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Gene Modification of Transforming Growth Factor β (TGF-β) and Interleukin 10 (IL-10) in Suppressing Mt Sonicate Induced Osteoclast Formation and Bone Absorption

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Data Interpretation D
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Background: Osteoarticular tuberculosis is an osteolytic lesion caused by *Mycobacterium tuberculosis* (MTB). Inflammatory factors such as TNF-α play a critical role in anti-tuberculosis immunity by regulating osteoblast and osteoclast functions. Both TGF-β and IL-10 have immune suppression effects to downregulate secretion and release of inflammatory factors, such as TNF-α, that play roles in regulating osteoblast and osteoclast functions. This study thus investigated the effects of osteoclast with modified TGF-β and IL-10 gene expression on MTB-induced osteoclast formation and bone absorption.

Material/Methods: Bone marrow mononuclear cells were induced to differentiate into osteoblasts and osteoclasts *in vitro* to generate a co-culture system. MTB powder lysed by ultrasound (Mt sonicate) were added in gradients to observe osteoblast formation and osteoclast absorption. Cell apoptosis was measured by flow cytometry, while ELISA was used assess TNF-α, TGF-β, and IL-10. Viral vectors carrying TGF-β or IL-10 gene were used to transfect osteoclasts, followed by ELISA assay. Bone absorption and osteoblast apoptosis were compared among groups.

Results: Mt sonicate significantly facilitated osteoclast formation and bone formation. It upregulated contents of TNF-α, TGF-β, and IL-10, induced osteoblast apoptosis, enhanced RANKL expression in osteoblasts, and decreased OPG expression. Overexpression of TGF-β and/or IL-10 significantly decreased its upregulation effect on TNF-α by Mt sonicate, and hindered Mt sonicate-induced osteoblast apoptosis, osteoclast formation, and bone absorption.

Conclusions: Overexpression of TGF-β and IL-10 significantly inhibits TMB-induced TNF-α synthesis and release, suppresses osteoblast apoptosis, and hinders osteoclast formation and bone absorption.

MeSH Keywords: Gene Expression Profiling • Receptors, Interleukin-10 • Tuberculosis, Avian

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Background

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB) [1]. Osteoarticular tuberculosis (OAT) is an MTB-induced, blood- or lymph-transmitted disease that can cause inflammatory infiltration of osteoarticular tissues, bone necrosis, and bone absorption [2]. OAT mainly occurs in bone joints with sufficient supply, heavy loads, and more activity. OAT frequently shows an atypical and invasive course, with long persistence and chronic progression, and features bone destruction and osteolytic lesion. OAT accounts for about 3% of total TB cases and is the most prevalent extra-pulmonary TB (more than 30% incidence). Physiologically, normal bone growth requires a balanced dynamic between osteoblasts and osteoclasts. OA is a prevalent osteolytic disease. When certain pathological mechanisms disrupts the functional balance between osteoblasts and osteoclasts, making the compensatory effects of osteoblasts lower than the bone absorption ability of osteoclasts, OAT-induced osteolysis occurs [3]. Once having TB infection, the body can initiate the anti-TB immune response involving multiple immune cells and inflammatory factors, to achieve MTB clearance and manage MTB. Multiple studies have shown that immune cells that participate in the anti-TB immune response can regulate onset and progression of OAT and modify biological functions of osteoblasts and osteoclasts. Transforming growth factor-β (TGF-β) has an immune suppression function and can downregulate secretion of inflammatory factors such as TNF-α, IL-1, and IFN-γ, as well as playing a critical role in mediating osteoblast proliferation/differentiation [4] and apoptosis [5]. Interleukin-10 (IL-10) is a Th2 anti-inflammatory factor that can inhibit the release of inflammatory factor or anti-TB immunity, in addition to its critical roles in regulating differentiation and function of osteoblasts/osteoclasts [6,7]. This study thus investigated the role of osteoclasts with TGF-β and IL-10 gene modification in MTB-induced osteoclast formation and bone reabsorption.

Material and Methods

Major reagent and material

α-MEM, DMEM, and fetal bovine serum (FBS) were purchased from Hyclone (USA). StemPro Osteogenesis Differentiation Kits were purchased from Gibco (USA). Tartrate-resistant acid phosphatase (TRAP) staining kits were purchased from Beyotime (China). ELISA kits for TNF-α, TGF-β, and IL-10 were purchased from RayBiotech (USA). Human recombinant M-CSF was purchased from R&D Systems. MTB strain H37rv was provided by the China Drugs and Biological Product Analysis Institute (China). PCR primer was synthesized by Sangon (China). Real-time fluorescent PCR and SYBR Green dye were purchased from Toyobo (Japan). Rabbit anti-human nuclear factor κB receptor activator ligand (RANKL), rabbit anti-human osteoprotegerin (OPG), and mouse anti-human NFATc1 monoclonal antibody were purchased from Abcam (USA). Mouse anti-human histone H3.1 was purchased from CST (USA). Adenovirus carrying TGF-β or IL-10 (Ad-TGF-β-EGFP, Ad-IL-10-EGFP, or Ad-EGFP) was purchased from Cygene (China).

MTB lysis preparation

Suspensions of MTB H37rv strain were lysed by intermittent ultrasound at 4°C for 60 min. The lysis buffer was centrifuged at 4000 rpm for 60 min and filtered with a 0.22-μm pore size filter. Filtrate was prepared into lyophilized powders (Mt Sonicate) kept at –4°C for further use.

Preparation of bovine cortical bone

Freshly prepared bovine limb cortical bones were prepared into 50×50 mm cubes, which were polished and fixed in 5% glutaraldehyde for 2 h. Bone pieces were then sectioned into 10-μm-thick slice. After dehydration in gradient ethanol, 0.25 mol/L ammonia was used for ultrasound rinsing, followed by γ-radiation sterilization. Bone samples were kept in PBS containing 2% penicillin-streptomycin for further use.

Induction of osteoblast

Bone marrow samples were collected from patients undergoing ilium implantation. Tissue samples were diluted in 2 volumes of α-MEM. After centrifugation, the supernatant was discarded. Cell precipitation was re-suspended in α-MEM containing 10% FBS and 1% penicillin-streptomycin and was incubated in a 37°C chamber with 5% CO_2. Minor amounts of fibroblast-like attached cells were observed on days 6~7. Cells were passed when covering all fields. P2-generation cells were cultured under the induction with the StemPro Osteogenesis Differentiation Kit for osteoblasts. At day 21 of induction, differentiated culture and 2% alizarine red S staining were performed to determine osteoblast differentiation.

Osteoclast induction and Mt Sonicate processing

Bone marrow was diluted in 2× volume of α-MEM medium. After centrifugation, cells were re-suspended in α-MEM containing 10% FBS, 20 ng/mL M-CSF and 1% penicillin-streptomycin. Bone marrow cells were inoculated into 24-well plates with/without cortical bone slices (5×10^6 cells per well). Cells were divided into an Mt sonicate treatment group (25, 50, or 100 μg/mL) and a negative control group. Culture medium was changed every 3 days, with 9 days of continuous culture.

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TGF-β and IL-10 gene expression in osteoclast

During the induced incubation of osteoclast, concentrated adenovirus particles were added on day 6 (MOI=300) and were adjusted to 8 mg/L final concentration with polybrene. Medium was changed 6 h after transfection until day 9. Osteoclasts were divided into a control, blank plasmid (Ad-EGFP) transfection group; an Ad-TGF-β-EGFP transfection group; an Ad-IL-10-EGFP group; and an Ad-TGF-β-EGFP + Ad-Il-10-EGFP transfection group.

Establishment of co-culture system

Osteoclasts induced by DMEM were inoculated onto the upper surface of the Transwell chamber containing 10% FBS. Osteoclasts from all treated groups were inoculated into the lower chamber for 72-h co-culturing. ELISA was used to assess the levels of TNF-α, TGF-β, and IL-10.

Observation of osteoclast and bone absorptive lacunae

Cells in a 24-well plate without bone slices were cultured until day 9. Culture medium was discarded. A TRAP staining kit was used for observation under an inverted microscope. All mega-cells with TRAP-positive staining and containing more than 3 nuclei were regarded as osteoclasts.

Cells cultured in 24-well plate containing bone slices were cultured until day 9. Bone slices were removed and rinsed in 0.25 mol/L ammonia under ultrasound for 3 min. Following PBS washing, 0.1% toluidine blue was used for 3~5-min staining, followed by 1% HCl-ethanol differentiation, acetone dehydration, and room-temperature drying. The number of bone absorptive lacunae was counted under a microscope. We randomly selected 20 bone absorptive lacunae from each bone slice for calculating average area.

qRT-PCR assay

Total RNA was extracted from cells. cDNA was synthesized by random and oligodT primers. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase. Primer sequences were: RANKL-forward: 5'-CAACA TATCG TTGGA TCACA GCA-3'; RANKL-reverse: 5'-GACAG ACTCA CTTTA TGGGA ACC; OPG-forward: 5'-GCGCT CGTGT TTCTG GACA-3'; OPG-reverse: 5'-AGTAT AGACA CTCGT CACTG GTG-3'; β-actin-forward: 5'-GAACC CTAAG GCCAA C-3'; β-actin-reverse: 5'-TGTCA CGCAC GATTT CC-3'. In a total of 10μL PCR reaction system, we added 5.0 μL 2XSYBR Green Mixture, 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL cDNA, and ddH_2O. Reaction conditions were: 95°C denature for 5 min followed by 40 cycles each containing 95°C for 15 s and 60°C for 1 min. ABI ViiA7 fluorescent quantitative PCR cycler was used for collecting data.

Western blot

Proteins were extracted by routine methods and were separated in SDS-PAGE followed by membrane transfer. The membrane was blocked in 5% defatted milk powder for 60 min followed by 4°C overnight incubation in primary antibody (RANKL at 1: 200, OPG at 1: 200, NFATc1 at 1: 100, Histone H3.1 at 1: 200, and β-actin at 1: 500). After washing out, secondary antibody was added for 60-min room-temperature incubation. ECL reagent was added to develop the film, which was exposed and images scanned.

ELISA for inflammatory factor contents

ELISA was performed following the manual's instructions. We added 100 μL standard samples with gradient dilutions of TNF-α, TGF-β, or IL-10 into a 96-well plate with antibody coating. The plate was incubated at room temperature for 2.5 h followed by 100 μL 1XWash Solution (4 times) prior to 100 μL biotin-labelled secondary antibody for 60-min room-temperature incubation. After removing secondary antibody, 100 μL 1XWash Solution was added into each well (4 repeats), followed by 100 μL Streptavidin solution for 45-min room-temperature incubation. We then added 100 μL 1XWash Buffer for 4 washing cycles. Each well was then mixed with 100 μL TMB One-Step Substrate Reagent for 30 min at room temperature. We then added 50 μL Stop Solution into each well. Absorbance value at 450 nm wavelength was immediately measured.

Statistical analysis

SPSS18.0 was used for data input and statistical analysis. Measurement data are presented as mean±standard deviation. The *t* test or analysis of variance (ANOVA) was used to compare measurement data between groups. Statistical significance was defined at $p<0.05$.

Results

Mt Sonicate treatment enhanced osteoclast function

With higher Mt Sonicate concentration, number of osteoclast was gradually increased, accompanied by significantly more absorptive lacunae and absorptive area (Table 1). Western blot results showed minimal nuclear NFATc1 protein expression in untreated osteoclasts, while Mt sonicate treatment significantly enhanced the expression of NFATc1 protein in osteoclast nuclei, indicating that Mt Sonicate significantly enhanced differentiation of osteoclasts (Figure 1).

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Table 1. Effects of different Mt Sonicate concentrations on osteoclast function.

Mt Sonicate (μg/mL)	0	25	50	100
Osteoclast count	2.32±0.64	19.34±4.23*	46.29±5.16*	71.23±8.36*
Bone absorptive lacunae	2.03±0.58	17.85±3.76*	43.31±4.68*	68.54±7.86*
Absorptive area (μm²)	83.51±7.92	3259.16±114.94*	72651.58±181.37*	13492.25±305.41*

* p<0.05 compared to negative control (0 μg/mL) group.

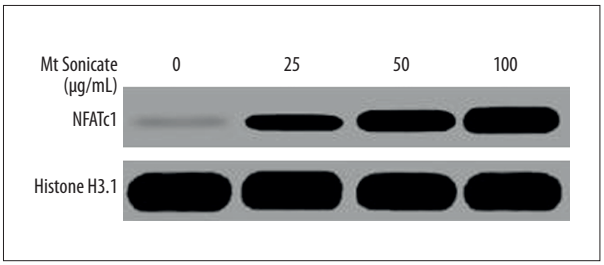

Figure 1. Western blot for nuclear NFATc1 protein expression.

Mt Sonicate significantly downregulated contents of TNF-α, TGF-β, and IL-10

Treatments using different concentrations of Mt Sonicate all significantly upregulated the level of inflammatory factor TNF-α. The high-concentration (100 μg/mL) group had a more than 17.8-fold increase of TNF-α compared to that of the control group. Mt Sonicate treatment also elevated contents of anti-inflammatory factors, including TGF-β and IL-10, but with smaller amplitude than that of TNF-α potentiation; the 100 μg/mL group had about 5.6-fold and 4.3-fold increase of TGF-β and IL-10, respectively (Figure 2). These results showed that Mt Sonicate processing initiated immune response and elevated the release of inflammatory factors. Although the expression of anti-inflammatory factors also increased, the relative content of inflammatory factors still dominated and the immune suppression induced by anti-inflammatory factors were noticeable.

Mt Sonicate decreased OPG expression and enhanced RANKL expression in osteoblasts and induced their apoptosis

By 2% alizarine red S staining, the differentiation of osteoblast was approximately finished within 21 days of induced culture. In the center of osteoblasts, red-stained calcified nodules were observed (Figure 3A). Those undifferentiated cells, however, showed negative results of 2% alizarine red S staining (Figure 3B). qRT-PCR results showed that Mt Sonicate processing significantly upregulated RANKL mRNA expression in osteoblasts and decreased OPG mRNA expression (Figure 3C). Western blot test results showed that Mt Sonicate processing significantly upregulated RANKL protein expression inside

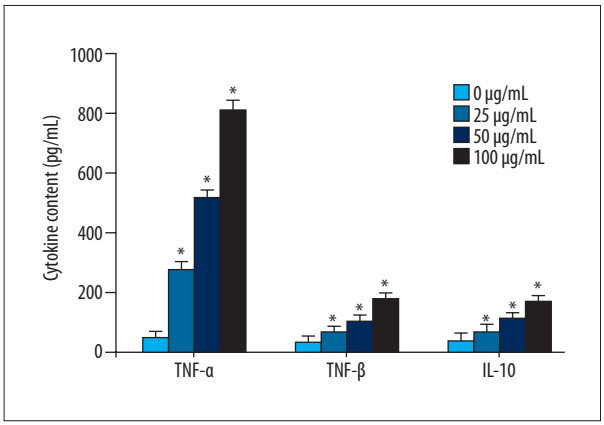

Figure 2. ELISA for TNF-α, TGF-β, and IL-10 contents. *, p<0.05 compared to negative control group.

osteoblasts and decreased OPG protein expression (Figure 3D). Flow cytometry results showed that Mt Sonicate processing significantly enhanced apoptosis of osteoblasts (Figure 3E).

Enhancement of TGF-β and IL-10 expression weakened absorption potency of osteoclasts and apoptosis of osteoblasts

ELISA assay showed that overexpression of TGF-β and IL-10 gene significantly enhanced the content of cytokines, including TGF-β and IL-10, in the supernatant of the co-culture system, and decreased TNF-α (Figure 4A). Western blot results showed that overexpression of TGF-β or IL-10 gene decreased RANKL protein expression inside osteoblasts and elevated OPG expression significantly (Figure 4B). Flow cytometry results showed that overexpression of TGF-β or IL-10 remarkably decreased apoptotic rate of osteoblasts (Figure 4C). Further testing showed that overexpression of TGF-β and IL-10 genes significantly decreased osteoclast formation and weakened their bone-absorption ability (Table 2).

Discussion

TB is a severe disease that can be life-threatening and is also a major public health issue worldwide. TB is contagious and can

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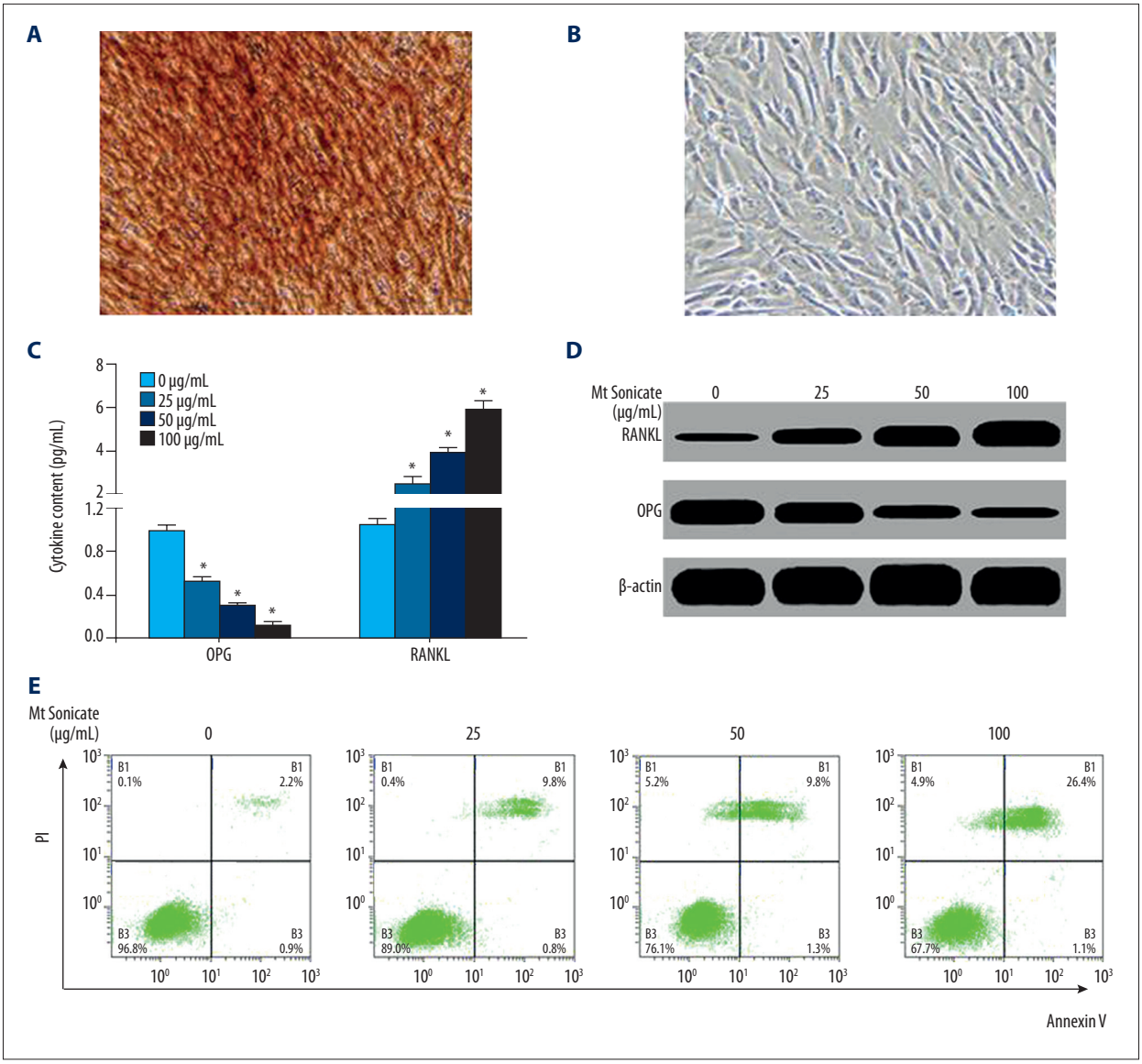

Figure 3. Mt Sonicate decreased OPG expression in osteoblasts, enhanced RANKL expression, and induced cell apoptosis. (**A**) 2% alizarine red S staining for osteoblast identification after induced differentiation. (**B**) 2% alizarine red S staining for undifferentiated cells; (**C**) qRT-PCR for OPG and RANKL mRNA expression inside osteoblasts; (**D**) Western blot for OPG and RANKL protein expression in osteoblasts; (**E**) Flow cytometry for osteoblast apoptosis. *, p<0.05 compared to 0 μg/mL.

be transmitted by the respiratory system. The larger susceptible population increases the incidence of TB. It is estimated that more than 30% of the world population has been infected with MTB, with approximately 10 million newly diagnosed patients and more than 2 million deaths [8]. China is one of the regions with the highest TB prevalence, having the second highest TB incidence worldwide and accounting for approximately 15% of all cases worldwide. A WHO report on TB in 2014 showed that approximately 9 million people were infected by TB in 2013, including 5.6 million newly diagnosed cases and 1.5 million deaths. China has more than 10% of all infectious cases worldwide [9]. TB persists for many years and is leading cause of infectious disease deaths. The mortality rate of TB is the second highest among all infectious diseases in humans, only lower than acquired immune deficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV). In recent years, due to increased numbers of AIDS or other immunodeficiency patients, TB infection as secondary disease has increased [10]. TB mainly infects pulmonary tissues, but may also induce OAT via blood-borne transmission to bone and joint tissues [11].

The maintenance of healthy bone structure, morphology, and bone volume requires the dynamic balance between osteoblasts

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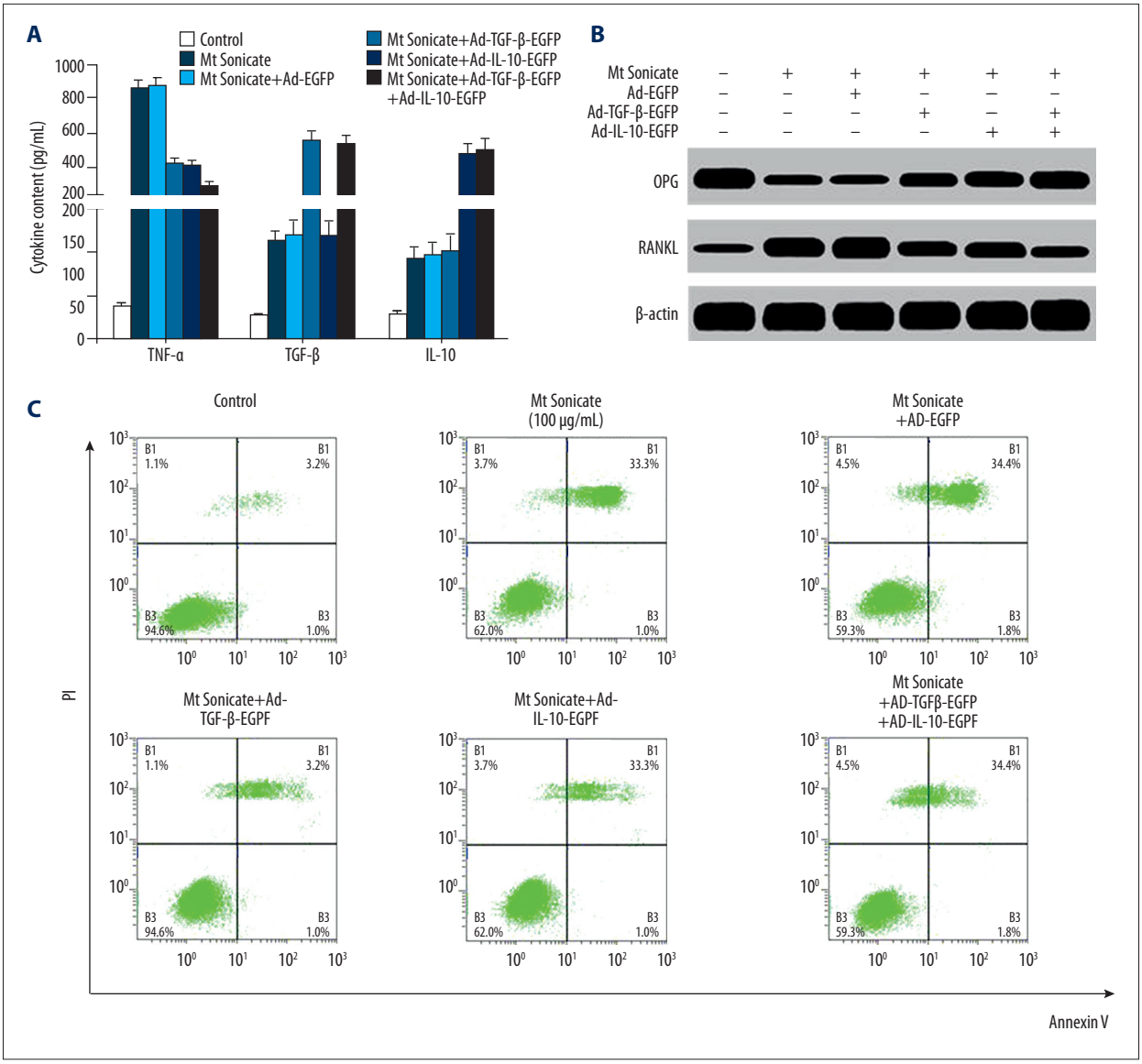

Figure 4. Enhanced TGF-β and IL-10 expression weakened osteoblast apoptosis. (**A**) qRT-PCR for OPG and RANKL mRNA expression in osteoblasts; (**B**) Western blot for OPG and RANKL protein expression in osteoblasts; (**C**) Flow cytometry for osteoblast apoptosis.

Table 2. Enhanced TGF-β and IL-10 expression affected osteoclast cell formation and bone absorption.

Group	Osteoclast count	Bone absorptive lacunae count	Bone absorptive area (μm²)
Control	2.37±0.15*#	2.11±0.19*#	69.26±5.91*#
Mt Sonicate	66.54±5.87	63.72±6.08	12834.72±198.67
Ad-EGFP	63.59±6.04	61.29±5.87	12756±201.55
Ad-TGF-β-EGFP	38.56±6.33*#	35.66±7.26*#	7546.85±109.93*#
Ad-IL-10-EGFP	36.62±5.96*#	33.54±6.17*#	6856.64±87.67*#
Ad-TGF-β-EGFP + Ad-IL-10-EGFP	28.32±4.36*#	25.19±63.85*#	4961.52±59.63*#

* p<0.05 compared to Mt Sonicate group; # p<0.05 compared to Ad-EGFP group.

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and osteoclasts. When osteoclast function exceeds compensation by osteoblasts, bone destruction and absorption follows [12]. Immune responses of the host plays a critical role in regulating MTB growth, managing infection, and immune clearance [13]. During the initial phase of MTB infection, inflammatory responses such as abundant activation of macrophages, MTB phagocytosis, and large amounts of TNF-α release play the major role in immune clearance. Previous studies showed that various proteins in MTB can bind with body lipid to induce late-onset hyper-sensitive reaction, leading to synthesis and release of TNF-α, as well as activation and proliferation of mononuclear macrophages [14]. RANKL is mainly expressed by osteoblasts, bone marrow matrix cells, and activated T cells, and plays critical roles in the osteoclast differentiation and maturation axis RNAKL/RANK/OPG [15]. RANK induces osteoclast differentiation and maturation via coupling with downstream signal molecules [16]. NFATc1 is one of the most potent transcriptional factors in response to RANKL stimuli in osteoclasts [17]. RANKL secreted by osteoblasts can bind with RANK in osteoclast precursors to upregulate expression of NFATc1, which is critical for osteoclast differentiation, via TRAF6 to initiate the downstream NF-κB signal pathway [18]. OPG is a soluble bite receptor of RANKL and is mainly secreted by osteoblasts and lymphocytes to competitively inhibit RANKL-RANK binding, thus inhibiting osteoclast maturation and differentiation and weakening the absorption function of osteoclasts [19]. Previous studies showed that TNF-α can facilitate osteoclast differentiation and upregulation absorption via multiple mechanisms [20,21]. The present study showed that Mt Sonicate treatment significantly increased osteoclast formation and enhanced its bone-absorption potency. Li et al. found that MTB treatment significantly enhanced osteoclast formation and bone absorption [22], which is in line with our results. Western blot analysis showed that Mt Sonicate treatment remarkably elevated NFATc1 protein expressions in osteoclast nuclei, indicating that Mt Sonicate can facilitate osteoclast differentiation. ELISA results showed that Mt Sonicate treatment significantly upregulated content of inflammatory factor TNF-α and enhanced synthesis and release of anti-inflammatory factors TGF-β and IL-10. The amplitude of increase in these inflammatory factors, however, was significantly lower than that of anti-inflammatory factors, indicating the dominance of inflammatory response, while immune suppression induced by anti-inflammatory factors is being dominated. Al-Attiyah et al. showed that MTB treatment significantly upregulated synthesis and release of TNF-α in peripheral blood mononuclear cell (PBMC) [23], consistent with our results showing that Mt Sonicate enhanced TNF-α content in the co-culture system. TNF-α can regulate functions of osteoblasts and osteoclasts via multiple mechanisms, making it an important bone metabolism regulatory factor. Son et al. showed that TNF-α directly affects osteoblasts to induce apoptosis [24]. The present study observed that Mt Sonicate treatment significantly induced apoptosis of osteoblasts in the co-culture system, which was probably related to the enhanced synthesis and release of TNF-α after Mt Sonicate treatment. This study also showed that Mt Sonicate could significantly enhance RANKL expression in osteoblasts and presents inhibitory effects against OPG expression, indicating that MTB can induce osteoblast formation via interfering osteoblast function. Hofbauer et al. demonstrated that TNF-α indirectly facilitated osteoclast differentiation and maturation via upregulating M-CSF and RANKL expression in osteoblasts [25]. Goto et al. found that TNF-α can facilitate RNAKL expression in a co-culture system containing bone marrow adipocyte and osteoclast precursors, thus enhancing differentiation and maturation of osteoclast precursors towards matured cells [21]. TNF-α can also directly facilitate formation of RANKL-induced osteoclast and enhance bone absorption. The present study found that Mt Sonicate treatment significantly induced RANKL expression in induced osteoblast, in agreement with Holbauer et al. [25] and Goto et al. [21].

TGF-β is an anti-inflammatory factor with immune-suppression functions. It can decrease immune response induced by T cells [26] and macrophages [27] via suppressing secretion and release of inflammatory factors, including TNF-α, IL-1, and IFN-γ. In addition to weakened inflammatory response, TGF-β also plays a role in mediating osteoblast proliferation, differentiation [4], and apoptosis [5]. IL-10 is a Th2 type anti-inflammatory factors and is produced by activated macrophages and T lymphocytes. It can inhibit release of inflammatory factors and anti-TB immune response, and plays critical roles in regulating differentiation and function of osteoblasts/osteoclasts [6,7]. The present study observed that the overexpression of TGF-β and/or IL-10 gene could upregulate TGF-β and IL-10 factor contents and decrease inflammatory factor TNF-α content in the co-culture system, demonstrating the anti-inflammatory role of TGF-β and IL-10. Moreover, overexpression of TGF-β and IL-10 genes also significantly decreased apoptosis of osteoblasts, decreased RANKL expression, decreased osteoclast formation, and weakened the bone-absorption ability of osteoclast. Rahman et al. showed that TGF-β is an important regulatory factors during osteoblast differentiation and bone formation [4]. Minuto et al. also showed that TGF-β can stimulate bone cell proliferation and differentiation, with close correlation with bone formation [28]. Noda et al. found a significant faciliatory role of TGF-β in bone formation [29]. Marcelli et al. found that TGF-β could induce the differentiation of mesenchymal cells into osteoblasts and facilitate bone mineralization [30]. Dufour et al. found that TGF-β affected PI3K/Akt signal pathway activity and regulated expressions of Bcl-2 and phosphor-Bad to achieve inhibition of osteoblast apoptosis [5]. The present study observed that overexpression of TGF-β significantly hindered osteoblast apoptosis in the Mt Sonicate-induced co-culture system, probably sharing

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common mechanism as observed by Dufour et al. [5]. Liu et al. found that IL-10 could decrease RANKL expression and inhibit osteoclast formation and bone absorption via enhancing OPG expression [7]. Mohamed et al. showed that IL-10 significantly suppressed expression of NF-κB p50, phosphorylated JNK, NFATc1, c-Fox, and c-Jun in RANKL-induced osteoclasts, thus inhibiting differentiation and bone absorption potency of osteoclasts [6]. The present study established an osteoclast model with TGF-β and IL-10 gene modification and found that overexpression of TGF-β and IL-10 inside osteoclasts remarkably inhibited synthesis and release of TNF-α under TMB induction, and decreased osteoblast apoptosis, osteoclast formation, and bone-absorption ability. However, whether TGF-β and IL-10 gene modification achieve their effects on osteoblasts and osteoclast functions directly or by influencing TNF-α synthesis and release was not assessed in the present study and thus requires further research.

Conclusions

The overexpression of TGF-β and IL-10 in osteoclasts significantly inhibits TMB-induced TNF-α synthesis and release, suppresses osteoblast apoptosis, and weakens osteoclast formation and bone absorption ability.

Conflict of interest

None.

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