

## Original Article

# The role of TNF- $\alpha$ and IFN- $\gamma$ in the formation of osteoclasts and bone absorption in bone tuberculosis

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**Abstract:** Bone-joint tuberculosis is one lytic bone lesion caused by *Tubercle bacilli*. Tumor necrosis factor (TNF)- $\alpha$  interferon (IFN)- $\gamma$  can mediate bone formation and osteoclasts. This study aimed to investigate the effect of these factors in the effect of *Tubercle bacilli* on osteoclast formation and bone absorption. Bone marrow mononuclear cells were separated and generated for osteoblast-osteoclast co-culture system. The effect of Mt sonicate on osteoclast formation and bone absorption was observed, with the correlation analysis between TNF- $\alpha$  and IFN- $\gamma$  concentration. Real-time PCR was employed to detect mRNA expression of receptor activator NF- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG) in osteoblasts. Immunofluorescence was used to quantify NFATc1 protein level. Antibody against TNF- $\alpha$  and IFN- $\gamma$  was applied for observing bone absorption. Mt sonicate significantly enhance osteoclast formation and bone absorption and facilitate secretion of TNF- $\alpha$  and IFN- $\gamma$ . It can also elevate mRNA expression of RANKL and OPG in osteoblast and decrease NFATc1 in osteoclast. The block of TNF- $\alpha$  or IFN- $\gamma$  significantly weaken Mt sonicate-induced osteoclast formation and bone absorption, facilitate OPG expression, and decrease RANKL and NFATc1 expression. *Tubercle bacilli* induced release of TNF- $\alpha$  and IFN- $\gamma$  is important for bone absorption. TNF- $\alpha$  and IFN- $\gamma$  interfere the interaction between osteoclast and osteoblast, causing bone destruction, via the modulation on the expression of OPG and RANKL in osteoblast.

**Keywords:** Tuberculosis, bone-joint, inflammatory cytokine, TNF- $\alpha$ , IFN- $\gamma$

## Introduction

Tuberculosis is one severe infectious disease caused by *Tubercle bacilli* (TB) [1]. Osteoarticular tuberculosis (OAT) can be spread to bone and joint tissues by TB, causing bone destruction and absorption featured with inflammatory infiltration. OAT has atypical asymptomatic and invasive manifestation, as lesion is featured with bone destruction and lysis, which has persistence and slow inhalation [2]. The healthy morphology and normal bone volume depend on the dynamic balance between osteoblast and osteoclast. When certain pathological factor makes the enhancement of osteoclast function beyond the compensatory mechanism of osteoblast, bone absorption and destruction will occur. There are multiple studies regarding the mechanism of destruction by pulmonary tuberculosis. However, the detailed mechanism of bone destruction during OAT is still unclear.

Host immune function plays important roles on TB growth. Once infected with TB, body will initiate related immune reaction involving multiple immune cells and inflammatory factors to manage infection [3, 4]. With the invasion of TB, innate immune cells including macrophage, NK cells and dendritic cells are rapidly activated to release multiple inflammatory factors such as interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-1, inducing protective inflammatory response for exerting immune clearance [5]. The over-production of protective anti-inflammatory factors, however, can interfere with the immune clearance against TB. In OAT lesion featured with tuberculous granuloma, both protective and pathological abnormality immune response co-exists, but having large difference regarding induced immune cells and inflammatory factors [6]. Generally speaking, protective immunity is mainly achieved via mac-

rophage activation by CD4 Th cells via IFN- $\gamma$ , while pathological abnormality reaction is induced by delayed hypersensitive T cells via TNF- $\alpha$  and IL-1 [6]. Various inflammatory factors involving in anti-TB immunity, also play important roles in mediating osteoblast and osteoclast functions. Multiple studies revealed that both TNF- $\alpha$  and IFN- $\gamma$  could regulate differentiation and biological functions of osteoblast and osteoclast via multiple pathways [7]. Whether TNF- $\alpha$  and IFN- $\gamma$  plays a role in OAT caused by TB, is still unclear.

### Materials and methods

#### *Reagent and materials*

Low-glucose DMEM,  $\alpha$ -MEM, FBS and osteoblast induction differentiation medium (Gibco, US); Anti-tartaric acid phosphatase (TRAP) staining kit (Beyotime, China); ELISA kits for human TNF- $\alpha$  and IFN- $\gamma$  (eBioscience, US); Human recombinant M-CSF (Peprotech, US); TB strain H37rv (Pharmaceutical Biological Products Analysis Institute, China); PCR primer (Sangon, China); Fluorescent quantitative PCR kit (Toyobo, Japan); Rabbit anti-human NFATc1 monoclonal antibody (Abcam, US); Rabbit anti-TNF- $\alpha$  and mouse anti-IFN- $\gamma$  body (R&D, US).

#### *Processing of TB*

Mycobacterium tuberculosis H37rv suspensions were lysed under intermittent ultrasound at 4°C for 60 min. Bacterial mixture was then centrifuged at 2 500 rpm for 60 min, followed by filtration through 0.22  $\mu$ m filter, aliquoted, and frozen to prepare lyophilized powder, which were kept at 4°C for further use.

#### *Preparing calf cortical bone*

Fresh cortical bones from calf limbs were cut into bone fraction (50 mm  $\times$  50 mm) and were smoothing. After fixation in 5% glutaraldehyde for 2 h, bone fraction was cut into sclerite, which was dehydrated in gradient ethanol. 0.25 mol/L ammonia was used to wash sclerite for three times. After  $\gamma$ -irradiation for sterilization, sclerite was immersed in 1000 U/mL streptomycin/penicillin (in PBS) and was kept at 4°C for storage.

#### *Induction of osteoblast*

5 mL bone marrow samples were drawn from ilium bone grafting patients, and were mixed

with 10 mL  $\alpha$ -MEM basic medium. After centrifugation at 1 200 rpm for 5 min, the supernatant was discarded.  $\alpha$ -MEM medium containing 10% FBS and 1% streptomycin/penicillin was added to re-suspend cell spheres, which were then incubated at 37°C chamber with 5% CO<sub>2</sub> perfusion. With changing medium every three days, few fibroblast-like attachment cells can be observed. Cells were further incubated until reaching complete fusion (PO), 0.05% trypsin was used to digest cells for passage. When P1 generation cell reaches confluence of 60%~80%, osteoblast induction differentiation medium was used for changing every three days in 14-day induction differentiation incubation. The basic formation of osteoblast can be observed by Alizarin red S staining. Osteoblasts were further passed until P2 generation for further experiments.

#### *Osteoclast induction and Mt sonicate processing*

Double volumes of  $\alpha$ -MEM medium were used to dilute bone marrow, and were centrifuged at 1 200 rpm for 5 min. Supernatant was discarded, with the addition of  $\alpha$ -MEM medium containing 10% FBS, 20 ng/mL M-CSF and 1% streptomycin/penicillin to re-suspend cells. After adjusting  $1 \times 10^7$ /mL concentration, cells were seeded into 24-well plate with or without cortical bone (0.5 mL each). Mt sonicate treatment groups at different concentrations (0  $\mu$ g/mL, 0.1  $\mu$ g/mL, 1  $\mu$ g/mL, 10  $\mu$ g/mL and 100  $\mu$ g/mL) were prepared for changing medium every 72 h in 8 consecutive days. In addition, to better observe the effect of TNF- $\alpha$  and IFN- $\gamma$  on osteoblast and osteoclast functions under Mt sonicate treatment, we further replenished blocker groups including anti-TNF- $\alpha$  (50 ng/mL) and/or anti-IFN- $\gamma$  (100 ng/mL), in parallel to control group using 100  $\mu$ g/mL Mt sonicate, to test related indexes.

#### *Establishment of co-culture system*

Low-glucose DMEM medium containing 10% FBS was added into the upper chamber of Transwell for inoculation of induced P2 osteoblast. Transwell chambers were then placed into 24-well chamber containing osteoclasts after treating using different concentrations of Mt sonicate. After 72 h incubation, culture medium in the lower chamber was collected to quantify TNF- $\alpha$  and IFN- $\gamma$  using ELISA approach.

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**Table 1.** TNF- $\alpha$ , IFN- $\gamma$ , osteoclast counting and bone absorption under Mt sonicate (N = 10)

Group	Osteoclast	Bone absorption lacunae	Bone absorption area ( $\mu\text{m}^2$ )	TNF- $\alpha$ (pg/mL)	IFN- $\gamma$ (pg/mL)
0 $\mu\text{g}/\text{mL}$	2.61 $\pm$ 0.91	2.45 $\pm$ 0.87	92.13 $\pm$ 8.62	97.56 $\pm$ 10.46	84.31 $\pm$ 12.45
0.1 $\mu\text{g}/\text{mL}$	4.23 $\pm$ 1.02	4.04 $\pm$ 1.11	98.72 $\pm$ 10.17	104.31 $\pm$ 11.34	88.43 $\pm$ 11.87
1 $\mu\text{g}/\text{mL}$	9.56 $\pm$ 2.67 <sup>a</sup>	7.67 $\pm$ 2.33 <sup>a</sup>	325.67 $\pm$ 36.95 <sup>a</sup>	228.78 $\pm$ 21.66 <sup>a</sup>	138.56 $\pm$ 15.75 <sup>a</sup>
10 $\mu\text{g}/\text{mL}$	21.54 $\pm$ 4.53 <sup>a</sup>	19.45 $\pm$ 3.88 <sup>a</sup>	3617.56 $\pm$ 77.59 <sup>a</sup>	356.73 $\pm$ 31.82 <sup>a</sup>	257.55 $\pm$ 30.64 <sup>a</sup>
100 $\mu\text{g}/\text{mL}$	65.25 $\pm$ 9.21 <sup>a</sup>	62.64 $\pm$ 10.43 <sup>a</sup>	15293.73 $\pm$ 291.48 <sup>a</sup>	768.26 $\pm$ 41.45 <sup>a</sup>	448.24 $\pm$ 39.61 <sup>a</sup>

Note: <sup>a</sup>, P < 0.05 compared to 0  $\mu\text{g}/\text{mL}$  group.

### Observation of bone absorptive lacunae and osteoclast

Cells in 24-well plate without cortical bone were incubated for 8 days. Culture medium was discarded, followed by the staining by TRAP kit. Under inverted microscope, those large cells with more than three nuclear with TRAP-positive staining were identified as osteoclasts. Five fields were randomly selected under 10X objective to count the number of osteoclasts. The average number across five fields represented the osteoclast number in this sample. Those cells cultured in 24-well plate with cortical bone were cultured for 8 days, and were washed in ultrasound with 0.25 mol/L ammonia for 3 min to remove attached cells. PBS was used to re-wash cells, followed by 0.1% toluidine blue staining for 3~5 min. 1% HCl-ethanol was used for differentiation, followed by acetone dehydration and drying under room temperature. Microscopic observation was used to calculate the number of bone absorptive lacunae. 20 randomly selected bone absorptive lacunae were calculated for average areas.

### qRT-PCR

Total RNA was extracted from cells. cDNA was then synthesized using random primers and oligdT primers under reverse transcription. Using cDNA as the template, PCR amplification was performed using TaqDNA polymerase using primers (RNAKL-forward, 5'-CAACA TATCG TTGGA TCACA GCA-3'; RNAKL-reverse, 5'-GAC-AG ACTCAC TTTAT GGGAA CC-3'; OPG-forward, 5'-GCGCTCGTGGTTTCTGGACA-3'; OPG-reverse, 5'-AGTAT AGACA CTCGT CACTG GTG-3'; GAPDH-forward, 5'-GGAGC GAGAT CCCTC CAAAA T-3'; GAPDH-reverse, 5'-GGCTG TTGTC ATACT TCTCA TGG-3'. In a total of 10  $\mu\text{l}$  reaction system, 4.5  $\mu\text{l}$  2XSYBR Green Mixture, 0.5  $\mu\text{l}$  forward/reverse primers (5  $\mu\text{M}/\text{L}$ ), 1  $\mu\text{l}$  cDNA and 3.5  $\mu\text{l}$

ddH<sub>2</sub>O were added. Reaction conditions were: 95°C denature for 5 min, followed by 40 cycles each containing 95°C for 15 sec, and 60°C for 1 min. Data were collected from ABI ViiA7 fluorescent quantitative PCR cycler, and were analyzed by 2 <sup>$\Delta\Delta\text{Ct}$</sup>  method.

### Western blotting

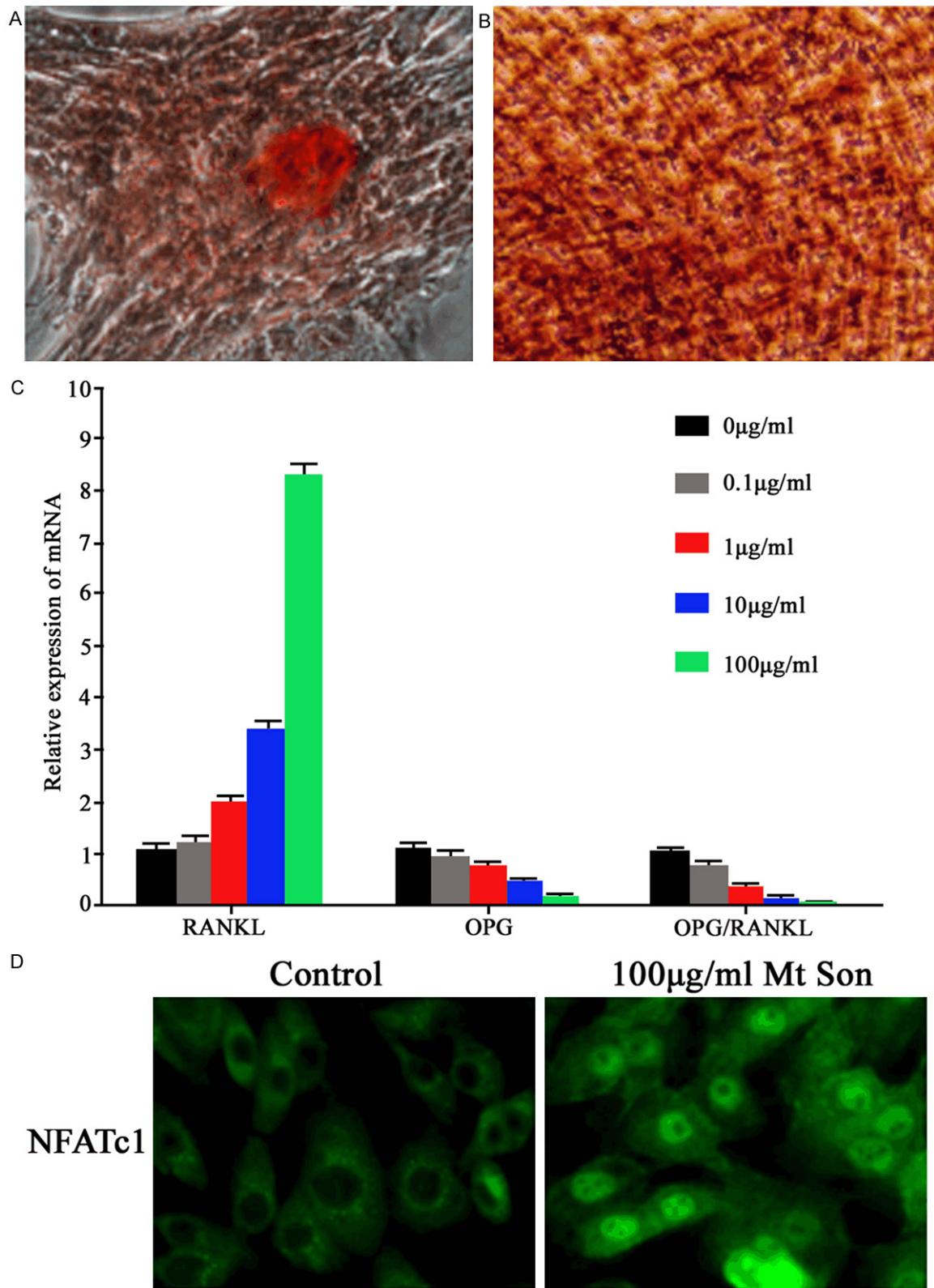
Nuclear proteins were extracted, separated in SDS-PAGE, and were transferred to PVDF membrane. The membrane was blocked in 5% defatted milk powder for 60 min at room temperature, and was added with primary antibody at 4°C overnight. Secondary antibody was then added for 60-min incubation. ECL method was used to develop and expose the membrane. Expression of nuclear NFATc1 proteins in osteoclasts was then quantified.

### Immunofluorescence

Cells in the lower chamber were washed in cold PBS twice and were fixed in 4% paraformaldehyde. After washing three times in PBS, 0.25% Triton X-100 was added for 20-min processing, followed by PBS washing. Blocking was performed using PBS containing 1% BSA at room temperature for 30 min. Rabbit anti-human NFATc1 antibody was added for overnight incubation at 4°C. On the next day, secondary antibody was added for dark incubation for 60 min. After PBS washing three times, mounting solution containing DAPI was applied for nuclear staining and observation under fluorescent microscope.

### Statistical processing

SPSS 18.0 software was used for data retraction and statistical analysis. Measurement data were presented as mean  $\pm$  standard deviation (SD). Student t-test or ANOVA was used



**Figure 1.** TB effect on OPG, RANKL and NFATc1 expression. A. P0 generation of osteoblasts under 2% ARS staining; B. P2 generation of osteoblasts under 2% ARS staining; C. mRNA expression assay of OPG and RANKL by osteoblast; D. Immunofluorescence assay of NFATc1 expression in osteoclast.

**Table 2.** Effects of TNF- $\alpha$  and IFN- $\gamma$  on osteoclast formation and bone absorption (N = 5)

Group	Osteoblast count	Bone absorption lacunae count	Bone absorption area ( $\mu\text{m}^2$ )
Control	3.56 $\pm$ 1.20 <sup>a</sup>	3.12 $\pm$ 0.99 <sup>a</sup>	88.43 $\pm$ 7.83 <sup>a</sup>
Mt Sonicate	58.54 $\pm$ 10.38	55.35 $\pm$ 9.88	14583.56 $\pm$ 252.89
Anti-TNF $\alpha$	31.61 $\pm$ 8.36 <sup>a</sup>	29.56 $\pm$ 8.21 <sup>a</sup>	3845.91 $\pm$ 78.66 <sup>a</sup>
Anti-IFN $\gamma$	37.67 $\pm$ 9.73 <sup>a</sup>	35.42 $\pm$ 7.87 <sup>a</sup>	4521.87 $\pm$ 95.82 <sup>a</sup>
Anti-TNF $\alpha$ + Anti-IFN- $\gamma$	22.87 $\pm$ 7.35 <sup>a</sup>	19.53 $\pm$ 6.84 <sup>a</sup>	2391.34 $\pm$ 76.46 <sup>a</sup>

Note: <sup>a</sup>, P < 0.05 compared to Mt sonicate group.

for between-group comparison. A statistical significance was defined when P < 0.05.

### Results

#### *Effect of Mt sonicate treatment on TNF- $\alpha$ , IFN- $\gamma$ and osteoclasts*

After treatment using different concentrations of Mt sonicate, the number of osteoclasts in all groups except 0.1  $\mu\text{g}/\text{mL}$  group was significantly higher than control group (P < 0.05). With increasing concentration of Mt sonicate, the number of osteoclasts was gradually elevated with significant difference between groups (P < 0.05). The number of bone absorptive lacunae and bone absorption area were also increased with Mt sonicate treatment in a dose-dependent manner. Moreover, TB can also stimulate the synthesis and secretion of TNF- $\alpha$  and IFN- $\gamma$  from bone marrow mononuclear cells (P < 0.05 except 0.1  $\mu\text{g}/\text{mL}$  group, **Table 1**). We performed correlation analysis between TNF- $\alpha$  and IFN- $\gamma$  with average values of osteoclast counting, bone absorptive lacunae and bone absorption area. Results showed significantly positive correlation between TNF- $\alpha$  or IFN- $\gamma$  concentration and osteoclast counting (r = 0.697, r = 0.619), bone absorptive lacunae (r = 0.731, r = 0.604) and bone absorption area (r = 0.742, r = 0.707, P < 0.05 in all cases).

#### *Expression of OPG, RANKL in osteoblast and NFATc1 in osteoclast by BT*

In the microenvironment of bones, activation and differentiation of osteoclasts mainly depend on the expression ratio of osteoprotegerin (OPG) against receptor activator NF- $\kappa\text{B}$  ligand (RANKL) in osteoblast, as the balance between OPG and RANKL determines the differentiation and maturation of osteoclasts. This study established a co-culture system for the differ-

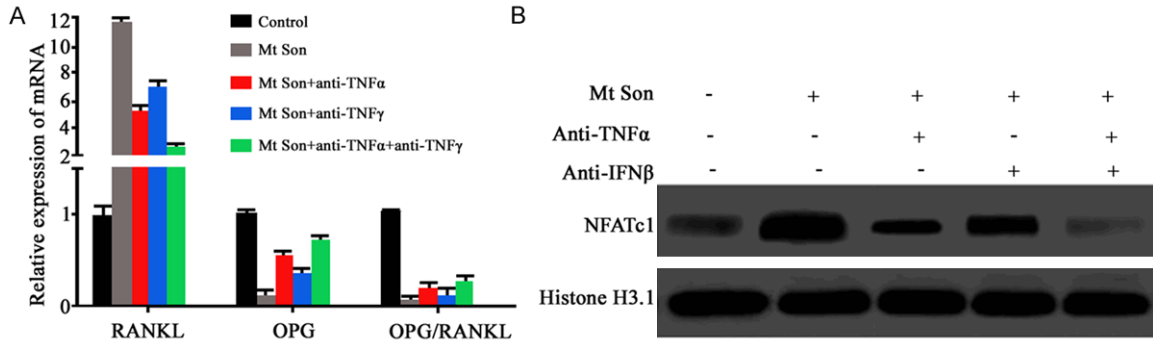
entiation of osteoblast and osteoclast, to observe the effect of TB on expression of OPG and RANKL in osteoblast. After 14-day induction culture, differentiated osteoblasts were formed, as shown by calcification nodules in the center under 2% ARS staining (**Figure 1A**). After passage to P2, almost all cells were stained as red

(**Figure 1B**), suggesting successful differentiation. Real-time PCR showed Mt sonicate treatment could significantly increase mRNA expression of RANKL in osteoblast and simultaneously decrease OPG expression, thus decreasing OPG/RANKL ratio with higher concentration of Mt sonicate (**Figure 1C**). This study also compared NFATc1 expression and found 100  $\mu\text{g}/\text{mL}$  Mt sonicate treatment could significantly potentiate nuclear expression of NFATc1 (**Figure 1D**).

#### *Blockage of TNF- $\alpha$ and IFN- $\gamma$ weakened TB-induced osteoclast formation and bone formation*

ELISA results showed that TB could significantly elevate the synthesis and secretion of TNF- $\alpha$  and IFN- $\gamma$  in addition to its induction of osteoclast formation and facilitation of bone absorption, suggesting that over-production of TNF- $\alpha$  and IFN- $\gamma$  might exert certain effects on TB-induced bone destruction. This study further replenished antibody to block the biological effect of TNF- $\alpha$  and IFN- $\gamma$ , to observe the influence on biological functions and related indexes by 100  $\mu\text{g}/\text{mL}$  Mt sonicate. Results showed that the blocking of TNF- $\alpha$  or IFN- $\gamma$  could weaken Mt sonicate-induced bone destruction effect, with the most potent inhibitory effect after dual blockage (P < 0.05 in both cases, **Table 2**). Real-time PCR results showed that the blockage of TNF- $\alpha$  and/or IFN- $\gamma$  could inhibit RANKL expression in osteoblast to different extents, and elevate OPG expression simultaneously. The OPG/RANKL ratio was also increased to different extents (**Figure 2A**). Western blotting results showed that Mt sonicate could remarkably facilitate nuclear expression of NFATc1 in osteoclasts, as consistent to those from immunofluorescence. The blocking of TNF- $\alpha$  or IFN- $\gamma$  also significantly depressed

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**Figure 2.** Effect of TNF- $\alpha$  and IFN- $\gamma$  on the expression of related factors in osteoblast and osteoclast. A. Real-time PCR assay for mRNA expression of OPG and RANKL in osteoblast; B. Nuclear expression of NFATc1 in osteoclast by Western blotting.

cellular expression of NFATc1, as the lowest level occurred under dual blocking condition (Figure 2B).

### Discussion

TB is one major challenge for the public health world widely, especially in underdeveloped countries such as India and China. There are about 9 million newly diagnosed TB patients, causing about 2 million deaths annually [8]. The major target organ of TB is pulmonary tissues. Once the dispersion to bone and articular tissues via vascular system, OAT can be caused [2]. OAT is the most common non-pulmonary tuberculosis, as it occupies about 1%~3% of total TB [9-11], and about 10%~15% of no-pulmonary tuberculosis [12]. OAT is often occurred in children and young people, with the involvement of spine and limb joints. Without timely treatment, it may eventually lead to dysformation of spine or limb joints, formation of fistula, limited movement function or even paralysis, thus severely affecting patient's health and life quality. Innate immunity is the primary defense mechanism against TB. After the invasion of TB, body will initiate anti-TB immune response involving multiple immune cells and inflammatory factors to manage the infection [3, 4]. Innate immune cells including macrophage, NK cells and dendritic cells are rapidly activated to release multiple inflammatory factors such IFN- $\gamma$  and TNF- $\alpha$ , the former of which further facilitate the formation of inflammatory granulosa to counteract TB infection [13]. At early stage of body infection of TB, protective inflammatory response featured with abundant activation of macrophage for phagocytosis of TB

and release of TNF- $\alpha$  play primary roles in immune clearance [5]. IFN- $\gamma$  is one important regulatory factor for macrophage activation. During innate immune stage, IFN- $\gamma$  secreted by NK cells can activate and recruit macrophage to recognize and endocytosis of TB, thus exerting anti-infection potency [14]. However, the over-activation of such protective inflammatory response may lead to abnormal immune response via intervention on the clearance of TB by host. In OAT lesion featured with tuberculosis granuloma, both protective and abnormal pathological immune response exists. Generally speaking, protective immunity is mainly achieved via macrophage activation by CD4 Th cells via IFN- $\gamma$ , while pathological abnormality reaction is induced by delayed hypersensitive T cells via TNF- $\alpha$  [6]. Previous study has shown that multiple proteins of TB could bind with body lipids to initiate delayed hypersensitive response, causing focal increase of TNF- $\alpha$  and activation/proliferation of mononuclear macrophage [15]. Therefore TB is one immune related disease, with the involvement of IFN- $\gamma$  and TNF- $\alpha$ .

Under physiological condition, normal growth of bone depends on the homeostasis between functions of osteoblast and osteoclast. OAT is one disease featured with bone lysis, thus requiring the breakdown of such balance to cause the overwhelming of bone absorption potency by osteoclast against compensatory ability of osteoblast, leading to bone lysis. Multiple inflammatory factors involved in anti-TB immunity also participate in the regulation of osteoblast and osteoclast functions. Multiple studies showed that IFN- $\gamma$  and TNF- $\alpha$  could reg-

ulate both differentiation and biological function of osteoblast/osteoclast via various pathways [7]. However, current knowledge about their expressional profile and functions in TB lesion and bone metabolism mainly comes from pulmonary TB, non-TB infection of bone-joints and rheumatoid arthritis, with little exploration in OAT. The detailed mechanism of bone destruction by TB during OAT is still unclear.

The destruction and absorption of bones depend on the function of osteoclast, which comes from mononuclear/macrophage lineage. Under certain inducing differentiation conditions, osteoclasts with bone destruction/absorption potency can be formed [16]. The differentiation of maturation of osteoclast requires the participation of multiple cells and molecules, with the most important roles of M-CSF and RANKL. Therefore, this study selected bone marrow mononuclear cells and treated with M-CSF to facilitate its differentiation towards macrophage, followed by the exploration of the effect of TB on osteoclast differentiation. Results showed that, compared to control group, Mt sonicate at various dosages significantly facilitate the formation of osteoclast with elevated bone absorption potency, proving the bone destruction by TB as consistent with Boyce et al [17]. This study also showed that Mt sonicate treatment could remarkably facilitate the synthesis and secretion of IFN- $\gamma$  and TNF- $\alpha$  by bone marrow mononuclear cells, as similar to Al-attiyah et al, who reported the abundant secretion of IFN- $\gamma$  and TNF- $\alpha$  by the stimulation of peripheral mononuclear cells by MTB-related antigens [18]. Correlation analysis revealed positive correlation between TNF- $\alpha$  or IFN- $\gamma$  concentration and osteoclast counting, bone absorptive lacunae and bone absorption area, suggesting roles of higher IFN- $\gamma$  and TNF- $\alpha$  contents in bone destruction by TB.

RANKL is one important component in the known RANKL/RANK/OPG axis for differentiation and maturation of osteoclast. It is mainly expressed by osteoblast, bone marrow matrix and activated T cells [19]. RANKL is one critical receptor on osteoclast progenitors. Under the stimulus of RANKL signal molecules, RANK receptor couples with downstream signal molecules to induce maturation and activation of osteoclast via classical or alternative signal

pathways. OPG is mainly secreted by osteoblast and lymphocytes, and is one indispensable molecule during the differentiation and activating regulation of osteoclast. OPG is one soluble receptor of RANKL, and can inhibit RANKL-RANK via competitive binding to suppress differentiation and maturation of osteoclast and thus decrease bone destruction/absorption [20]. The ratio of OPG/RANKL plays one critical role in maturation and differentiation of osteoclast. Although various cells could express OPG and RANKL, in the microenvironment of bones, the alignment of osteoblast and osteoclast progenitor makes the direct role of OPG and RANKL by osteoblast during osteoblast-osteoclast signal transduction [21]. NFATc1 is one of the most potent factors inside osteoclast after RANKL stimulus [22]. RANKL from osteoblast binds with RANK receptor on osteoclast progenitor cells, and can up-regulate NFATc1, which is one critical nuclear transcriptional factor for osteoclast differentiation, via NF- $\kappa$ B signal pathway downstream of TRAF6 to potentiate c-Fos expression [23]. This study thus employed one co-culture system including osteoblast and osteoclast, to observe if Mt sonicate could affect osteoclast via osteoblast. Results showed that Mt sonicate could remarkably up-regulate RANKL expression in osteoblast in addition to the down-regulation of OPG, thus suppressing OPG/RANKL ratio, leading to potent expression of NFATc1 in osteoclast and more cell formation.

Hofbauer et al demonstrated that TNF- $\alpha$  could facilitate expressions of M-CSF and RANKL of osteoblast, thus indirectly facilitating differentiation and maturation of osteoclast [24]. Yarilina et al found that TNF- $\alpha$  could directly initiate c-Jun and NF- $\kappa$ B signaling pathways of macrophage for its differentiation into osteoclast [25]. Goto et al revealed that TNF- $\alpha$  facilitated RANKL expression in bone marrow adipocytes-osteoclast progenitor co-culture system, thus enhancing the differentiation of osteoclast progenitor toward mature cells [26]. TNF- $\alpha$  could also directly facilitate the formation of RANKL-induced osteoclast formation to increase bone absorption [15]. Redlich et al reported the involvement of TNF- $\alpha$  in various bone destruction caused by inflammatory diseases [27]. The role of IFN- $\gamma$  in osteoclast is still inconclusive. Kohara et al found the inhibition of osteoclast differentiation by IFN- $\gamma$  treatment

[28], while Ayon Haro et al demonstrated that IFN- $\gamma$  treatment after bacterial LPS-induced bone destruction/absorption could significantly accelerate the formation of osteoclast and activity of bone absorption [29], suggesting that the effect of IFN- $\gamma$  on osteoclast might depend on the existence of other factors, which can be potentiated by IFN- $\gamma$ . This study showed that Mt sonicate remarkably elevate bone absorption potency in addition to increasing contents of IFN- $\gamma$  and TNF- $\alpha$ . With past knowledge of IFN- $\gamma$  and TNF- $\alpha$  in regulating functions of osteoblast/osteoclast, it was suggested that IFN- $\gamma$  and TNF- $\alpha$  might be pathogenic factors for TB-induced bone destruction. The blocking of IFN- $\gamma$  or TNF- $\alpha$  significantly depressed osteoclast formation and bone absorption, with most potent effects occurred under dual blocking. Meanwhile, OPG/RANKL expression in osteoblast, and NFATc1 protein expression in osteoclast nucleus were all partially rescued by such blocking, in addition to the recovery of OPG/RANKL ratio. Results indicated the important role of IFN- $\gamma$  and TNF- $\alpha$  contents in TB-induced osteoclast formation and bone absorption. It is worth noticing that this study also revealed higher potency on inhibition of osteoclast formation and bone absorption after blocking of TNF- $\alpha$  compared to the blocking of IFN- $\gamma$ , probably related with the major role of TNF- $\alpha$ . Regarding the role of IFN- $\gamma$  in TB's effect on osteoclast formation, this study proposed that IFN- $\gamma$  might exert a synergistic effect on TNF- $\alpha$ -induced osteoclast formation, as TNF- $\alpha$  release has been proved as one bone destruction factor in TB infection. Besides inflammatory factors, various protein contents in Mt sonicate could also disrupt the balance of osteoblast/osteoclast, leading to bone lysis. Moreover, LPS of TB could also cause bone absorption, while elevated IFN- $\gamma$  has synergistic and accelerating roles on LPS-induced bone lysis, as demonstrated by Ayon Haro et al [29].

In summary, the elevation of cytokines including IFN- $\gamma$  and TNF- $\alpha$  after TB infection is one important reason underlying TB-induced bone lysis and OAT. Abundant synthesis and release of IFN- $\gamma$  and TNF- $\alpha$  could interfere with OPG/RANKL expression in osteoblast, and interrupt the interaction between osteoblast and osteoclast, eventually leading to bone destruction.

### Disclosure of conflict of interest

None.

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