Interleukin-1 induces receptor activator of nuclear factor-κB ligand-independent osteoclast differentiation in RAW264.7 cells

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Abstract. Interleukin-1 (IL-1) is a pro-inflammatory cytokine which induces bone destruction in various diseases, such as osteoporosis and rheumatoid arthritis. RAW264.7 cells are frequently used in studies as osteoclast precursors, however it remains unclear whether IL-1 can induce osteoclast differentiation from RAW264.7 cells without the stimulation of receptor activator of nuclear factor-κB ligand (RANKL). Hence, the present study aimed to investigate the effects of IL-1 on the formation of osteoclasts from RAW264.7 cells. The cell viability was determined via the Cell Counting Kit-8 (CCK-8) assay. Protein and gene expression were measured by western blotting and reverse transcription-quantitative PCR, respectively. Tartrate-resistant acid phosphatase (TRAP) staining and the resorption pit assay were performed to determine the formation and activity of osteoclasts. A significantly increased quantity of osteoclasts were found in the IL-1 group compared with the control group, and also in the RANKL+IL-1 group compared with the RANKL group. In addition IL-1 significantly increased both the protein and mRNA expression of specific genes associated with osteoclastogenesis, including nuclear factor of activated T cells cytoplasmic 1, matrix metalloprotein-9, cathepsin K and TRAP. The findings of the present study suggested that IL-1 can induce osteoclast differentiation and upregulate the quantity of osteoclasts differentiated from RAW264.7 cells. These results may lay a foundation for further study of diseases involving inflammation-associated bone loss. The combined blockade of IL-1 and RANKL may be effective for the prevention of inflammatory bone loss.

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Introduction

Bone mass is maintained in a perfect balance between osteoclastic bone resorption and osteoblastic bone formation in the standard physiological situation (1). Overactivation of osteoclasts is a significant cause of excessive bone loss in diseases, such as osteoporosis (1). Studies on osteoclast differentiation have revealed that several cytokines, including macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB ligand (RANKL), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) regulate the differentiation process subtly (2,3).

Osteoclasts are short-lived and terminally differentiated cells that cannot be passaged and they are relatively difficult to obtain (4). To date, RAW264.7 cells and bone marrow-derived macrophages (BMMs) are widely adopted in studies to differentiate into osteoclasts (4-6). RAW264.7 cells are murine macrophage cells that need RANKL to complete osteoclast differentiation and are a type of osteoclast precursors, which at a later stage of differentiation are comparable with BMMs (7).

In vitro osteoclast generation (osteoclastogenesis) consists of several steps: i) Hematopoietic macrophage differentiation into osteoclast precursors induced by M-CSF; ii) precursor cells development into mononuclear osteoclasts in the presence of RANKL, IL-1, etc.; iii) mononuclear preosteoclasts fusion into multinuclear osteoclasts; and iv) activation and maturation of osteoclasts. The underlying mechanisms of osteoclastogenesis have been partly unveiled, including the RANKL-signaling pathway and RANKL-independent signaling pathway (8).

RANKL/RANK interaction has been considered a canonical pathway of osteoclastogenesis (9,10). RANKL is a TNF ligand superfamily member (2). RANKL binds to the receptor nuclear factor-κB (RANK) and recruits TNF receptor-related factors, such as TRAF6, and initiates a downstream signaling cascade (11). This downstream signaling cascade promotes expression of several osteoclastic transcriptional factors, such as nuclear factor of activated T cells 1 (NFATc1) and induces the expression of osteoclast-associated genes, including matrix metalloprotein-9 (MMP-9), cathepsin K (CTSK), tartrate-resistant acid

phosphatase (TRAP), hence, the RANKL/RANK axis is essential for osteoclastogenesis (12).

IL-1 has an essential role in various bone diseases which are associated with overactivation of osteoclasts, including osteoporosis, rheumatoid arthritis and periodontal disease (13-15). Besides indirectly stimulating osteoblast/stromal cells, IL-1α and IL-1β can act on specific steps of osteoclast differentiation *in vitro* by binding to IL-1RI with equal affinity (16). IL-1α and IL-1β facilitate the cell fusion of mononuclear and activation of multinucleated osteoclasts, but are not involved in the differentiation of osteoclast precursors to mononuclear osteoclasts (17).

Previous published studies have revealed that although IL-1 can activate osteoclast maturation and enhance bone resorption, it alone cannot initiate the process of osteoclast differentiation from osteoclast precursors (18,19). Kim *et al* (20) however, suggested that IL-1 can induce osteoclast differentiation without the interaction of RANKL/RANK in the context of appropriate microenvironmental conditions. The present study aimed to provide stronger evidence for whether IL-1 could induce osteoclastogenesis without the stimulation of RANKL by performing several qualitative and quantitative experiments using the RAW264.7 macrophages cell line.

Materials and methods

Cell culture and treatment. The RAW264.7 cell line, a murine macrophage cell line, was obtained from the Zhong Qiao Xin Zhou Biotechnology Co., Ltd. The cells were cultured in α-MEM (Gibco; Thermo Fisher Scientific Inc.) supplemented with antibiotics (1% penicillin/streptomycin) and 10% fetal bovine serum (FBS; Gibco Thermo Fisher Scientific Inc.). In every assay, the cells were classified into 4 groups: i) Control (untreated cells); ii) IL-1 (10 ng/ml) (20,21); iii) RANKL (50 ng/ml); and iv) IL-1 (10 ng/ml)+RANKL (50 ng/ml). Soluble RANKL (PeproTech Inc.) or IL-1 (PeproTech Inc.) was added to the culture medium at room temperature 12 h after RAW264.7 cells were seeded into wells. First, the IL-1 and/or RANKL solution was added into fresh medium, then it was used to replace the old medium. The cells were cultured in a 5% CO_2 humidified incubator at 37˚C and the medium was refreshed every other day.

Cell viability assay. Cell viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc.) according to the manufacturer's protocol. RAW 264.7 cells were cultured in a 96-well plates at a density of $1x10^3$ cells/well. The former medium was replaced by 110 μl of fresh α-MEM containing 10 μl CCK-8 solution for 2 h prior to determination of cell viability. Subsequently, a wavelength of 450 nm was used to determine the cell viability. The CCK-8 assay was performed on the 4 previously mentioned cell groups every 24 h (0, 24, 48, 72 and 96 h).

TRAP staining assay. RAW264.7 cells were seeded at a density of $1.5x10^4$ cells/well into 24-well culture plate with a matched cell slide in each well. After cell culture for 4 days, the cells were first washed with PBS 3 times and then fixed for 45 sec at 4˚C. The TRAP staining kit (Nanjing Fengfeng Biomedical Technology Co., Ltd.) was used to count the number of mature osteoclasts. The staining process was accomplished at 37˚C in the dark for 45 min. The cell culture plate was observed under an inverted light microscope and TRAP-positive cells (≥3 nuclei/cell) were identified as mature osteoclasts. A total of 5 random views were selected and the amount of mature osteoclasts was counted manually. Subsequently, the measurement of TRAP activity was detected at 540 nm wavelength and the results were presented as expression related to control.

Western blotting. RAW264.7 cells were seeded into 6-well plates ($1.5x10^5$ cells/well) and then incubated for 4 days after stimulation with IL-1 and/or RANKL as aforementioned. Subsequently, proteins were extracted with RIPA buffer (Nanjing Fengfeng Biomedical Technology Co., Ltd.) and the protein concentration was quantified using a bicinchoninic acid protein assay kit (Bioworld Technology Inc.). Protein (30 μg/lane) was loaded onto 10% SDS PAGE gels and was transferred onto the PVDF membrane (Merck & Co., Inc.). The membranes were blocked for 1 h at room temperature with 5% skimmed milk and then incubated with primary antibodies for MMP-9 (cat. no. 10375-2-AP; 1:1,000; ProteinTech Group Inc.), TRAP (cat. no. 10325-1-AP; 1:3,000; ProteinTech Group Inc.), anti-IL-1RI (cat. no. orb499639; 1:2,000; ProteinTech Group Inc.), NFATc1 (cat. no. 8032S; 1:1,000; Cell Signaling Technology, Inc.), β-actin (cat. no. 20536-1-AP; 1:4,000; ProteinTech Group Inc.) or CTSK (cat. no. 11239-1-AP; 1:1,000; ProteinTech Group Inc.) at 4˚C overnight. β-actin was used as the loading control. Subsequently, the membrane was washed with TBS with 0.1% Tween-20 (TBST) 3 times and incubated for 1 h at room temperature with horseradish peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG secondary antibody (cat. no. SA00001-2; 1:6,000; ProteinTech Group Inc.). The membrane was washed 3 times with TBST at room temperature and soaked in the enhanced chemiluminescence kit (Santa Cruz Biotechnology Inc.). Finally, the bands were detected by the Tanon Imaging System (Tanon Science and Technology Co., Ltd.).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. RAW264.7 cells were seeded into 6-well plates ($1x10^5$ cells/well). Following 4 days of stimulation with IL-1 and/or RANKL as aforementioned, total intracellular RNA was obtained by acid guanidinium thiocyanate-phenol-chloroform method (22), the whole extraction process was completed on ice to avoid degradation, and single-stranded cDNA was synthesized using the Prime Script RT kit (Takara Biotechnology Co, Ltd.) according to the manufacturer's protocol. qPCR was performed using a SYBR Green-1 kit (Takara Biotechnology Co, Ltd.) and the ABI 7500 real-time PCR system (Thermo Fisher Scientific Inc.). The thermocycling conditions were as follows: Initial denaturation for 30 sec at 95˚C; 40 cycles of denaturation for 10 sec at 95˚C and extension for 30 sec at 60˚C. The relative mRNA expression of each gene was calculated using the $2^{-\Delta\Delta Cq}$ method (23) and was normalized to GAPDH (6). The primers were purchased from Generay Biotech Co., Ltd. The primer sequences used were as follows: MMP-9 forward, 5'-GCAGAGGCATACTTGTACCG-3' and reverse, 5'-TGATGTTATGATGGTCCCACTTG-3'; CTSK forward, 5'-GTTACTCCAGTCAAGAACCAGG-3' and reverse, 5'-TCTGCTGCACGTATTGGAAGG-3'; GAPDH

forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-GGGGTCGTTGATGGCAACA-3' (24); TRAP forward, 5'-CTTGCGACCATTGTTAGC-3' and reverse, 5'-TTCTCGTCCTGAAGATACTG-3'; and NFATc1 forward, 5'-CAACGCCCTGACCACCGATAG-3' and reverse, 5'-GGCTGCCTTCCGTCTCATAGT-3' (25).

Resorption pit assay. Corning Osteo Assay Surface 96-well plates (Corning Inc.) were used to examine the ability of bone resorption. The plates are coated with inorganic polystyrene, which is a bone biomimetic synthetic surface (26,27). The RAW264.7 cells were seeded into a 96-well plate ($1x10^3$ cells/well) and treated with IL-1 (10 ng/ml), RANKL (50 ng/ml), or IL-1 (10 ng/ml)+RANKL (50 ng/ml) as aforementioned. The IL-1/RANKL solution was re-added on the 4th day when medium was refreshed. After 8 days of stimulation, cells were removed using 10% sodium hypochlorite solution and stained with 1% toluidine blue at room temperature for 30 min. Subsequently, the plates were washed 3 times with distilled water and the area of resorption pit was photographed using a light microscope. The relative level of resorption area was measured though pixels area analysis via ImageJ software (Version 1.8.0; National Institutes of Health). The resorption area for the treatment groups was normalized by using the pixel area in the control group (27,28).

Statistics. Each experiment was performed at least 3 times and the data were presented as the mean ± standard deviation (SD). The results were analyzed using one-way analysis of variance with subsequent post hoc Tukey's tests using SPSS 26 software (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability of RAW264.7 cells is not altered by IL-1/RANKL. Effects of IL-1 and/or RANKL on the cell viability was examined by a CCK-8 assay following stimulation. The RAW264.7 cells were treated with or without IL-1 (10 ng/ml) and/or RANKL (50 ng/ml) for 4 days and no significant differences were found between groups at the indicated days ($P>0.05$) (Fig. 1).

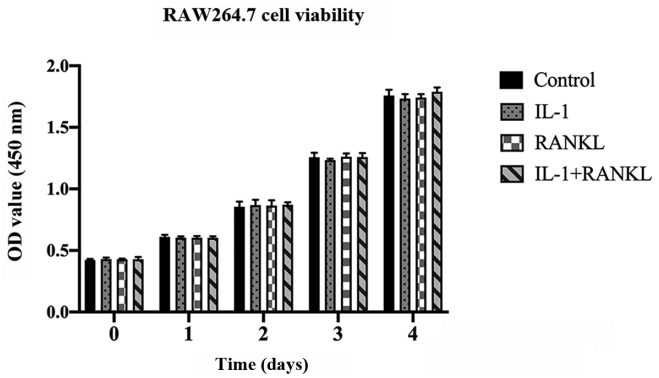

Figure 1. IL-1/RANKL does not affect the cell viability of RAW264.7 cells. The cells were treated with or without IL-1 (10 ng/ml) and/or RANKL (50 ng/ml). The CCK-8 assay was used to measure the cell viability at indicated time points (days 0, 1, 2, 3 and 4). No statistical difference was found between groups. OD, optical density; IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand-independent; control, untreated cells.

Osteoclast formation is promoted by IL-1/RANKL. Osteoclast formation was evidenced by $TRAP^+$ multinuclear cells. Significantly more $TRAP^+$ cell were formed after stimulation of IL-1 or RANKL compared with the control group ($P<0.01$), while there was no statistically significant difference between the IL-1-treated and RANKL-treated group ($P>0.05$) (Fig. 2A and B). The quantity of $TRAP^+$ osteoclasts was significantly higher in the 2-stimulus group (IL-1+RANKL) compared with the single-stimulus groups (IL-1/RANKL) ($P<0.01$) (Fig. 2A and B). The results of TRAP activity (Fig. 2C) were in agreement with the staining results ($P<0.01$) (Fig. 2A and B).

Expression of osteoclastogenesis-specific genes and proteins are elevated in IL-1/RANKL-treated cells. To further demonstrate the role of IL-1 in osteoclast differentiation, the expressions of osteoclastogenesis-related genes (NFATc1, MMP-9, CTSK and TRAP) and IL-1RI were examined by western blotting and RT-qPCR analysis 4 days after stimulation. RAW264.7 cells exhibited substantial protein expression of IL-1RI in the control group and compared with other groups, the IL-1+RANKL group showed the highest expression of IL-1RI (Fig. 3B). Compared with the control group, IL-1 promoted the protein and gene expression of MMP-9, NFATc1, CTSK and TRAP ($P<0.01$), which was comparable to the results of RANKL-treated group ($P<0.01$) (Fig. 3A and C-G). In addition, compared with the IL-1-treated group, the expressions of osteoclastogenesis-specific genes were significantly higher in the 2-stimulus group ($P<0.05$ for TRAP and CTSK expression, $P<0.01$ for NFATc1 and MMP-9 expression) (Fig. 3A and C-G).

The area of bone resorption is increased in IL-1/RANKL-treated cells compared with the control group. IL-1 significantly increased the bone resorption area compared with the control group ($P<0.01$), meanwhile, no significant difference was found in the area of resorption between the IL-1-treated and RANKL-treated groups ($P>0.05$) (Fig. 4A and B). In addition, the bone resorption area in the IL-1+RANKL group was also significantly increased compared with that in the single stimulus groups ($P<0.01$) (Fig. 4).

Discussion

As one of the highly important factors that causes osteoporosis, the excessive activation of osteoclasts has been studied *in vivo* and *in vitro* for a long time (1-3). RAW264.7 cells are widely used as osteoclast precursors, because like BMMs, they also originate from a hematopoietic lineage (29). In addition, compared with BMMs, RAW264.7 cells are easily accessible and sensitive to the stimulation of RANKL (30). In the present study, to clarify the direct effect of IL-1 on osteoclast differentiation, RAW264.7 cells were used as osteoclast precursors and TRAP staining and bone resorption assay were used to examine the osteoclast differentiation and activity, respectively. In the present study, a significantly increased quantity of osteoclasts was not only observed in the IL-1 group compared

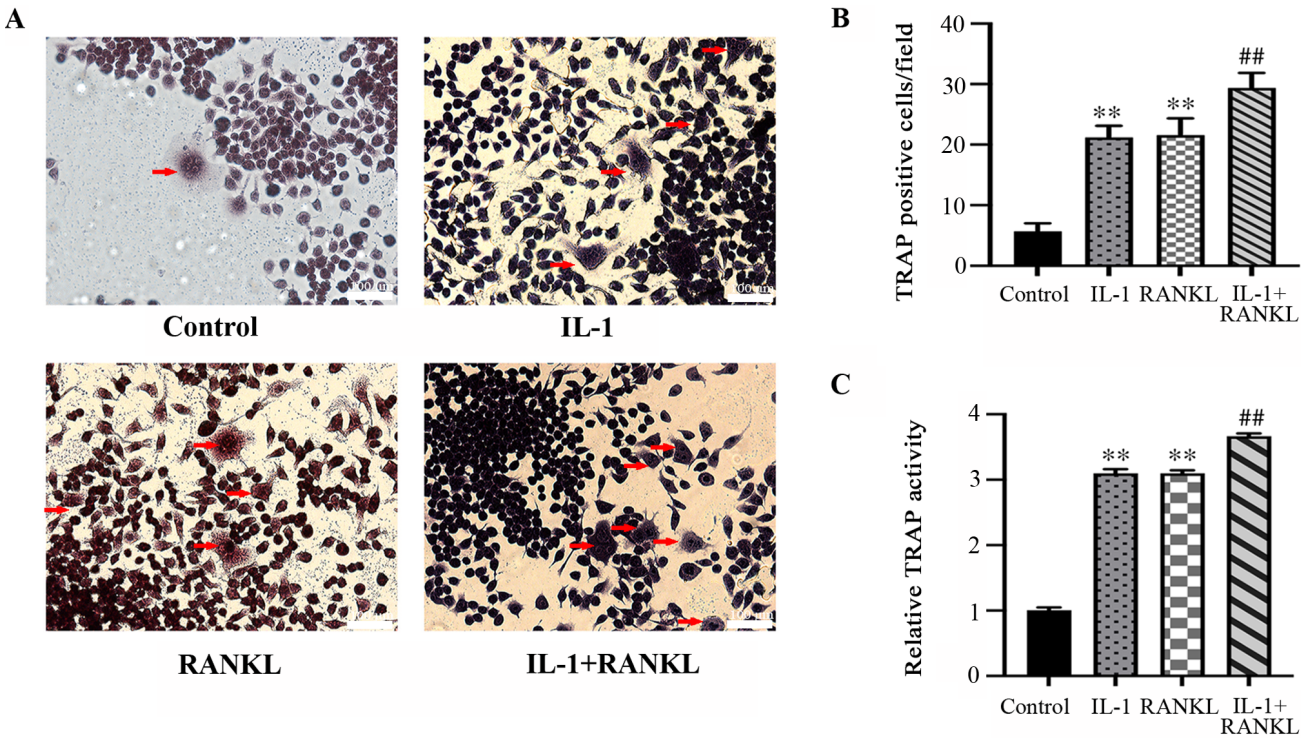

Figure 2. IL-1 promotes RAW264.7 cells osteoclastogenesis. Following 12 h of incubation, culture medium with IL-1 (10 ng/ml), RANKL (50 ng/ml), or IL-1 (10 ng/ml)+RANKL (50 ng/ml) was added to RAW264.7 cells. (A) RAW264.7 cells were stained 4 days after the stimulation using a TRAP kit according to the manufacturer's instructions and observed under alight microscope. Scale bar=100 μm. Red arrows indicate mature osteoclasts. (B) TRAP-positive osteoclasts with ≥3 nuclei were identified and counted in 5 random fields. (C) TRAP activity was detected at 540 nm and the results were presented as expression related to control. **P<0.01 compared with the control group, $^{\#\#}$P<0.01 compared with IL-1-treated or RANKL-treated alone. OD, optical density; IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand-independent; control, untreated cells; TRAP, tartrate-resistant acid phosphatase.

with the control group, but also in the IL-1+RANKL group compared with the IL-1/RANKL group. The present study demonstrated that IL-1 can induce osteoclastogenesis in a RANKL-independent way and upregulate the osteoclast differentiation in RAW264.7 cells in the presence of RANKL.

It is now acknowledged that activation of RANK pathway is essential for osteoclast differentiation (1). Substitutes for RANKL include IL-1, transforming growth factor β and IL-6 (8). However, the function of IL-1 in osteoclast differentiation remains controversial. IL-1 can enhance the capacity of mature osteoclasts in bone resorption, which is supported by previous studies (8,31) and the results of the present study. However, IL-1 could not induce osteoclast differentiation from BMMs partly due to the insufficient expression of IL-1RI (20). In the present study, RAW264.7 cells exhibited substantial protein expression of IL-1RI.

Osteoclasts are the unique cells which can resorb bone. An excessive increase in osteoclast differentiation leads to several bone-resorptive diseases, such as osteoporosis (1). Jimi *et al* (32) concluded that IL-1 can bind to putative IL-1 receptors on osteoclast-like cells leading to an induction of a NF-κB-like factor. Wei *et al* (31) discovered IL-1 can enhance the expression of RANKL in bone marrow stromal cells and directly stimulate differentiation of osteoclast precursors. However, Watanabe *et al* (33) found that the formation of osteoclasts was suppressed by IL-1β via decreasing M-CSF production and increasing osteoprotegerin production. The cause of conflicting results regarding the effect of IL-1 on the differentiation and proliferation of osteoclasts may lie in the different cells and induction methods that were used in each study.

Lorenzo *et al* (34) demonstrated that there was no significant bone loss in IL-1RI-deficient mice after ovariectomy, which is a widely used osteoporosis model relevant to menopause (35,36). Osteoclast formation and bone resorption area are decreased in ovariectomized mice treated with IL-1 receptor antagonist compared with the sham control group (36). The results suggested that IL-1 is an important cytokine in bone loss associated with a decline in estrogen (34-36). However, studies using IL-1RI-deficient mice to explore the effects of IL-1 on bone metabolism have revealed controversial results. Bajayo *et al* (37) reported the loss of bone mass in IL-1RI-deficient mice, indicating that IL-1 receptor signaling pathway is also an important regulator of bone mass and bone remodeling. On the contrary, Vargas *et al* (38) demonstrated that the bone volume and number of osteoclasts of humeri in IL-1RI-deficient mice are normal compared with the control group.

RANKL induces the gene and protein expression of NFATc1 by activating Ca^{2+} signals from the immunoreceptor tyrosine-based activation motif pathway (39). The expression of NFATc1 is significantly increased by auto-amplification (40) and subsequently, the expression of osteoclast-related genes, such as TRAP, CTSK and MMP-9 are induced by NFATc1 (41). It was also reported in the aforementioned study, that NFATc1-deficient stem cells stimulated with RANKL did not differentiate into osteoclasts, while the upregulation of NFATc1 led to efficient osteoclast

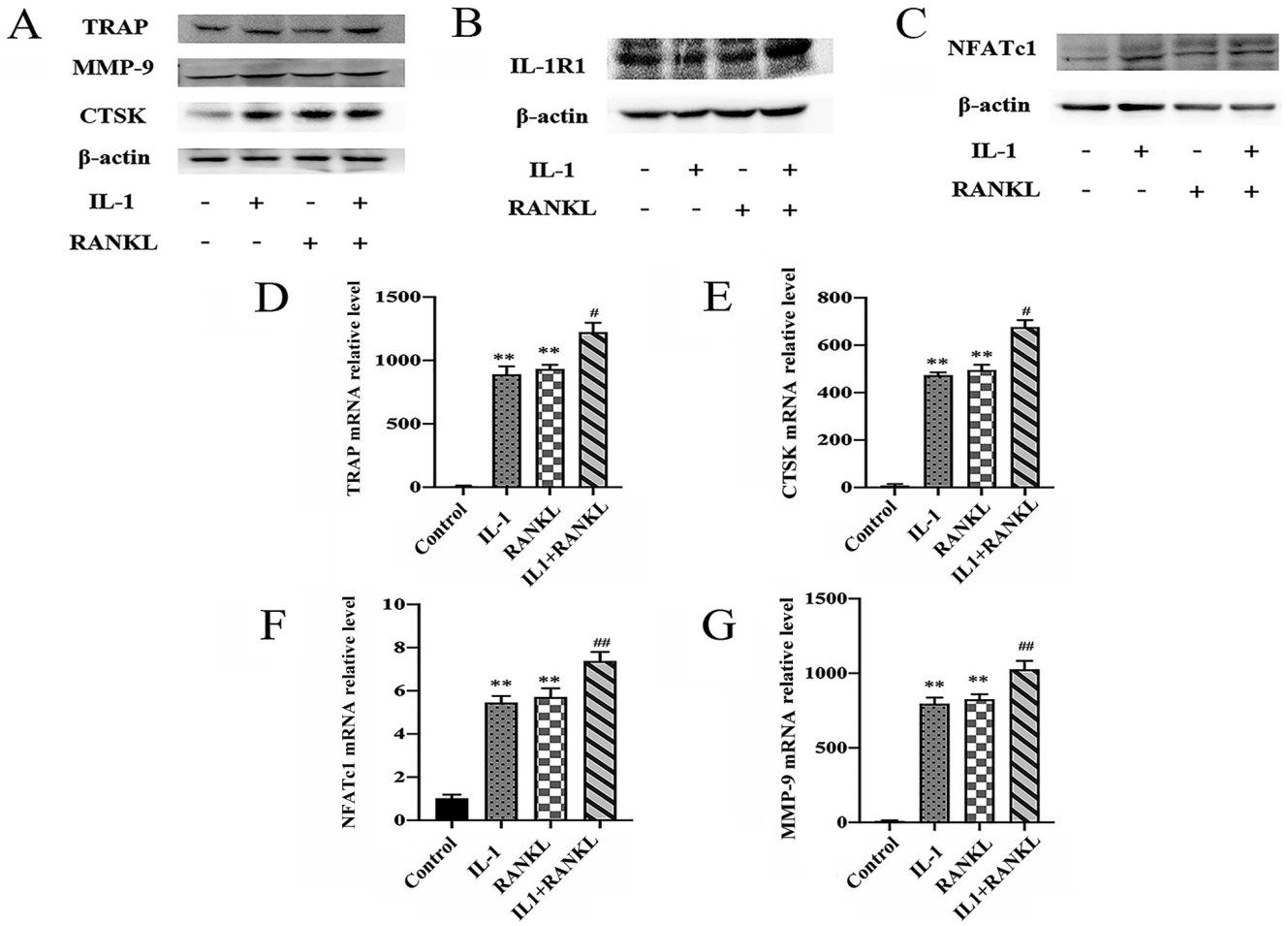

Figure 3. IL-1 promotes the expression of osteoclastogenesis-specific genes. RAW264.7 cells were treated with IL-1 (10 ng/ml) and/or RANKL (50 ng/ml) for 4 days. (A) Expressions of osteoclastogenesis-specific genes [MMP-9, CTSK, TRAP and NFATc1 (C)] and IL-1RI (B) were determined by western blotting. The exposure time of TRAP, IL-1RI and MMP-9 was 10 sec, the exposure time of NFATc1 was 60 sec and the exposure time of other blots was 5 sec. IL-1RI and NFATc1 were from different gels, TRAP, MMP-9 and CTSK were from the same gel. (D-G) RT-qPCR expression in the 4 groups of RAW 264.7 cells (control, IL-1, RANKL and IL-1+RANKL) of (D) TRAP (E) NFATc1, (F) CTSK and (G) MMP-9. Relative gene expression was normalized to GAPDH. **P<0.01 compared with the control group, $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with IL-1-treated or RANKL-treated alone. IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand-independent; control, untreated cells; RT-q, reverse transcription-quantitative; NFATc1, nuclear factor of activated T cells cytoplasmic 1; MMP-9, matrix metalloprotein-9; CTSK, cathepsin K; TRAP, Tartrate-resistant acid phosphatase; IL-1RI, IL-1 receptor I.

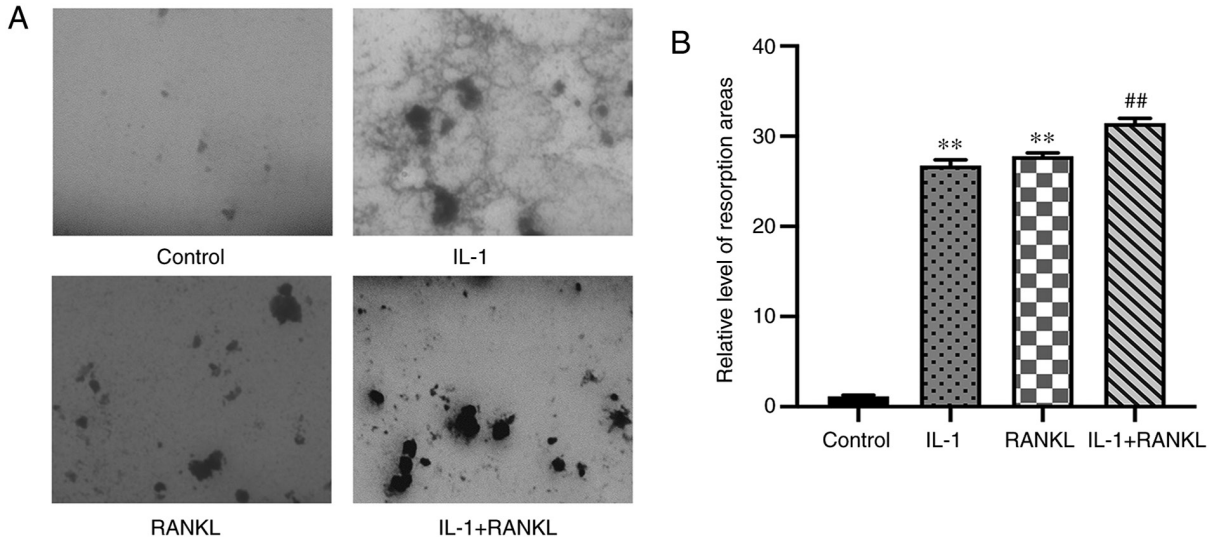

Figure 4. IL-1 promotes the area of bone resorption pit. (A) Images of RAW264.7 cells on Corning Osteo Assay Surface treated with or without IL-1 (10 ng/ml) and/or RANKL (50 ng/ml) for 8 days. Magnification (x40). (B) Relative level of resorption area was measured using Image J Plus software. **P<0.01 compared with the control group, $^{\#\#}$P<0.01 compared to IL-1-treated or RANKL-treated alone. IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand-independent; control, untreated cells.

differentiation without the stimulation of RANKL (41). Hence, NFATc1 may function as a master switch in activating target genes expression of downstream of RANKL in the terminal stage of osteoclast differentiation. In the present study, RAW264.7 cells expressed nearly no NFATc1 without the stimulation of IL-1 and RANKL, which suggested that IL-1 or RANKL is important for initiating the expression of NFATc1 during the process of osteoclast differentiation. It has been reported that IL-1 can upregulate the induction of osteoclasts in the presence of RANKL (20,42), and the present study found that RAW264.7 cells expressed NFATc1 on stimulation with IL-1 and that there was a potential interaction between IL-1RI, NFATc1 and RANKL. Further studies are needed for investigating the interaction between IL-1RI, NFATc1 and RANKL and to confirm that IL-1 is a requisite for bone remodeling.

Normally, dentine or bone slices are used to evaluate osteoclast differentiation and function (20,43), however, they are difficult to handle and easily damaged. The Corning Osteo Assay Surface represents a convenient and reproducible substitute for slices (26,27). Hence, in the present study the bone resorption area was observed and calculated using the Corning Osteo Assay Surface. The present study demonstrated that there was no statistically significant difference between osteoclasts and the area of the resorption induced from IL-1 and RANKL-treated cells. In contrast, Kim *et al* (20) reported that the number of osteoclasts and the relative intensity of resorption pit on dentine slices formed in IL-1/IL-1RI-treated cells was less compared with RANKL-treated cells. The aforementioned results suggested that the activation of both signaling pathways are important in osteoclast formation and function and the difference in their findings may be due to the different osteoclast precursors used in the 2 studies.

There are several limitations of the present study. Firstly, the osteoclast precursors used, although RAW264.7 cells have been widely used as osteoclast precursors to study osteoclast differentiation they are not identical to BMMs in the human body. Secondly, the optimum *in vitro* concentration of IL-1 was not determined in the present study. Hence, the results of the present study are not completely applicable to osteoclasts derived from human body. Lastly, the pathways involved and the potential interaction between IL-1RI, NFATc1 and RANKL were not investigated in the present study. Further studies are needed to investigate all the aforementioned points and to verify the finding of the present study.

In conclusion, the present study to the best of our knowledge for the first time demonstrated that IL-1 can induce osteoclast differentiation from RAW264.7 macrophages and the results may provide groundwork for the study of diseases involving in the bone loss associated with inflammation.

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Availability of data and materials

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

Authors' contributions

JT conceived and designed this study, RoL, ZF, WL, RuL, XX and SY performed the experiments. RoL and ZF confirmed the authenticity of all the raw data and drafted the manuscript. WL, RuL, XX and SY reviewed the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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