# Triptolide inhibits the function of TNF-α in osteoblast differentiation by inhibiting the NF-κB signaling pathway

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**Abstract.** Chronic inflammation often delays fracture healing or leads to bone nonunion. Effectively suppressing pathological inflammation is crucial for fracture healing or bone remodeling. Triptolide, which is a diterpenoid epoxide, is the major active component of the Thunder God Vine, *Tripterygium wilfordii*. The aim of the present study was to investigate the role of triptolide in osteoblast differentiation and explore the molecular mechanisms of triptolide in fracture healing. Alkaline phosphatase (ALP) activity was used to evaluate osteoblast differentiation. ALP activity was measured via histochemical staining and western blotting was used to determine the expression of factors associated with inflammation. C2C12 cells were initially treated with 200 ng/ml bone morphogenetic protein (BMP)-2 alone for 3 days, which caused a significant increase in ALP activity (P<0.01). However, treatment with tumor necrosis factor (TNF)-α significantly decreased the ALP activity (P<0.05). Notably, treatment with the chronic inflammatory cytokine TNF-α significantly decreased the effect of BMP-2 in C2C12 cells compared with BMP-2 treatment alone (P<0.01). C2C12 cells were treated with increasing concentrations of BMP-2 or TNF-α for 3 days. The results demonstrated that TNF-α treatment significantly inhibited BMP-2-induced osteoblast differentiation in a dose-dependent manner (P<0.01). The role of triptolide in BMP-2-induced osteoblast differentiation was also examined. Cells were treated with BMP-2, BMP-2 + TNF-α alone, or BMP2 + TNF-α with increasing concentrations of triptolide (4, 8 or 16 ng/ml). After 3 days, the results of ALP activity revealed that triptolide significantly reversed the TNF-α-associated inhibition of osteoblast differentiation (P<0.01). Western blotting analysis demonstrated that triptolide markedly inhibited the phosphorylation of nuclear factor-κB, therefore suppressing the effects of TNF-α. In summary, triptolide is able to reverse the TNF-α-associated suppression of osteoblast differentiation, suggesting that triptolide treatment may have a positive effect on bone remodeling and fracture repairing.

## Introduction

Chronic inflammation significantly delays fracture healing and can lead to nonunion of the bones (1,2). The normal physiological inflammatory reaction is one of the body's principal defense reactions, which is beneficial to fracture healing (3,4). However, chronic inflammation caused by infection or other factors, is detrimental to fracture healing (5,6). Various inflammatory factors, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, are able to stimulate osteoclast differentiation from monocyte-macrophages (7,8). A number of previous studies have reported that infection stimulates the activation of osteoclast differentiation, as well as the molecular mechanisms that trigger it (9,10). However, bone/fracture healing is a proliferative physiological process and requires a lot of new osteoblasts to accelerate the synthesis and calcification of bone, as well as the increase of bone volume and density (11).

Osteoblasts are derived from mesenchymal stromal cells, which are responsible for bone matrix synthesis and subsequent mineralization (12). Chronic persistent inflammation inhibits bone formation and osteoblast differentiation. Abbas *et al* (13) previously reported that TNF-α is able to inhibit pre-osteoblast differentiation via its type-1 receptor. TNF-α is able to activate nuclear factor (NF)-κB via canonical signaling pathway. NF-κB is a transcription regulator and serves an important role in the process of inflammation response. NF-κB contains two subunits, p50 (NF-κB1) and p65 (RelA) (14). Inhibitor of kappa B (IκB)α is able to bind to the submits of NF-κB to inhibit DNA binding activity (15). Inflammatory factors, such as TNF-α and lipopolysaccharide, are able to activate IκB kinase, which phosphorylates IκB proteins, leading to their ultimate degradation and the liberation of nuclear factor (NF)-κB (16,17). NF-κB subsequently translocates into the cell

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nucleus and binds with its downstream proteins and promoters to activate the expression of targeted genes (18).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily, which were first extracted from mineralized bone in 1960s and found to be able to induce heterotopic bone growth. BMPs are secreted proteins present in extracellular locations and can be detected in the serum (19). BMP2, for example, induces the differentiation of mesenchymal cells into chondrocytes. It has previously reported that BMP2 gene was successfully transfected into bone stromal cells, which showed the strong osteogenesis ability (20). BMP2 is the most effective osteoblast differentiation-inducing factor and is able to promote both *in vivo* and *in vitro* osteoblast differentiation and the formation of heterotopic bone (20). Huang *et al* (21) reported that BMP-2 is a novel differentiation factor capable of inducing osteoblast differentiation and bone formation through BMP-2-induced BMP/Smad signaling. Additionally, the mechanism by which BMP-2 induces osteoblast differentiation requires BMP-2-induced activation of the phosphatidylinositol 3-kinase and Akt serine/threonine kinase pathway (22,23).

Triptolide is one of the major active components extracted from the Thunder God Vine, *Tripterygium wilfordii*, and is a diterpenoid epoxide with molecular formula of $C_{20}H_{24}O_6$ (24-26). In the present study, it was indicated that 100 ng/ml BMP-2 was able to effectively stimulate the differentiation of bone cell precursors in the C2C12 cell line. The underlying molecular mechanism of triptolide in osteoblast differentiation was also investigated.

## Materials and methods

*Cell line and agents*. The C2C12 cell line was purchased from Biowit Technologies Ltd. (Shenzhen, China). C2C12 is a mouse myoblast cell line, and C2C12 cells typically differentiate into contractile myotubes and produce characteristic muscle proteins. Treatment with BMP-2 causes a shift in the differentiation pathway from myoblastic to osteoblastic cells. In the present study, C2C12 cells were treated with BMP-2 (10426-H01H-10; Sinobiological, Inc., Beijing, China) in order to produce osteoblast cells. C2C12 cells were cultured with Dulbecco's Modified Eagle Medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an atmosphere containing 5% $CO_2$ for 3 days. The culture medium was changed every 2 to 3 days and cells were passaged every 3 to 5 days once the cell culture plate was 80% covered. Triptolide was purchased from Sigma-Aldrich (Merck kGaA, Darmstadt, Germany) and the purity was reported to be >98% as determined by high performance liquid chromatography by the manufacturer. The amount of endotoxin in BMP2 was <1.0 EU/µg as determined by the manufacturer. Recombinant mouse TNF-α was obtained from BioLegend, Inc. (cat. no. 575202; San Diego, CA, USA), and the endotoxin level was <0.1 EU/µg (<0.01 ng/µg) protein as determined by the LAL method.

*Osteoblast differentiation and cell treatment*. All C2C12 cells were initially treated with 100 ng/ml BMP-2 and 5 ng/ml TNF-α. Osteoblast differentiation was assessed by testing the activity of alkaline phosphatase (ALP). The ALP kit (cat. no. AP0100-1KT) was obtained from Sigma-Aldrich (Merck kGaA) and ALP activity was measured according to the manufacturer's protocol. C2C12 cells were divided into 4 groups and treated with different stimulators. The four groups were as follows: BMP2 group, treated with 200 ng/ml BMP-2 for days; TNF-α group, treated with 5 ng/ml TNF-α for 3 days; BMP-2-TNF-α group, treated with 200 ng/ml BMP + 5 ng/ml of TNF-α for 3 days. Following treatment, the ALP activity for each group was determined according to the kit protocols. To investigate the role of triptolide, increasing concentrations (0, 4, 8 and 16 ng/ml) were used to treat C2C12 cells. After 3 days, ALP activity was determined and the role of triptolide was investigated.

*ALP detection*. Osteoblast differentiation was detected via an ALP detection assay. In one experiment, ALP activity was evaluated using histochemical staining. C2C12 cells were treated with BMP-2 at a concentration of 200 ng/ml and/or TNF-α at a concentration of 5 ng/ml for 3 days. The cells were stained with naphthol and AS-BI alkaline solution with Alizarin for 20 min at 37°C. In other experiment, C2C12 cells were treated with 100 ng/ml BMP-2 in the absence or presence of 5 ng/ml TNF-α and/or triptolide (4, 8 and 16 ng/ml) and cells were subsequently divided into groups treated for 72 h as outlined above. Additionally, to test the role of BMP-2 and TNF-α in osteoblast differentiation, C2C12 cells were treated with different concentrations of BMP-2 or TNF-α for 3 days. Firstly, cells were treated with 100, 200 or 300 ng/ml BMP-2 for 3 days and, based on the results of this experiment, 100 ng/ml was selected as the appropriate concentration of BMP-2 for further experiments. Cells were subsequently treated with 2.5, 5.0 or 10.0 ng/ml TNF-α for 3 days and ALP activity was determined as well. C2C12 cells were lysed and the cellular ALP activity in different groups was measured using a fluorometric detection kit with 4-methylumbelliferyl phosphate disodium substrate (Sigma-Aldrich; Merck kGaA). In the present study, the untreated cells were used as negative controls and the ALP activity of each sample was normalized by protein concentration. The experiment was repeated three times.

*Western blotting analysis*. Cells were washed three times with PBS buffer and lysed with lysis buffer (Tris 50 mmol/l, NP-40 1%, NaCl 150 mmol/l, EDTA 1 mmol, SDS 0.1%, sodium deoxycholate 0.25%; Beyotime Institute of Biotechnology, Haimen, China; cat. no. P0013B). Cells were subsequently centrifuged at 800 g for 10 min at room temperature, proteins (10 µl per lane) were separated by 10% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane at 400 mA for 1 h. The membrane was blocked with 5% nonfat milk in TBST buffer (Tris-HCl 50 mmol/l, , NaCl 150 mmol/l and 0.1% Tween) for 30 min at room temperature and subsequently incubated with primary antibodies at 4°C overnight followed by secondary antibodies at room temperature for 30 min. Between steps, the membrane was washed with TBST buffer for 5 min. Bands were detected using an enhanced chemiluminescence western blotting detection system (Pierce ECL Western Blotting Substrate; cat. no. 32106; Thermo Fisher Scientific, Inc.)
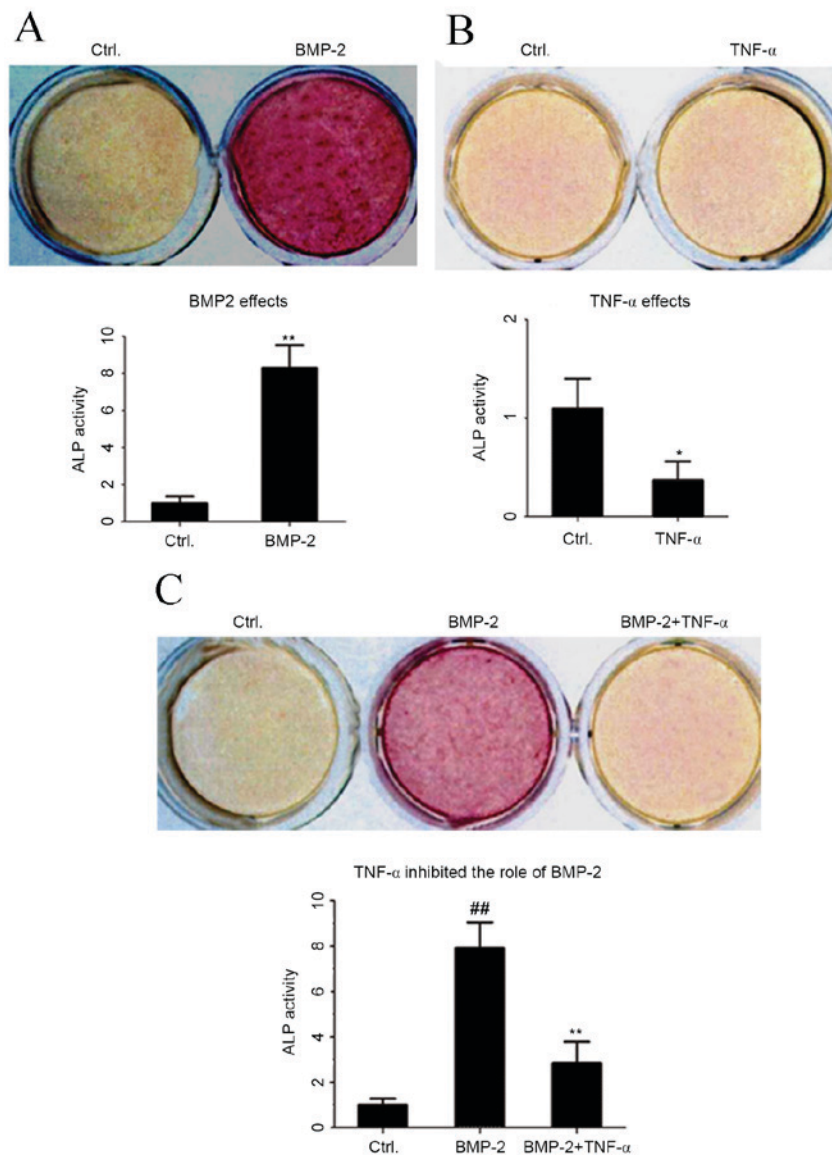
Figure 1. TNF-α significantly inhibited the BMP-2-induced osteoblast differentiation. (A) Effect of BMP-2 on osteogenic activity. C2C12 cells were treated with 200 ng/ml BMP-2 for 3 days and the ALP activity was assessed. $^{**}P<0.01$ vs. Ctrl group. (B) Effect of TNF-α on osteoblast activity. C2C12 cells were treated with 5 ng/ml TNF-α for 3 days and the ALP activity was determined. $^{*}P<0.05$ vs. Ctrl group. (C) TNF-α inhibited the effect of BMP-2 on osteogenic activity. C2C12 cells were treated with 200 ng/ml BMP-2 or 200 ng/ml BMP + 5 ng/ml TNF-α for 3 days and the ALP activity was determined. $^{##}P<0.01$ vs. Ctrl group, $^{**}P<0.01$ vs. BMP-2 group. TNF, tumor necrosis factor; BMP, bone morphogenetic protein; ALP, alkaline phosphatase; Ctrl, control.

according to the manufacturer's protocol. The antibodies used in the study were as follows: Rabbit polyclonal anti-NF-κB p65 (phospho S536; cat. no. ab86299; Abcam, Cambridge, UK), mouse monoclonal anti-β-Actin (C4; 200 µg/ml; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The secondary antibodies used were as follows: Goat anti-rabbit Immunoglobulin (Ig)G H&L (1:1,000; cat. no. ab6721; Abcam) and goat anti-mouse IgG H&L (1:1,000; cat. no. ab6789; Abcam). Protein bands were analyzed using ChemiDoc XRS version 1.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Statistical analysis*. All data are presented as the mean ± standard deviation from at least 3 independent experiments. Each experiment was performed in triplicate. Data were analyzed by one-way analysis of variance using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

*TNF-α significantly inhibits BMP-2-induced osteoblast differentiation*. ALP is often used to determine the progress of osteoblast differentiation. ALP activity was significantly increased following treatment with BMP-2 compared with the control ($P<0.01$; Fig. 1A), whereas TNF-α treatment significantly decreased ALP activity compared with the control ($P<0.05$; Fig. 1B). Cells treated with BMP-2 in combination with TNF-α were also evaluated, and the results demonstrated that the ALP activity was significantly decreased compared with BMP-2 treatment alone ($P<0.01$; Fig. 1C).
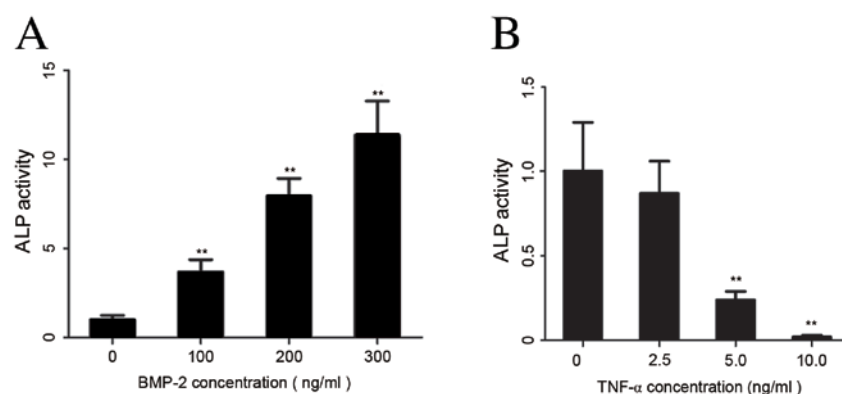
Figure 2. TNF-α inhibited BMP-2-induced osteoblast differentiation in a dose-dependent manner. (A) C2C12 cells were treated with 100, 200 or 300 ng/ml BMP-2 for 3 days and ALP activity was evaluated. **P<0.01 vs. untreated cells. (B) C2C12 cells were treated with 100 ng/ml BMP-2 + 2.5, 5.0 or 10.0 ng/ml TNF-α for 3 days and ALP activity was assessed. **P<0.01 vs. BMP-2 group (0 ng/ml TNF-α). TNF, tumor necrosis factor; BMP, bone morphogenetic protein; ALP, alkaline phosphatase.

*TNF-α inhibits BMP-2-induced osteoblast differentiation in a dose-dependent manner.* To further determine the role of BMP-2 and TNF-α on osteoblast differentiation, increasing concentrations of BMP-2 or TNF-α were used to treat C2C12 cells for 3 days. Cells were treated with 100, 200 or 300 ng/ml BMP-2 for 3 days and the ALP activity was found to be 3.68±0.69, 7.94±0.98, 11.37±1.90, respectively (Fig. 2A). ALP activity was significantly increased at all concentrations of BMP-2 compared with untreated cells (all P<0.01). Based on the results of this experiment, 100 ng/ml was selected as the appropriate concentration of BMP-2 for further experiments. Cells were subsequently treated with 2.5, 5.0 or 10.0 ng/ml TNF-α for 3 days and ALP activity was found to be 0.87±0.19, 0.24±0.05 and 0.02±0.01, respectively (Fig. 2B). ALP activity was significantly decreased by TNF-α treatment at a concentration of ≥5.0 ng/ml compared with untreated cells (P<0.01), therefore 5.0 ng/ml TNF-α was used for all further experiments.

*Triptolide significantly antagonizes the inhibition role of TNF-α in osteoblast differentiation.* Triptolide is one of the main active components in the Chinese herb *Tripterygium wilfordii*, which has been demonstrated to possess anti-inflammatory properties (27-29). Furthermore, the anti-inflammatory effects of triptolide have been demonstrated to be involved in NF-κB signaling pathway (30). In order to further investigate the role of triptolide in osteoblast differentiation, C2C12 cells were treated with 100 ng/ml BMP-2 and 5 ng/ml TNF-α with or without increasing concentrations of triptolide (4, 8 or 16 ng/ml) for 3 days. The results demonstrated that triptolide significantly increased ALP activity in a dose-dependent manner compared with the BMP-2-TNF-α group (Fig. 3; P<0.01 at all doses of triptolide).

*Triptolide effectively inhibits the phosphorylation of NF-κB.* To investigate the underlying molecular mechanism of triptolide in osteoblast differentiation, the role of triptolide in the NF-κB signaling pathway was assessed. TNF-α treatment markedly increased the phosphorylation of NF-κB in a time-dependent manner (Fig. 4A). The amount of p-NF-κB was most markedly increased 1 h following treatment with 5 ng/ml TNF-α,
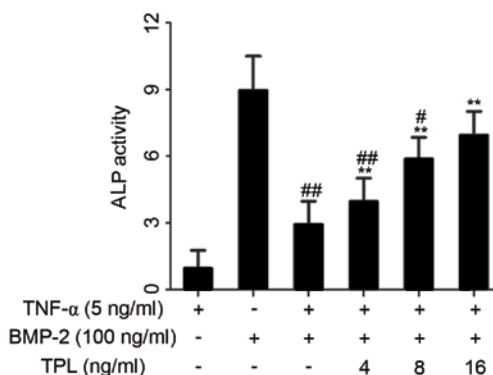


Figure 3. TPL significantly attenuated TNF-α-induced inhibition of osteoblast differentiation. C2C12 cells were treated with 100 ng/ml of BMP-2 and 5 ng/ml of TNF-α with or without triptolide (4, 8, 16 ng/ml) for 3 days and ALP activity was assessed. **P<0.01, vs. BMP-2-TNF-α group and #P<0.05, ##P<0.01, vs. BMP-2 group. TPL, triptolide; TNF, tumor necrosis factor; BMP, bone morphogenetic protein; ALP, alkaline phosphatase.
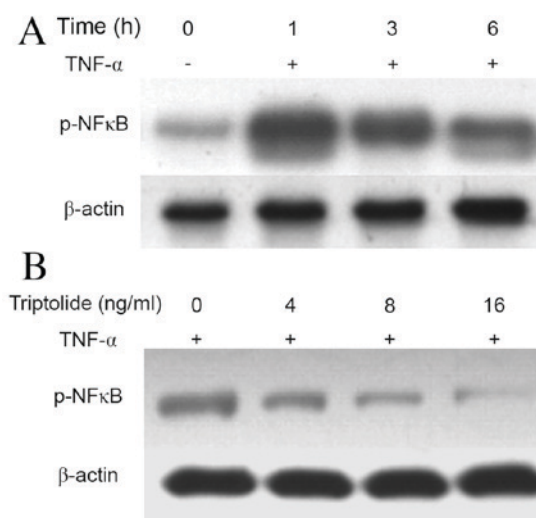


Figure 4. Triptolide effectively inhibited the phosphorylation of NF-κB. (A) TNF-α treatment had a dynamic effect on the phosphorylation of NF-κB. (B) Levels of p-NF-κB gradually decreased with increasing concentrations of triptolide. The levels of p-NF-κB were determined by western blotting analysis. NF, nuclear factor; TNF, tumor necrosis factor; p, phosphorylated.

whereas levels of phosphorylated NF-κB decreased slightly at 3 and 6 h after TNF-α treatment. Based on these results, 1 h was selected as the appropriate time for TNF-α treatment in conjunction with increasing concentrations of triptolide, Triptolide treatment was demonstrated to markedly downregulate levels of phosphorylated NF-κB in a dose-dependent manner (Fig. 4B). The results of the present study demonstrate that the anti-inflammatory role of triptolide in the process of osteoblast differentiation is involved in the inhibition of phosphorylation of NF-κB.

## Discussion

When a bone is broken or fractured, the mesenchymal stem cells in nearby bone marrow are stimulated and activated by various cytokines in the extracellular matrix to migrate to the injury site to develop and differentiate into osteoblasts (31). It has been reported that osteoblast differentiation is induced by c-Jun N-terminal kinase and extracellular signal-related kinase-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells (32). Cytokines such as TNF-α have a dual action in bone formation and are important in various skeletal diseases, including rheumatoid arthritis, inflammation and osteolysis (33). TNF-α is a potent pro-inflammatory cytokine and is ubiquitously distributed throughout the body (34).

BMPs have been reported to regulate osteoblast formation and osteogenesis, which also promotes gene expression by bone-specific transcription factors (35). Under normal circumstances, pluripotent muscle-derived C2C12 cells develop and differentiate into muscle cells, however treatment with BMP-2 causes them to differentiate into osteoblasts (36). Thus, C2C12 cells have been widely used to study osteoblast differentiation in early stage. In the present study, BMP-2 was used to promote osteoblast differentiation and ALP activity was increased in BMP-2 treated cells. Conversely, ALP activity was significantly decreased in cells treated with TNF-α. This demonstrates that BMP-2 is able to restore normal osteogenesis and promote osteoblast differentiation, whereas TNF-α suppresses the role of BMP-2.

Triptolide is a diterpenoid lactone compound with the molecular formula $C_{20}H_{24}O_6$ and molecular weight 360.16 that is extracted from *Tripterygium wilfordii* (37). It has previously been used as a treatment for leprosy and rheumatoid arthritis and exhibits strong pharmacological activity with anti-inflammatory, anti-fertility and immunomodulatory effects (38,39). In the present study, the role of triptolide in osteoblast differentiation was investigated. The results revealed that triptolide was able to significantly suppress TNF-α-induced inhibition during osteoblast differentiation in a dose dependent manner. It is therefore suggested that triptolide may have potent and positive effects to promote the osteoblast differentiation in the therapy of bone regeneration and rheumatoid arthritis. The results of the present study may provide the basis for clinical use of triptolide to treat bone fractures.

Suppressing chronic inflammation is helpful for fracture healing and bone remodeling. In the present study, the role of triptolide in osteoblast differentiation and fracture healing was explored. The results clearly demonstrate that triptolide is able to significantly reverse TNF-α-associated inhibition of osteoblast differentiation, suggesting that, as an anti-inflammatory compound, triptolide treatment may suppress chronic inflammation and have a positive effect on bone remodeling and fracture repair.

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