
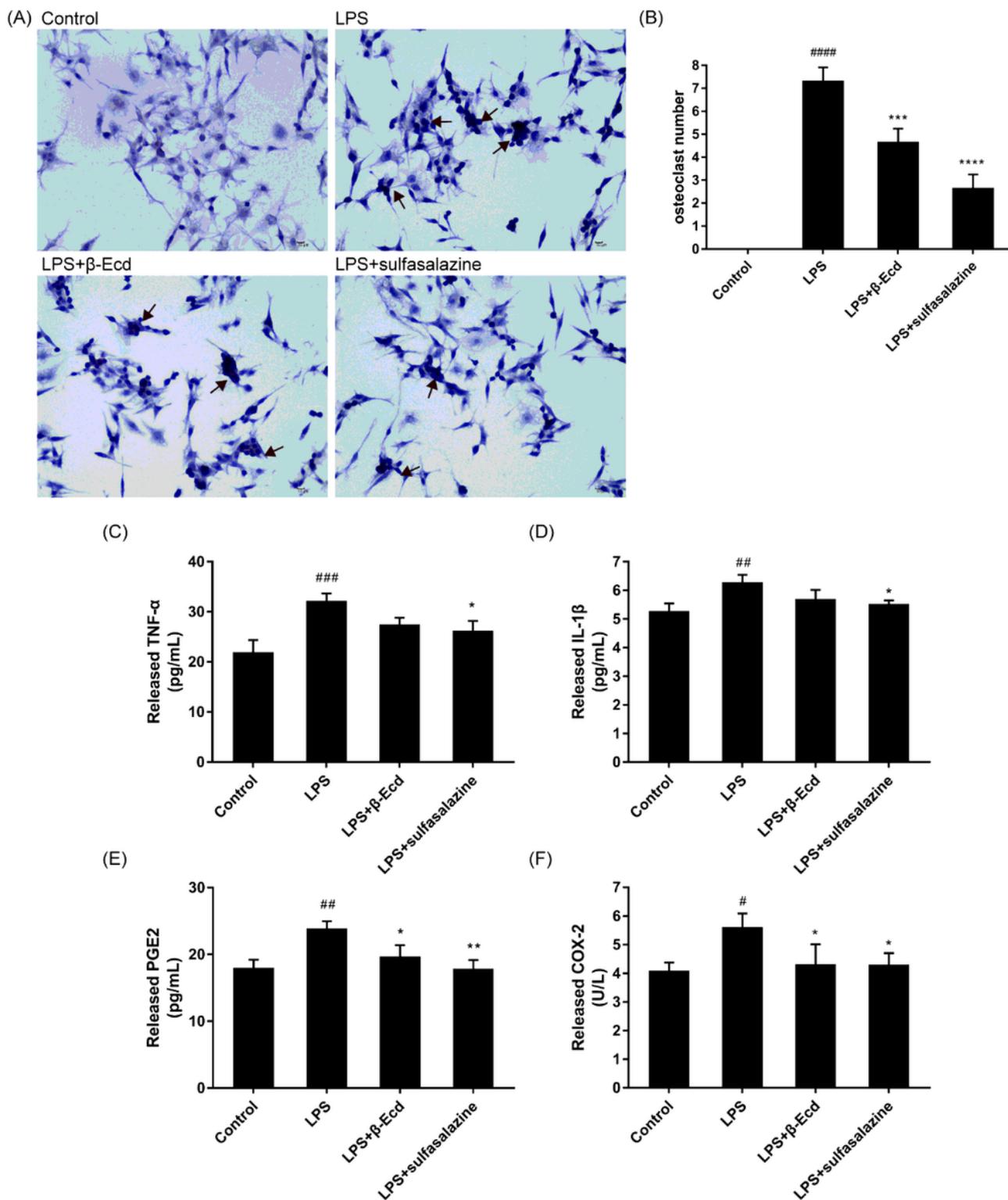


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**Figure 6**

Effect of inhibition NF- $\kappa$ B pathway on LPS-induced osteoclast formation and inflammation. (A) After RAW264.7 cells were cultured for 24 h, cells were treated with 40  $\mu$ M  $\beta$ -Ecd plus LPS or sulfasalazine plus LPS for 72 h. Cells were stained for TRAP staining and cells containing more than three nuclei were counted as osteoclast under IX70 microscope. Bars=10 $\mu$ m. (B) The numbers of osteoclasts were counted per field of microscope and the number of control group was zero. The expressions of pro-inflammatory

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