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The alterations of molecular repertoire of the RANKL-induced osteoclastogenesis in the M1 macrophage-derived inflammatory milieu

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Inflammation have been linked to bone diseases such as osteoporosis or bone destruction. However, whether M1 inflammatory stimuli exert a stimulatory or inhibitory effect on the differentiation of osteoclasts remained controversial. Also, how inflammatory milieu influence cell proliferation and survival during osteoclastogenesis have not been determined. Here we reported the molecular repertoire alterations of RANKL-stimulated osteoclastogenesis from RAW264.7 at different stages in the inflammatory environments. Adding conditioned medium collected from LPS-stimulated macrophage, which are the primary source of extracellular inflammatory mediators, resulted in a biphasic change in cell number among differentiating preosteoclasts. The inflammatory milieu induced a transient proliferation of preosteoclasts during the initial 48 h, which was followed by a significant decline in cell numbers from the fourth day onwards. Proliferation-related AKT and ERK were transiently activated in the inflammatory environments, which also upregulated the expressions of *c-myc*, a major transcription factor for osteoclast differentiation, and pro-inflammatory genes, such as *Tnf-α* and *Nos2*. Following prolonged exposure to an inflammatory environment, undifferentiated osteoclast precursors undergo apoptosis. Our findings suggest that short-term inflammatory exposure transiently promotes the proliferation and differentiation of preosteoclasts, whereas long-term exposure leads to apoptosis, potentially due to the enhancement of inflammatory signals.

Keywords Inflammation, Inflammatory milieu, Pro-inflammatory cytokines, Osteoclastogenesis

Chronic inflammation has been widely recognized as an important risk factor of chronic inflammatory-bone metabolic disorders, such as rheumatoid arthritis (RA), periodontitis and periprosthetic loosening. Inflammation triggered excessive bone loss yet limited bone formation[1,2], possibly through changing the functions and differentiation of osteoclasts and osteoblasts[3]. The alterations of osteoclast and osteoblast under inflammatory environment have not been fully understood. Recent research revealed that dysregulated immune cells induced alterations in osteoclastogenesis[4,5]. Under inflammatory conditions, immune cells affect the differentiation and functions of bone cells and osteoclasts through paracrine effects. Previous studies reported that γδ-T cells inhibit osteoclastogenesis through the production of interferon gamma (IFN-γ)[6]. Inflammatory environments stimulate the production of ligand of receptor activator of NF-κB ligand (RANKL) from B and

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T cells, subsequently enhance the differentiation of osteoclasts[7]. Macrophages outnumber B and T cells being the most abundant immune cells found in the synovial membrane in osteoarthritis[8] and in synovial fluid in rheumatoid arthritis[9]. Additionally, macrophages, which serve as the major sources of pro-inflammatory mediators in pro-inflammatory state (M1 state), is the critical cellular constituent regulating osteocalstogenesis, bone resorption, and bone remodeling[10,11].

Pro-inflammatory cytokines secreted from M1 macrophages has been commonly considered as a positive regulator of osteoclast differentiation or bone resorption. The signaling pathways that drive the osteoclast differentiation overlap with the signaling pathways activated by pro-inflammatory cytokines, such as above-mentioned NF-κB and mitogen-activated protein kinase (MAPK) signaling pathway. Additionally, Tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) stimulate the production of Macrophage colony-stimulating factor 1 (M-CSF) and macrophage chemoattractant protein-1 (MCP-1) to attract osteoclasts, as well as enhancing activity of mature osteoclasts[12]. Additionally, TNF-α has been demonstrated to promote the secretion of RANKL from lymphocytes and endothelial cells[13], while IL-1β has been shown to induce the production of Prostaglandin E2 (PGE2) synthesis, both of which have been identified as indirect inducers of osteoclast formation[14,15]. TNF-α and prostaglandins play a pivotal role in osteoclast maturation. Osteoclast precursors require TNF-α in the presence of small amounts of RANKL in order to differentiate into mature osteoclasts[16]. IL-6 is another inflammatory cytokine that increase RANKL production, bone resorption, and PGE2 production[17,18]. Therefore, inflammation has been commonly considered as a positive regulator of osteoclast differentiation or bone resorption. However, in an inflammatory environment, cells are not affected only by the previously mentioned cytokines. Recently, Yamaguchi et al. reported that co-culture of M1 macrophages with RANKL-induced RAW264.7 cells significantly suppressed osteoclastogenesis[19]. Direct contact between M1 and osteoclast precursors was not required for this differentiation suppression, indicating engagement of soluble factors released from M1. Real-time PCR analyses showed that IFN-γ suppressed gene expression of Nuclear Factor of activated T cells 1 (*Nfatc1*), whereas IL-12 increased the apoptosis of osteoclasts, suggesting possible roles of IFN-γ or IL-12 in M1-mediated inhibition of osteoclastogenesis. These findings were confirmed in an in vivo ligature-induced mouse periodontitis model in which adoptive transfer of M1 macrophages showed a significantly lower level of bone loss and less tartrate-resistant acid phosphatase (TRAP)-positive cell induction than M0 or M2 macrophage transfer[20]. These results showed that the cellular alterations induced in inflammatory environments cannot be predicted from the effects of single cytokine. It should be the combination of cytokines with positive and negative effects.

Cell proliferation or death are often altered under inflammatory environments. Some pro-inflammatory cytokines, such as IL-6 are known to trigger cell proliferation, while other cytokine like IFN-γ could promote cell death. TNF-α induces either proliferation or apoptosis depending on other environmental factors. The proliferation and survival of differentiating preosteoclasts during osteoclastogenesis has not been characterized. In this study, we applied conditioned medium collected from lipopolysaccharide (LPS)-primed macrophage culture to mimic the inflammatory milieu that occurred in the inflammatory sites. We aimed to characterize the cell proliferation or death during osteoclastogenesis. Our results provided a different scenario for the cell fate of osteoclast under inflammatory environments.

Materials and methods

Cell culture and treatment

The murine macrophage cell line RAW264.7 subclone 2 was obtained from American Type Culture Collection (ATCC, ATCC® TIB-71™). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) penicillin–streptomycin solution (P/S), and incubated at 37 °C in 5% CO_2 humidified air. The medium was changed every 3 days. To obtain osteoclasts, RAW264.7 cells were grown for 6 days in DMEM containing 10% FBS, 1% P/S and 50 ng/ml mouse receptor activator of nuclear factor-kappa β ligand (mRANKL, PeproTech, Rocky Hill, NJ, USA)[21]. During the osteoclast differentiation process, exogenous IL-6 (50 ng/ml) and IFN-γ (20 ng/ml) were added as supplements to the normal culture medium.

Tartrate-resistant acid phosphatase (TRAP) staining

The RAW 264.7 cells were seeded at a density of 5×10^4 cells/well in a 24-well tissue culture plate and induced to differentiate into osteoclasts in accordance with the previously described methodology. Following a six-day incubation period, the cells were fixed and stained using the TRAP activity staining kit (386A-1KT, Sigma-Aldrich) in accordance with the manufacturer's instructions. The presence of dark red TRAP-positive cells was observed, and the number of multinucleated TRAP-positive cells with more than three nuclei was quantified.

MTT assay

RAW264.7 were plated into 96-well plates and grown in differentiation medium for indicated days. Cells were treated with RANKL and with or without IL-6 for 24, 48 or 72 h. Subsequently, the medium was removed, and cells were cultured in 100 µL fresh medium containing 10% 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) for 4 h at 37 °C. The supernatant was removed, and the formazan crystals were dissolved in 100 µL of Dimethyl sulfoxide (DMSO). Absorbance was recorded at 595 nm by using a microplate reader[21].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay

The cells were collected 3 or 24 h after treatment separately. Total RNA was extracted using RNAzol reagent (MRC, Molecular research center, Inc., USA), according to the manufacturer's protocol. cDNA was synthesized using a IQ2 MMLV RT-Script kit (Bio-Genesis Technologies Inc., TW) according to manufacturer's protocol.

qPCR was performed using SYBR-Green (Applied Biosystems, Thermo Fisher Scientific, Inc., USA), and data collection was conducted using an ABI 7300 (Applied Biosystems; Thermo Fisher Scientific, Inc., USA). The PCR cycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 s, 58 °C or 53 °C for 20 s and 72 °C for 40 s, a final extension step of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. No template control and no amplification control were performed to control the extraneous nucleic acid contamination and specificity, and fluorescence background. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. Gene expression was calculated using the delta-delta Ct method. Primer sequences were listed in Table 1.

Western blot

The cells were collected 2 h after treatment and lysed with protein lysis buffer. Protein samples were electrophoresed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (NuSep, Bio-Rad) and were transferred onto polyvinylidene difluoride membranes. The blots were cut based on the standard band positions, then incubated with the appropriate antibodies including anti-phospho-Akt(Ser473) rabbit antibody (1:2000, #4060, Cell Signaling), Akt(pan) rabbit antibody (1:1000, #4691, Cell Signaling), anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit antibody (1:500, #9101, Cell Signaling), anti-p44/42 MAPK (Erk1/2)(137F5) rabbit antibody (1:1000, #4695, Cell Signaling), anti-phospho-IKKα/β (1:1000, #2697, Cell Signaling), anti-IKKα (1:1000, #11930, Cell Signaling), anti-IKKβ (1:1000, #8943, Cell Signaling) overnight at 4 °C, followed by incubation with an HRP (horseradish peroxidase)-labeled secondary antibody. The protein bands were visualized using the ECL method, and a semiquantitative analysis was conducted using ImageJ software. The cell lysate of cells that known expressing the target protein were used for positive/specificity control, while no primary antibody reacting experiment were used for negative/specificity control. Actin antibody results could also be considered as the positive control for each blot.

Animal study

C57BL/6 J mice (8–12-week-old; BioLASCO Taiwan Co., Ltd, Taiwan) were used in this study. They were maintained under a 12-h light–dark cycle at a temperature of 23 ± 2 °C and were provided with a standard diet and water ad libitum. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chengchi University. All animal experiments were conducted in accordance with IACUC regulations and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Every effort was made to minimize both the suffering and the number of animals involved in the experiments.

Primer name	Primer sequence
GAPDH- forward	GCACAGTCAAGGCCGAGAAT
GAPDH- reverse	GCCTTCTCCATGGTGGTGAA
Ki67- forward	ACCGTGGAGTAGTTTATCTGGG
Ki67- reverse	TGTTTCCAGTCCGCTTACTTCT
Tnf-α- forward	CCCTCACACTCAGATCATCTTCT
Tnf-α- reverse	GCTACGACGTGGGCTACAG
Nos2 forward	CTTTGCCACGGACGAGAC
Nos2- reverse	TCATTGTACTCTGAGGGCTGA
c-myc- forward	GCTGGACACGCTGACGAAA
c-myc- reverse	TCTAGGCGAAGCAGCTCTATTT
c-fos- forward	GGGGACAGCCTTTCCTACTA
c-fos- reverse	CTGTCACCGTGGGGATAAAG
Nfatc1- forward	GACCCGGAGTTCGACTTCG
Nfatc1- reverse	TGACACTAGGGGACACATAACTG
CTSK- forward	CTTCCAATACGTGCAGCAGA
CTSK- reverse	TCTTCAGGGCTTTCTCGTTC
TRAP- forward	GCTGGAAACCATGATCACCT
TRAP- reverse	GAGTTGCCACACAGCATCAC
MMP9- forward	GTTTTTGATGCTATTGCTGAGATCCA
MMP9- reverse	CCCACATTTGACGTCCAGAGAAGAA
Bad- forward	CTCCGAAGGATGAGCGATGAG
Bad- reverse	TTGTCGCATCTGTGTTGCAGT
Bax- forward	TGAAGACAGGGGCCTTTTTG
Bax- reverse	AATTCGCCGGAGACACTCG

Table 1. Primers for RT-qPCR in this study.

Generation of bone marrow derived macrophage (BMDM) and collection of conditioned medium

L929 conditioned medium is required for the generation of BMDM from bone marrow cells. Prior to collecting bone marrow cells, the L929 cells were cultivated for three passages following their recovery from cryogenic storage. The cells were seeded in high-glucose DMEM containing 10% FBS, 1 mM L-glutamine and 1% P/S at a density of 1×10^6 cells per 10 cm dish. The induction of BMDM followed the methods published previously with modification[22]. Following a five-day culture period, the media was harvested and stored at − 20 °C for further inducing BMDM from bone marrow cells. Bone marrow cells were extracted from the femurs and tibiae of C57BL/6 J mice aged between two and three months. The cells were treated with red blood cell lysis buffer (comprising 155 mM NH_4Cl, 12 mM $NaHCO_3$ and 0.1 mM EDTA) and a stop reaction was initiated with PBS. Subsequently, the cells were subjected to filtration through a cell strainer with a 70-micron pore size, after which the cell number was determined through counting. Subsequently, the bone marrow cells were seeded at a density of 5×10^6 cells per 10 cm dish in high glucose DMEM containing 10% FBS, 1% P/S and 20% previously collected L929 conditioned media. During the five-day differentiation process, the media were changed every other day. Following a five-day induction period, over 90% of the cultured cells exhibited expression of the myeloid marker CD11b. Upon completion of the differentiation process, the induction medium, which contained 20% L929 culture medium, was removed. The differentiated BMDM were then cultured in fresh high-glucose DMEM, supplemented with 10% FBS and 1% P/S, with or without the addition of LPS (1 μg/ml). Following a 24-h incubation period, the culture media were collected for further application to stimulate osteoclastogenesis. The cytokine components of the conditioned medium were identified by cytokine array Proteome Profiler Mouse Cytokine Array Kit (R&D systems). The medium collected from BMDM culture without LPS stimulation was added to the control group, while the medium from LPS-primed BMDM culture, which contains macrophage-secreted proinflammatory cytokines, was used as conditioned medium in this study.

Statistical analysis

Data were expressed as pooled mean ± standard error of the mean of at least three independent experiments (n = 3–9). Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA). Multiple or Student's t-tests were used to compare data from the control and treatment groups. Symbols were considered to indicate a statistically significant difference (*where $P < 0.05$, **where $P < 0.01$, ***where $P < 0.001$, ****where $P < 0.0001$).

Results

Different proinflammatory cytokines induces different cellular responses in preosteoclasts

Our understanding about how inflammatory environments affect bone resorption largely come from the stimulation of individual pro-inflammatory cytokines. However, different cytokines might produce different, or even opposite effects on osteoclast differentiation. Interleukin-6 (IL-6) and interferon-γ (INF-γ) are both key components in the inflammatory milieu. IL-6 mediates acute phase response in the inflammatory response, while INF-γ is the major cytokine secreted from helper T cell to activate macrophage for further cell-mediated immunity. RAW264.7 is a macrophage cell line, which can be used as an osteoclastogenesis and osteoclast cell model when induced with RANKL[23]. The differentiation of osteoclasts was confirmed through TRAP staining. Figure 1a illustrated the distinctive morphology of fused, polykaryon osteoclasts, demonstrating that preosteoclast RAW264.7 cells can be induced to differentiate into mature osteoclasts. Figure 1b demonstrated that the expression of all three bone resorptive genes, Cathepsin K (*Ctsk*), *Trap* and matrix metalloproteinase-9 (*Mmp9*) (all three genes, n = 6, $p < 0.0001$) was increased in the RANKL-treated differentiating osteoclasts compared to their expression in undifferentiated preosteoclasts (RAW264.7). These results indicate that 6 days of RANKL treatment has induced preosteoclast cells differentiated into mature osteoclasts. Figure 1c and d demonstrated opposite response of IL-6 and INF-γ on RANKL-treated RAW264.7 cells. When treated with IL-6, cell proliferation of differentiating preosteoclasts were transiently promoted to 65% viability increase at day2 ($p < 0.0001$, n = 9), but returned to comparable levels with that of control group, in which RAW264.7 cells only treated with RANKL (Fig. 1b). In contrast, INF-γ reduced cell viability starting from 24-h incubation. Longer exposure continuously decreased cell survival. The viability rate for INF-γ treatment was 53% (2-day), 22% (4-day), and 13% (6-day) when considered separately. ($P < 0.0001$, n = 9, Fig. 1c). These results demonstrated that the influence of inflammatory milieu might not be precisely predicted by the results of individual factors.

M1 macrophages-derived inflammatory milieu induced cell number changes during the RANKL-induced osteoclastogenesis in a biphasic manner

Macrophages and osteoclasts serve as major sources of the inflammatory mediators within bone tissue. Therefore, the pro-inflammatory molecules secreted from LPS-stimulated BMDM might represent the constitution of inflammatory environments. The components of the conditioned medium were analyzed using a cytokine array (Proteome Profiler Mouse Cytokine Array Panel A Kit, R&D Systems). The medium contained high expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β), chemokines (CCL2, CCL3, CCL5, CCL2/MCP1, CXCL10, slCAM-1/CD54, MIP-1B/CCL), and even M2 cytokines (CXCL1, MIP-2/CXCL2)[24]. In the subsequent experiments, RANKL-treated RAW264.7 cells were stimulated with 20% conditioned medium, which contained subacute levels of the cytokines. This treatment may serve to mimic the chronic inflammation in the bone cell niche.

Inflammatory milieu possibly affects a broad array of biological functions of bone cells, including cell proliferation, survival, the differentiation of osteoclast precursor cells and ultimately bone resorption. To monitor the fluctuation of cell numbers of preosteoclasts during the differentiation process, MTT assay was employed to assess the cell viability and proliferation. We compared the cell number change between RANKL treated

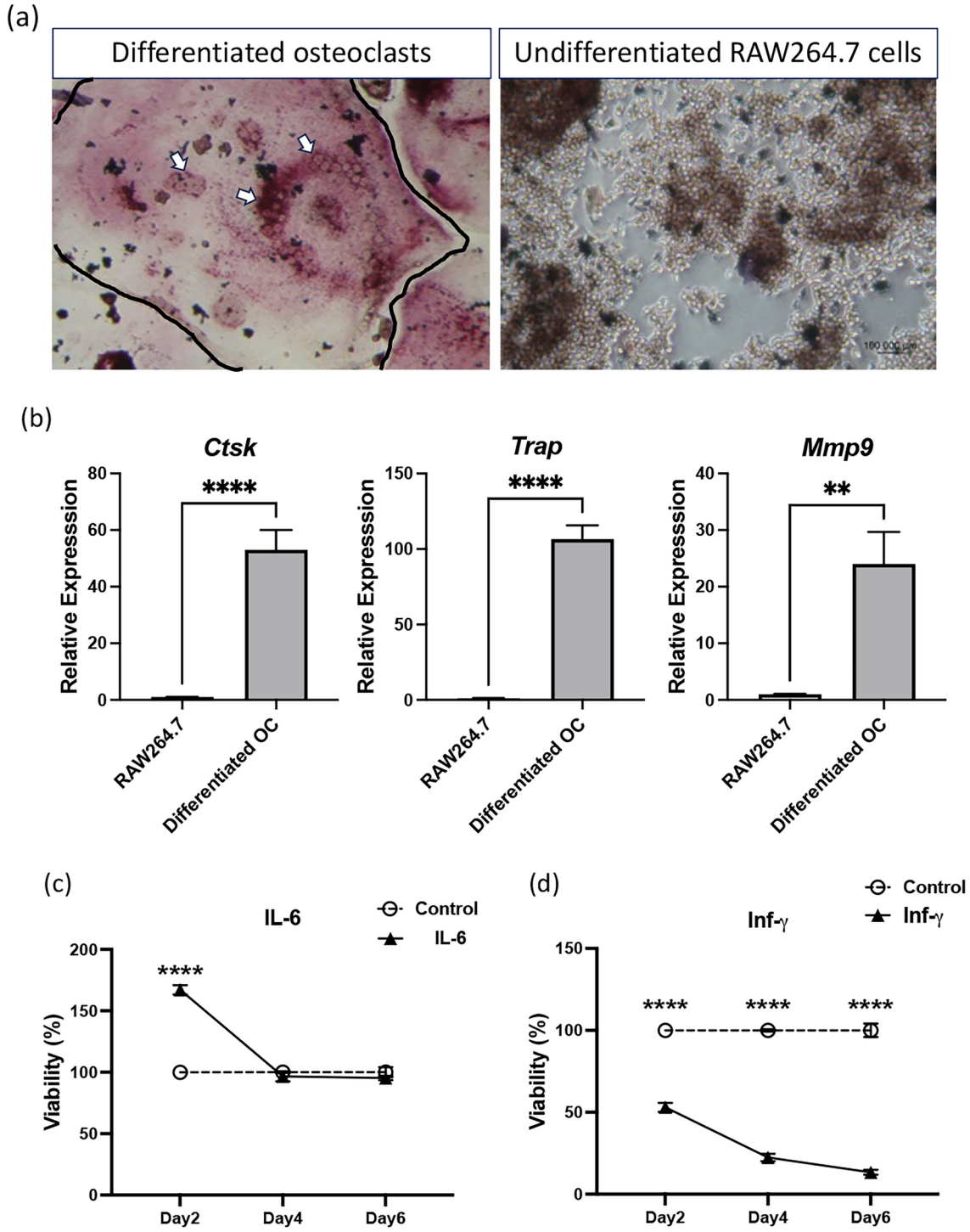

Fig. 1. Different proinflammatory cytokines induce distinct cellular responses in preosteoclasts. RAW264.7 cells were cultured in DMEM containing 10% FBS and 50 ng/mL RANKL to induce osteoclast differentiation. Following a six-day incubation period, the differentiation status of the RANKL-treated osteoclasts was confirmed through TRAP staining. (**a**) The TRAP staining was observed to be dark red, and the cells were found to be multinucleated (indicated by arrow, the black line depicted the boundary of one fused cell), which are both characteristics of mature osteoclasts. (**b**) The *Ctsk*, *Trap* and *Mmp9* three genes expression of preosteoclasts treated with/without RANKL for 6-day (RAW264.7: as control, without RANKL-induced differentiation; Differentiated OC: as differentiated osteoclasts, with RANKL-induced differentiation). Each column represents the mean ± standard error of the mean of at least three independent experiments. The effect of IL-6 and interferon-γ on cell viability in preosteoclasts. Preosteoclasts were treated with IL-6 (**c**) and interferon-γ (**d**) for 2, 4 and 6 days separately. Cell viability was determined using the resazurin reduction assay with 10% resazurin solution incubated for four hours. Error bars depict the standard error of the mean. ****$p < 0.0001$. Control: RAW264.7 cells with RANKL; IL-6: RAW264.7 cells with RANKL and IL-6 treatment; Inf-γ: RAW264.7 cells with RANKL and interferon-γ treatment.

RAW264.7 with or without the induction of conditioned medium. Our data revealed cell number changes in a biphasic manner. Figure 2a showed that the macrophage derived pro-inflammatory cytokines induced transient increase of cell number during the first two days of the treatment (the viability rate was 129%, $p = 0.0043$, n = 4), and induced cell loses thereafter. Until the fourth days after the exposure, the viable cells returned to the levels comparable to that of the control group. The time line is similar to the changes induced by IL-6. After six days of exposure, cell viability drastically dropped to 71% viability ($p = 0.0082$, n = 4). The expression levels of cell cycle marker Ki67 was determined to confirm whether cell proliferation was enhanced. Ki67 gene was transcriptionally upregulated 3 h after IL-6 stimulation ($p = 0.0334$, n = 9), but downregulated after 24 h exposure ($p = 0.0214$, n = 9, Fig. 2b). Both data suggested that the cell proliferation was immediately but transiently stimulated, and the promotion of cell proliferation ceased within 2 days. Longer treatment resulted in the decrease of cell counts starting from 4 days of incubation. The cell number of the differentiating osteoclasts further dropped from the six days, possibly due to lower cell survival after the proliferation-promoting phase.

M1 genes were upregulated in the RANKL treated macrophages in the inflammatory environments

As monocytic lineage cells, osteoclasts presumably could be activated by the pro-inflammatory cytokines. To examine whether pro-inflammatory signaling was upregulated by inflammatory milieu, the activation of NF-κB pathway was first determined. Figure 3a demonstrated that phosphorylated IKK-αβ levels transiently elevated upon condition medium stimulated ($p = 0.0376$, n = 3). The upregulation did not sustain longer than 2-h of incubation. The expressions of pro-inflammatory genes were also measured. Figure 3b showed that the expression of inflammatory mediator genes, such as *Tnf-a* and nitric oxide synthase 2 (*Nos2*) also transiently upregulated (*Tnf-a*: $p < 0.0001$(3 h), $p < 0.0001$(24 h), n = 3; *Nos2*: $p < 0.0001$(3 h), $p = 0.0103$(24 h), n = 3). It has been previously reported that the secretion of pro-inflammatory cytokines activate osteoclast and promote bone resorption. Our results revealed that pro-inflammatory signaling was transiently upregulated, and possibly stimulate the function and differentiation of osteoclasts.

Pro-inflammatory cytokines ectopically activated extracellular signal-regulated kinase (ERK) and serine/threonine kinase 1 (AKT) during the RANKL-induced differentiation

In addition to NF-κB pathway, ERK and AKT are also important signal pathways involving in osteoclast differentiation. Figure 4a and b demonstrated that ERK and AKT were both activated under M1 macrophage-derived inflammatory milieu. Similar to the activation status of NF-κB pathway, the phosphorylation levels of ERK signaling transiently upregulated upon the exposure to inflammatory milieu and the activation decreased after 2 h of incubation (0.5 h: $p < 0.0001$, n = 3). In comparison, Akt persisted in high levels for at least 48 h (0.5 h: $p = 0.0048$; 2 h: $p = 0.0001$; 6 h: $p = 0.0486$; 24 h: $p = 0.0007$, n = 3). The activation of both ERK and AKT are essential for the expression of critical osteoclastogenic regulator *c-myc*, *c-fos*, which in turn upregulate the expression of master regulator *Nfat1c*. Therefore, the expression levels of *c-myc*, *c-fos* and *Nfat1c* genes were also determined. Our data showed that the expressions of *c-myc* were significantly upregulated at both 3-h and 24-h exposure to inflammatory environments (*c-myc*: $p < 0.0001$(3 h), $p < 0.0001$(24 h), n = 3, Fig. 4c), which was correlated with the phosphorylation levels of AKT. On the other hand, the expression of *c-fos* was not modified at either time point. The expression of *Nfat1c* was observed to decrease following a three-hour exposure to an inflammatory environment, before returning to a level comparable to that of the control group following a 24-h exposure. (*Nfat1c*: $p = 0.0274$(3 h), n = 3, Fig. 4d and e). Our results suggested that the persistence of

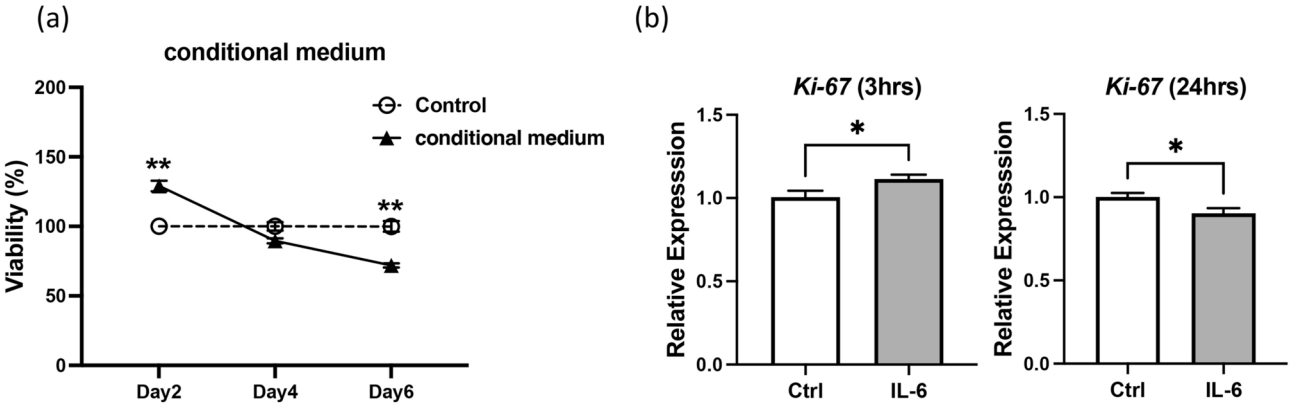

Fig. 2. During RANKL-induced osteoclastogenesis, the inflammatory milieu derived from M1 macrophages induced changes in cell numbers in a biphasic manner. The effect of conditional medium on cell viability in preosteoclasts for 2, 4 and 6 days separately (**a**). Each bar represents the mean ± standard error of the mean of at least three independent experiments. $^{**}p < 0.01$. Control: RAW264.7 cells with RANKL; conditional medium: RAW264.7 cells with RANKL and conditional medium. The *Ki-67* gene expression of preosteoclasts treated with RANKL and with/without IL-6 treatment for 3 and 24 h (**b**). Each column represents the mean ± standard error of the mean of at least three independent experiments. $^{*}p < 0.05$. Ctrl: RAW264.7 cells with RANKL; IL-6: RAW264.7 cells with RANKL and IL-6 treatment.

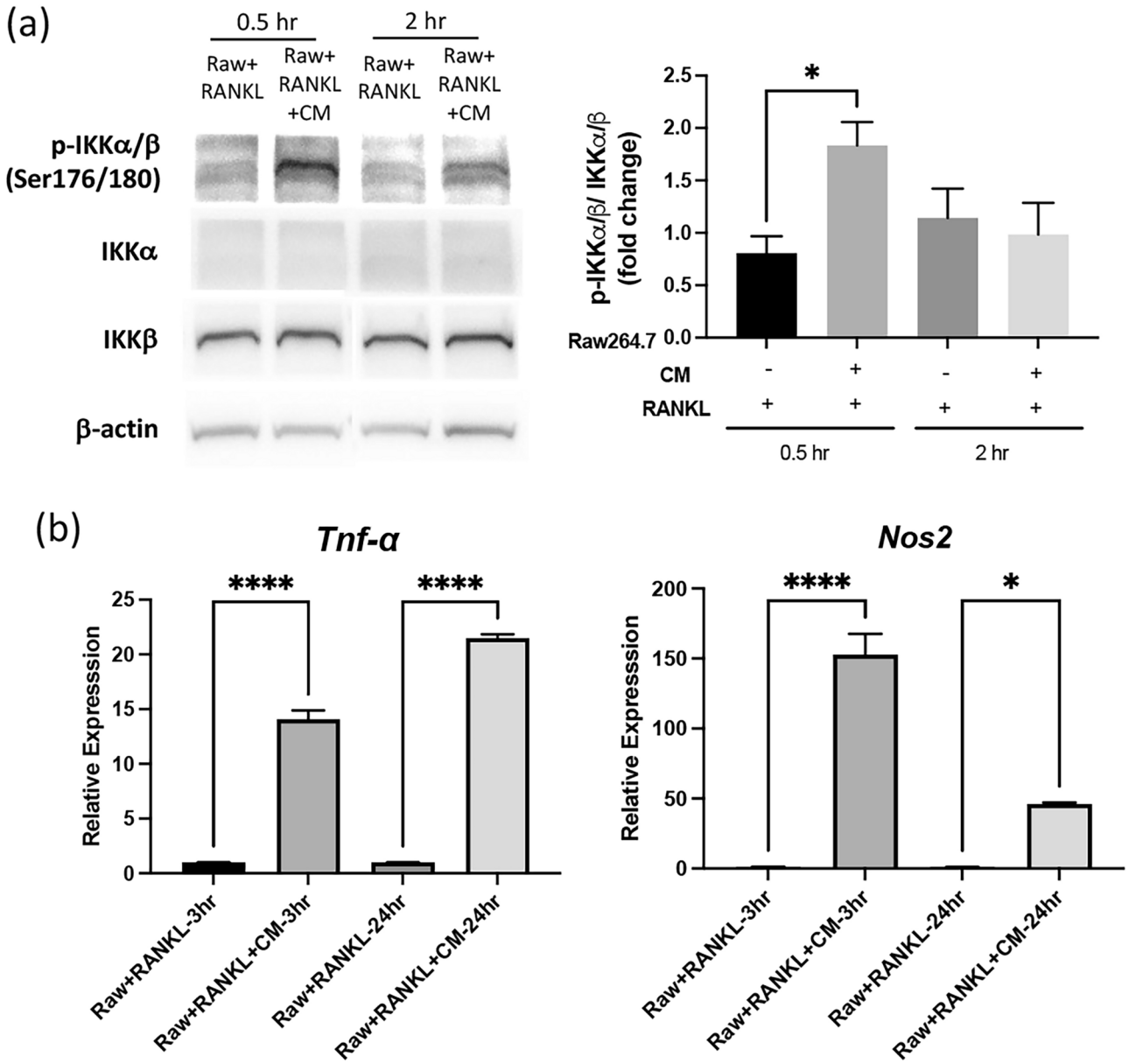

Fig. 3. The M1 genes showed an upregulation in macrophages treated with RANKL in inflammatory environments. The protein expressions of p-IKKα/β, IKKα and IKKβ in preosteoclasts treated with RANKL and with/without conditional medium for 0.5 and 2 h (**a**). Each column represents the mean ± standard error of the mean of at least three independent experiments. *$p < 0.05$. The *Tnf*-α and *Nos2* gene expression of preosteoclasts treated with RANKL and with/without IL-6 for 3 and 24 h (**b**). Each column represents the mean ± standard error of the mean of at least three independent experiments. *$p < 0.05$, ****$p < 0.0001$. CM: conditional medium; *Tnf*-α: tumor necrosis factor-alpha; *Nos2*: nitric oxide synthase 2.

ERK activation might be essential for upregulation of *c-fos* and *Nfat1c*. In addition, bone resorptive functions appeared not being significantly influenced under inflammatory environments.

The expressions of critical bone resorptive genes were temporarily enhanced

Furthermore, the expression of the major bone resorptive gene in differentiating osteoclasts at varying durations under proinflammatory cytokine stimulation was determined. A three-hour exposure to an inflammatory environment resulted in the upregulation of three bone resorptive genes: Cathepsin K (*Ctsk*), *Trap* and matrix metalloproteinase-9 (*Mmp9*) (*Ctsk*: $p = 0.0044$; *Trap*: $p = 0.0044$; *Mmp9*: $p = 0.0198$, n = 3, Fig. 5a). As the expression of all three genes was increased in comparison to their expression in undifferentiated preosteoclasts, the expression of all three genes was further augmented in comparison to those of RANKL-treated differentiating osteoclasts. In contrast, the augmentation of expression was not sustained following prolonged exposure to inflammatory environments (24-h exposure, Fig. 5b). The expression level of *Ctsk* is comparable to that of

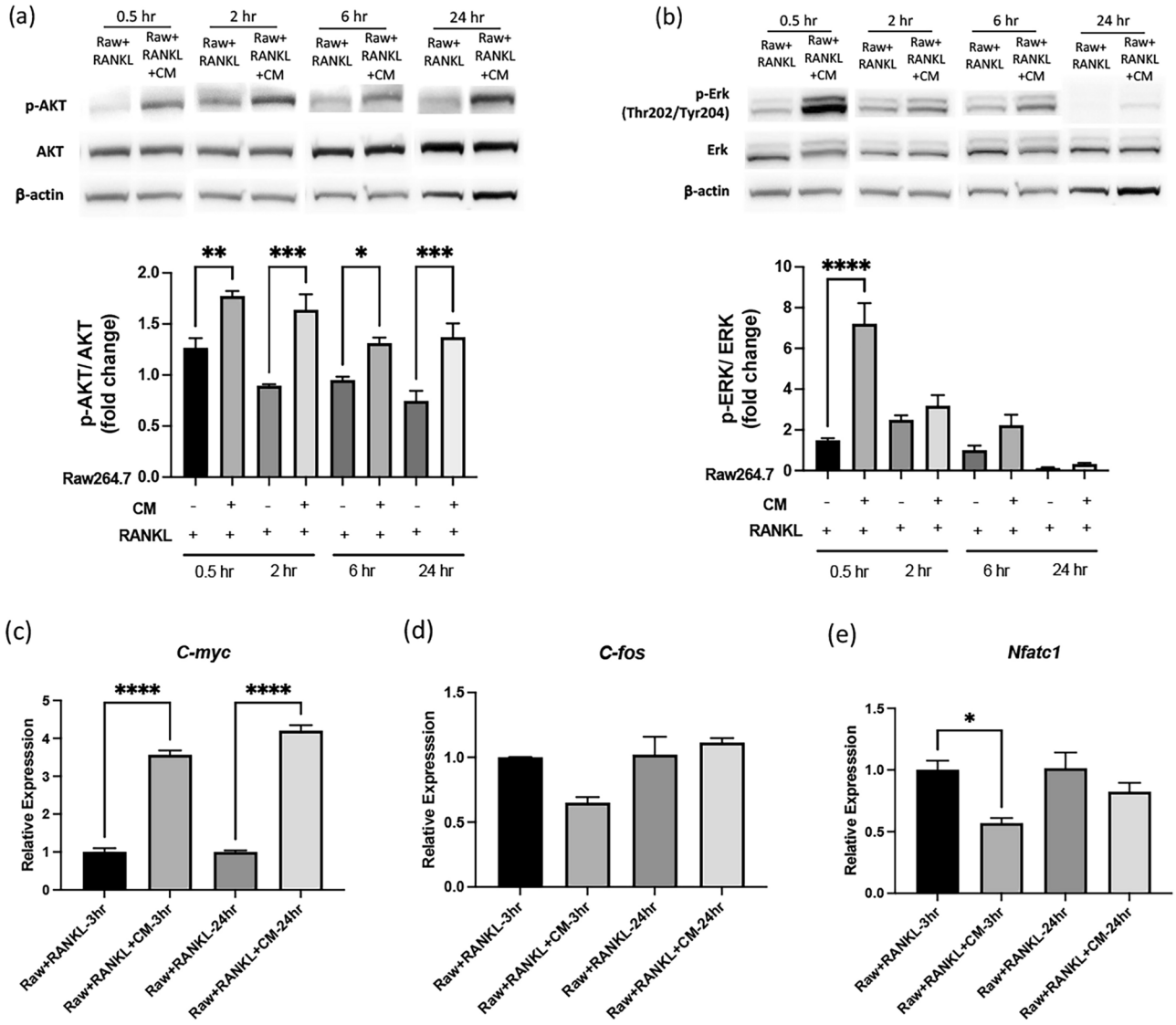

Fig. 4. Pro-inflammatory cytokines cause ectopic activation of ERK and Akt during RANKL-induced differentiation. The protein expressions of p-AKT and AKT in preosteoclasts treated with RANKL and conditional medium for 0.5, 2, 6 and 24 h (**a**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The protein expressions of p-ERK and ERK in preosteoclasts treated with RANKL and conditional medium for 0.5, 2, 6 and 24 h (**b**). ****$p < 0.0001$. The *C-myc* (**c**), *C-fos* (**d**) and *Nfatc1* (**e**) gene expression of preosteoclasts treated with RANKL and with/without conditional medium (CM) for 3 and 24 h separately. Each column represents the mean ± standard error of the mean of at least three independent experiments. *$p < 0.05$, ****$p < 0.0001$. CM: conditional medium; *Nfatc1*: Nuclear Factor of activated T cells 1.

differentiated osteoclasts, while the expression level of *Trap* is lower than that of differentiated osteoclasts (*Trap*: $p = 0.0308$, n = 3, Fig. 5b). However, the expression level of Mmp9 is the same as that at 3-h time point, showing an upward trend (*Mmp9*: $p < 0.0001$, n = 3, Fig. 5b). Our data demonstrated that the inflammatory milieu transiently promoted the expression of bone resorptive genes. As the expression of *Nfat1c* was not elevated, the gene upregulation may be mediated by pro-inflammatory signals.

Long incubation of proinflammatory cytokines triggered apoptosis of macrophages with or without RANKL induction

Our MTT data revealed that cell number of differentiating osteoclast was decreased after long term exposure to the inflammatory environments, indicating that the cell survival was compromised. To further examine whether the apoptosis was promoted, the expression of pro-apoptotic genes BCL2 associated agonist of cell death (*Bad*) and BCL2 associated X (*Bax*) were measured. Figure 6 revealed that the expression of *Bad* and *Bax* were not elevated at Day 2 (Fig. 6a). Consistent with the MTT results, the expression of those two genes were upregulated after 4 days exposure to the inflammatory environments (*Bad*: $p = 0.0167$; *Bax*: $p = 0.0055$, n = 3, Fig. 6b). The results indicated that chronic inflammation triggered apoptosis of the differentiating osteoclasts.

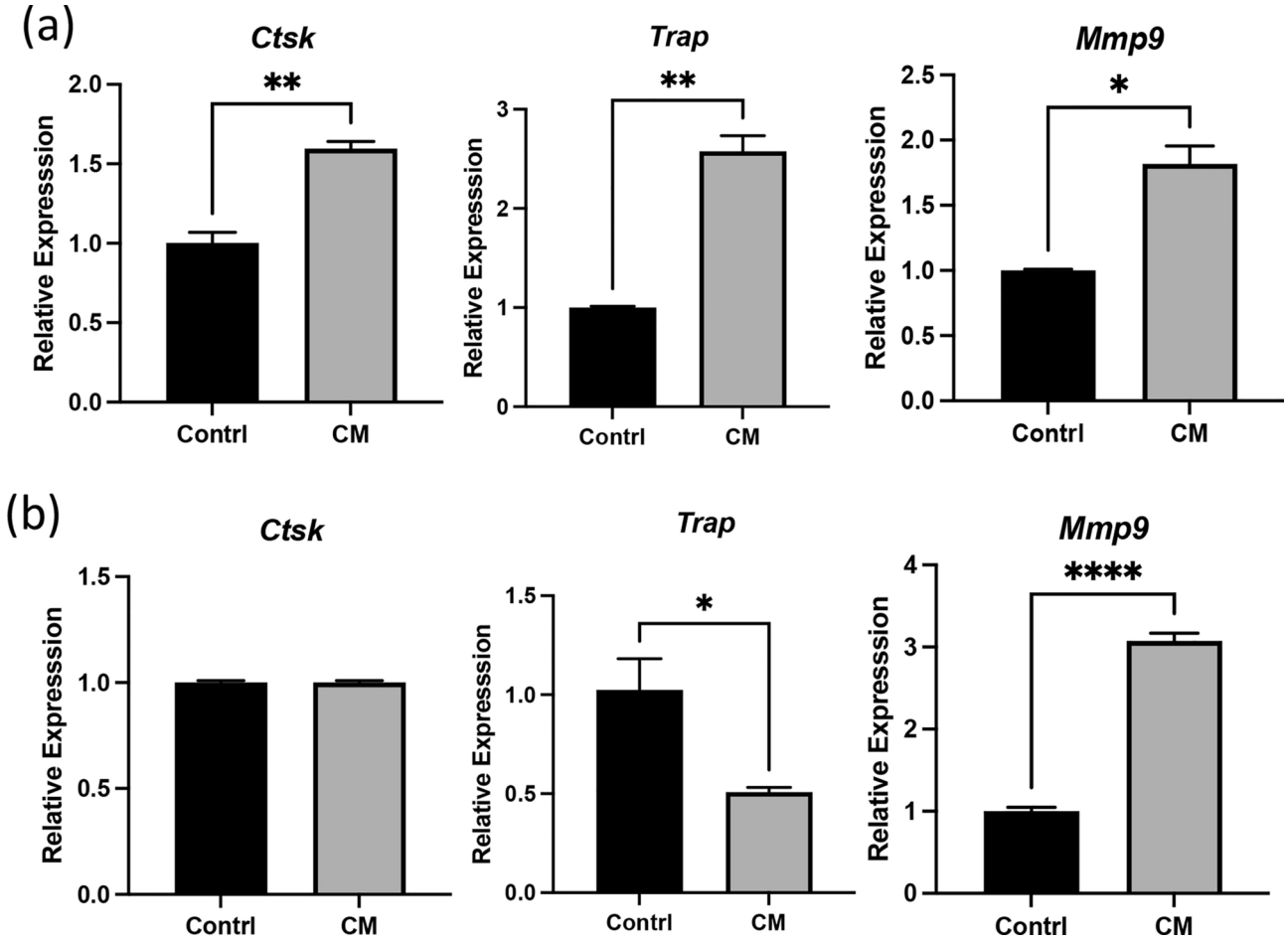

Fig. 5. The expression of critical bone resorption genes was temporarily increased. The *Ctsk, Trap* and *Mmp9* three genes expression of preosteoclasts treated with RANKL and with/without conditional medium (CM) for 3 (**a**) and 24 h (**b**) separately. Each column represents the mean ± standard error of the mean of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. CM: conditional medium; *Ctsk*: Cathepsin K; *Trap*: tartrate-resistant acid phosphatase; *Mmp9*: matrix metalloproteinase-9.

Discussions

Inflammation has been linked to the clinical conditions characterized by bone mass loss, such as osteoporosis and osteopenia. It is possible that these bone diseases are caused by bone cell death under the inflammatory environment. The cytokines in the LPS-primed macrophage conditioned medium with high expression included TNF-α, IL-1β, IL-6, CXCL1, CXCL10 and CCL5. Each of them triggers activation in monocytic lineage, but the actions vary in different type of cells. The activation stimulates cell proliferation. Additionally, many of these cytokines regulates cell proliferation or stimulate inflammatory cell death. Inflammation caused several types of cell death, such as necroptosis or pyroptosis, depending on cell types and conditions. TNF-α could induce pyroptosis through NALP-mediated inflammasome which activates caspase 1 and cleave gasdermin to generate pores on the plasma membrane. In parallel, TNF-α might activate RIPK1, which in turn phosphorylate RIPK3 and MLKL, and lead to necroptosis[30]. Our results revealed that cell proliferation was transiently promoted, as cell cycle marker Ki67 were upregulated. The signaling pathways that involved in enhancing cell proliferation, such as MAPK and PI3K pathways, were both activated. Also, a long exposure to inflammatory milieu triggered cell death of the differentiating osteoclasts. Upregulation of apoptotic pathways genes such as *Bax* and *Bak* indicated apoptosis was promoted in these differentiating osteoclasts. Whether other types of cell death were also elevated need further analysis.

How inflammatory environment regulate osteoclast differentiation positively or negatively remain in debate. Under inflammation environments, differentiated osteoclasts will be activated and bone resorption will be promoted[25]. Since several critical signaling pathways of pro-inflammatory responses are also critical for osteoclast differentiation, such as NF-κB, MAPK and PI3K pathways, inflammation used to be considered as a positive regulator of osteoclastogenesis[26]. Alternatively, Yamaguchi et al. demonstrated that the addition of the RAW264.7-derived M1 macrophage to RANKL-induced RAW264.7 culture reduced the number of TRAP-positive multinuclear osteoclasts. The authors also reported that the soluble factors secreted by M1 macrophages are responsible for the inhibition of osteoclastogenesis[19]. In this study, we found that the responses of differentiating preosteoclast changed with the degree of differentiation. Resembling to the responses of

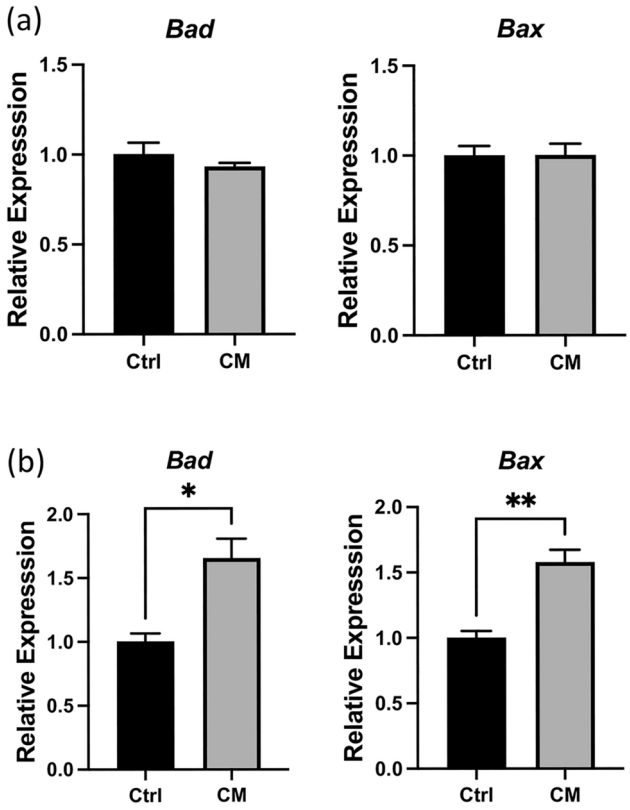

Fig. 6. Prolonged incubation with proinflammatory cytokines induced macrophage apoptosis with or without RANKL induction. The *Bad* and *Bax* genes expression of preosteoclasts treated with RANKL and with/without CM for 3 (**a**) and 24 h (**b**) separately. Each column represents the mean ± standard error of the mean of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. CM: conditional medium; *Bad*: BCL2 associated agonist of cell death; *Bax*: BCL2 associated X, apoptosis regulator.

the monocytic cells, undifferentiated preosteoclasts were stimulated to proliferation and secret more pro-inflammatory mediators. After RANKL-induced differentiation has initiated, the cell features also changed with time. At the beginning the inflammatory milieu positive regulate the expressions of the critical bone resorptive genes. In previous study the M1 macrophage is added to the differentiating osteoclasts after 3 days of RANKL stimulation. Downregulation of the *Ctsk* gene suggested the alternative cellular response in the osteoclasts with higher degree of differentiation. The reduced number of the differentiated osteoclast in the previous study could be attributed to the abrogation of the expression of bone resorptive genes or the induction of apoptosis.

RANKL stimulation initiates a series of changes in both gene expression landscape and cell features. Our previous observations showed that AMP-activated protein kinase (AMPK) interfered osteoclast differentiation and its expression was suppressed during the RANKL-induced osteoclast differentiation, but was induced in inflammatory environments to enhance bone resorption in differentiated osteoclasts[27]. The results demonstrated not only the differences of the regulations and the functions of between undifferentiated and differentiated osteoclasts, but also the complexity of regulatory machinery of osteoclast functions. Recently Toor et al. also reported the differences of transcription profiles between undifferentiated preosteoclast, early osteoclast differentiation and progressive osteoclast differentiation[28]. In early differentiation stage, genes for regulation of development process, cell morphogenesis, regulation of cell differentiation was upregulated, while genes for inflammatory responses, defense response, response to stress and immune system process were downregulated. During the progressive differentiation stage, the genes for generation of precursor metabolites and energy, small molecule metabolic process, organophosphate metabolic process, phosphate-containing compound metabolic process, and nucleotide metabolic process were upregulated, whereas the genes for immune responses stayed downregulated. The alterations imply the switch from the preosteoclast with macrophage-like characteristics to the differentiated osteoclast with metabolic process of various molecules. The changes of the cellular properties could also influence the cellular responses to the inflammatory mediators. Our results suggested that the inflammatory mediators induced the apoptotic responses in progressive differentiated osteoclasts.

It has been reported that distinct murine osteoclast precursor subsets or myeloid lineages respond differently to pro-inflammatory cytokines or the osteoclastogenesis-inducing cytokines[29]. For example, interleukin-1β stimulated proliferation of early blasts but enhanced bone resorption in multinucleated differentiated osteoclasts. Life span also varied among different subsets of osteoclasts or monocytic lineages. Alternatively, IFN-γ inhibits the early differentiation of osteoclasts by targeting the RANK–RANKL pathway, and. IFN-γ indirectly increases osteoclastic factors by activating immune responses[6]. Our results showed that the combination of pro-

inflammatory mediators induced the promotion of the pro-inflammatory responses, but possibly inhibited cell proliferation and bone resorption.

Data availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

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Author contributions

CSW and CYC carried out experiments. CHY and YPH analyzed the experimental results. CHY carried out data collection. YHC and SKC conceived, designed, coordinated the study, and prepared and reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chengchi University. All of the animal experiments were conducted in accordance with the IACUC regulations and Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines.

Additional information
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