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Coordinated effect of IL-17A and IL-27 on osteoclast differentiation of RANKL-stimulated RAW264.7 cells

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Orthodontic tooth movement is based on the response of biological tissue to mechanical loading. On both the compressed side and tensile side, stress is loaded onto cells by means of physical stimulation that causes cells to produce various cytokines that are involved in the promotion or suppression of osteoclast formation. During orthodontic tooth movement, alveolar bone resorption at the area of compression occurs through osteoclastic activity. In this study, we confirmed the coordinated effect of IL-17A and IL-27 on receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis, using RAW264.7 cells as osteoclast precursors. High expression of IL-17RC and IL 27-Rα was detected on RAW264.7 cells, and this expression was not altered by the stimulation of RANKL for 24 h. A coordinated effect of IL-17A and IL-27 suppressed osteoclast differentiation from RAW264.7 cells treated with RANKL without reduction of cell proliferation. Furthermore, the phosphorylation of c-Jun-N-terminal kinase (JNK) stimulated with RANKL was inhibited by the coordinated effect of IL-17A and IL-27. These data suggest the possibility that the coordinated effect of IL-17A and IL-27 inhibited the phosphorylation of JNK which is involved in the suppression of RANKL-induced osteoclast differentiation in RAW264.7 cells. Our findings provide new insights into the mechanisms of osteoclastogenesis. (J Osaka Dent Univ 2015 ; 49(1) : 61–68)

Key words : Interleukin-17A (IL-17A) ; Interleukin-27 (IL-27) ; Osteoclast differentiation

INTRODUCTION

Orthodontic treatment is done to obtain optimal occlusion through tooth movement. The treatment may take several years, and stress may occur during this time. Orthodontic tooth movement is associated with both mechanical and psychological stresses that may be associated with pain or discomfort. Measures that reduce the time of orthodontic treatment and accelerate the alveolar bone response would be welcomed by the patient. Tooth movement requires a bone remodeling that consists of bone resorption on the compressed side and bone formation on the tensile side of the tooth. Osteoclasts play a key role in modulating bone mass. During orthodontic tooth movement, alveolar bone resorption at the area of compression occurs through osteoclastic activity. Since osteoclast differentiation/activation is engaged in orthodontic tooth movement at the pressure side,[1] the investigation of osteoclasts is crucial for orthodontics.[2] However, the mechanism of osteoclast differentiation/activation in orthodontic tooth movement remains unknown.

It is well known that two essential cytokines are required for osteoclastogenesis : macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB (NF-κB) ligand (RANKL).[3] M-CSF induces the proliferation of osteoclast precursor cells and sustains their survival.[4] RANKL induces osteolast differentiation from osteoclast precursors and stimulates their bone resorption activity.[5] RANKL was detected in osteoblasts and periodontal ligament cells during experimental tooth movement.[6] RANKL plays a key role in osteoclast differentiation and activation.

RANKL-induced activation of RANK that is expressed on osteoclast precursors initiates the recruitment of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and leads to the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways, including c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38.[7] Furthermore, RANKL-stimulation initiates calcium signaling which is indispensable for the induction of the key transcription factor, nuclear factor of activated T cells (NFAT) c1, for osteoclast differentiation.[8] Then, NFATc1 regulates the expression of genes required for osteoclast differentiation, such as calcitonin receptor (CTR), tartrate resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP 9) and cathepsin K, which participate in the osteoclast phenotype and lead to the formation of bone resorption pits.[9]

Interleukin-17A (IL-17A) was originally identified as a characteristic cytokine secreted by activated T cells, i.e., Th 17 cells.[10] Since the identification of IL-17 A in 1995,[11] five other members of this family, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, have been identified based on amino acid sequence homology.[12] The IL-17 family consists of a subset of cytokines that participate in both acute and chronic inflammatory responses. The IL-17 receptor family, which consists of 5 members (IL-17RA, IL-17RB, IL-17RC, IL- 17RD and IL-17RE), is also identified by the typical structure homology among the members.

IL-17 receptors have been found on several cells and tissues.[3] IL-17A transduces the signals through a heterodimeric receptor complex composed of IL-17 RA and IL-17RC.[13] It has been reported that IL-17A induces RANKL production by osteoblasts.[14] In addition, recent evidence has indicated that IL-17A stimulates RANKL gene expression in osteoblasts and induces osteoclast differentiation in co-cultures of osteoblasts and bone marrow cells.[3] Recently, we investigated the direct effects of IL-17A on the differentiation of osteoclastogenesis using RAW264 cells as osteoclast precursors. The findings indicated that the differentiation was suppressed by the direct effects of IL-17A. Furthermore, IL-17A inhibits the phosphorylation of p38, which is a factor that suppresses the differentiation of osteoclastogenesis on RAW264 cells.[15] The details of this difference have not been well studied. However, it is clear that IL-17A is a crucial cytokine for osteoclastogenesis via the RANK-RANKL system.

Interleukin-27 (IL-27) is a recently discovered member of the IL-12 family of heterodimeric cytokines that also includes IL-12, IL-23 and IL-35.[16] IL-27 is made up of the p 28 subunit and the Epstein-Barr virus induced gene-3 (EBI-3) subunit.[17] IL-27 binds to a heterodimeric receptor that is comprised of the IL-27 Rα subunit (also termed WSX-1) and the gp 130 subunit. IL-27 Rα is the type I cytokine receptor family that is expressed in mast cells, endothelial cells, NK cells, macrophages, monocytes, B cells, dendritic cells, and native T cells.[18] The other subunit, gp 130 is also a type I cytokine receptor family. It is a ubiquitously expressed receptor chain that is shared with IL-6-family cytokines.[19] IL-27 is mainly produced by activated macrophages and dendritic cells.[20] IL-27 is known to have multifaceted actions during immune responses, with both activating and regulatory roles.[20] More recently, IL-27 has been shown to abrogate RANKL-mediated osteoclastogenesis through signal transducer and activator of transcription 1 (STAT1)-dependent inhibition of c-Fos.[17] It has also been reported that IL-27 inhibits human osteoclastogenesis by a direct mechanism that represses the responses of osteoclast precursors to RANKL.[16, 21]

Previous studies reported the direct effect of RANKL-induced osteoclast differentiation from osteoclast precursors in IL-17A or IL-27 alone. However, the mechanism of the coordinated effect of IL-17A and IL-27 on osteoclastogenesis has not been reported. In this study, we investigated this coordinated effect on RANKL-induced osteoclastogenesis and the molecular mechanisms of JNK in RAW264.7 cells.

MATERIALS AND METHODS

Cell culture

We used the murine monocyte/macrophage cell line RAW264.7 cells as osteoclast precursors that were obtained from DS Pharma Biomedical, Osaka, Japan. RAW264.7 cells were cultured in minimal essential medium alpha modification (α-MEM) (Wako Pure

Chemicals, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 μg/mL penicillin, 100 μg/mL streptomycin and 2 mM L-Glutamine (Wako Pure Chemicals). RAW264.7 cells were incubated at 37°C under 5% CO_2. Accutase (Innovative Cell Technologies, San Diego, CA, USA) was used to detach the cells.

Antibodies and regents

Anti-IL-17RC antibody and anti-IL-27 Rα antibody were obtained from R&D Systems, Minneapolis, MN, USA. Alexa Fluor 488 chicken anti-goat IgG (H + L) and Alexa Fluor 488 goat anti-rat IgG (H + L) were obtained from Invitrogen, Eugene, OR, USA. Anti-phospho-JNK antibody, anti-JNK antibody and anti-Actin were obtained from Santa Cruz Biotechnology, Dallas, TX, USA. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, Germany). Recombinant mouse RANKL was purchased from Wako Pure Chemicals. Recombinant mouse IL-17A was purchased from PeproTech, Rocky Hill, NJ, USA. Recombinant mouse IL-27 was purchased from R&D systems.

Flow cytometry

Expression of surface antigens was measured by flow cytometry. RAW264.7 cells were cultured in α-MEM in the presence or absence of RANKL (10 ng/mL) for 24 h. The cells were incubated at 4°C for 1 h with primary antibodies (Mouse IgG, IL-17RC and IL-27 Rα). The cells were washed, then incubated with a 2nd antibody (Alexa Fluor 488 chicken anti-goat IgG (H + L) or Alexa Fluor 488 goat anti-rat IgG (H + L)) at 4°C for 30 min in the dark. The cells were washed and analyzed using a FACSCalibur (BD Biosciences, Mountain View, CA, USA).

TRAP staining

RAW264.7 cells were seeded at 3×10^3 cells/well on a 96-well plate and incubated for 72 h in α-MEM containing 10% FBS, 20 ng/mL of IL-17A and/or 20 ng/mL of IL-27 in the presence of 10 ng/mL of RANKL. Osteoclast formation was assessed by TRAP staining. The cells were fixed with 10% formalin solution in phosphate buffered saline (PBS) for 1 min and 1% Triton X-100 in PBS for 30 sec. The fixed cells were treated with the TRAP staining solution for 10 min and observed under a light microscope. TRAP-positive cells with more than three nuclei were considered to be osteoclast-like cells. The osteoclast-like cells of each well were counted. Five wells were used for each condition, and the mean number of cells for each condition were recorded.

Cell proliferation assays

RAW264.7 cells were seeded at 3×10^3 cells/well on a 96-well plate and incubated for up to 72 h in α-MEM containing 10% FBS, 20 ng/mL of IL-17A and/or 20 ng /mL of IL-27 in the presence of 10 ng/mL of RANKL. Cell proliferation reagent WST-1 (Roche Diagnostics, Basel, Switzerland) was used to assess cell proliferation. Absorbance was measured at 450/650 nm using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

RAW264.7 cells (1×10^6 cells/sample) were treated with 20 ng/mL of IL-17A and/or 20 ng/mL of IL-27 in the presence of 10 ng/mL of RANKL for 15 min. Cells were solubilized with lysis buffer containing 50 mM Tris-HCl at pH 7.6, 0.5% TX-100, 300 mM NaCl, 5 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate by gentle rocking for 30 min at 4 °C. Insoluble material was removed by centrifugation and the supernatants were subjected to SDS-PAGE for Western blotting. Cell lysates were eluted by boiling in SDS-containing sample buffer. Equal amounts of each sample were subjected to 7% SDS/PAGE and transferred to PVDF membrane (Merck Millipore).

Gels were run at 150 V for 90 min. The membrane blocking was with 5% BSA (Nacalai, Kyoto, Japan) in TBS-T at 4°C overnight. The membrane was treated with anti-phospho-JNK for 1 h, and washed with TBS -T. The membrane was treated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody for 30 min, and washed with TBS-T. After incubation with antibodies, phosphorylated protein were de-

tected by Immobilon Western Chemiluminescent HRP Substrate for 2 min. The images were analyzed using ChemiDoc MP (Bio-Rad, Hercules, CA, USA). The membranes were stripped and reprobed with anti-JNK antibody. The results revealed that equal amounts of JNK were created by lysates obtained from each sample.

RESULTS

IL-17RC and IL-27 Rα expression not altered after RANKL stimulation on RAW264.7 cells

We first evaluated the expression of IL-17 R subunit IL-17RC and IL-27 R subunit IL-27-Rα on RAW264.7 by flow cytometry. As shown in Figs. 1 A and C, high IL-17RC and IL-27 Rα expression was detected on RAW264.7 cells. Next, we tested whether RANKL-stimulation could modulate IL-17RC and IL-27 Rα expression on RAW264.7 cells. Using the FACS analysis, RAW264.7 cells stimulated with RANKL at 10 ng /mL for 24 h were evaluated for the expression of IL-17RC and IL-27 Rα. IL-17RC and IL-27 Rα expression in RANKL-stimulated RAW264.7 cells was not altered above that compared with the expression of non-stimulated RAW264.7 cells (Figs. 1 B and D). These findings suggest that RANKL stimulation was not be involved in modulating the expression of IL-17 RC and IL-27 Rα.

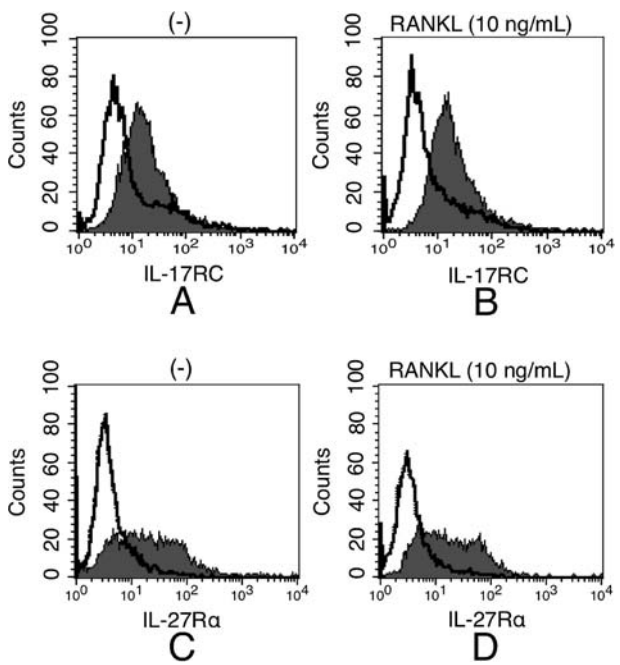

Fig. 1 Surface expression of IL-17 RC and IL-27 Rα on RAW 264.7 cells by flow cytometry. The cells were seeded at 1×10^5 cells/well on a 12-well plate and incubated for 24 h in the presence (B and D) or absence (A and C) of 10 ng/mL of RANKL. RAW 264.7 cells were stained with mAb specific for anti-IL 17RC (A and B closed histogram) and anti-IL-27 Rα (C and D closed histogram). Staining with anti-mouse IgG as a control is shown as an open histogram for ease of comparison.

Coordinated effect of IL-17A and IL-27 on RANKL-induced osteoclast differentiation of RAW 264.7 cells

To evaluate how the coordinated effect of IL-17A and IL-27 may control osteoclast physiology, we investigated its action on RAW264.7 cells. TRAP is a well-known enzyme that is widely acccepted as a histochemical marker of osteoclasts. TRAP activity can be significantly elevated by RANKL stimulation.[22] We investigated the coordinated effect of IL-17A and IL-27 on RANKL-induced osteoclast differentiation in RAW 264.7 cells. RAW264.7 cells were cultured in the presence of RANKL with the indicated concentration of IL-17A and/or IL-27. The cells were TRAP stained after 72 h and observed by light microscopy. The enzymatic TRAP activity measured at the end of the differentiation process was increased by RANKL stimulation in RAW264.7 cells. RAW264.7 cells were previously reported to differentiate into osteoclast-like TRAP-positive multinuclear cells on treatment with 10 ng /mL RANKL (Figs. 2 A and B). When IL-17A or IL-27 was treated alone in the culture with RANKL, it slightly decreased the RANKL-induced osteoclast differentiation in RAW264.7 cells under the conditions of this study (Figs. 2 A and B). However, the coordinated effect of IL-17A and IL-27 decreased the RANKL-induced osteoclast differentiation in RAW264.7 cells (Figs. 2 A and B). These results confirmed the potential of the coordinated effect of IL-17A and IL-27 to suppress osteoclast differentiation in RAW264.7 cells.

Coordinated effect of IL-17A and IL-27 on RAW 264.7 cell proliferation

To exclude the possibility that inhibition of the coordinated effect of IL-17A and IL-27 on TRAP staining was due to cytotoxicity, the viability of RAW264.7 cel-

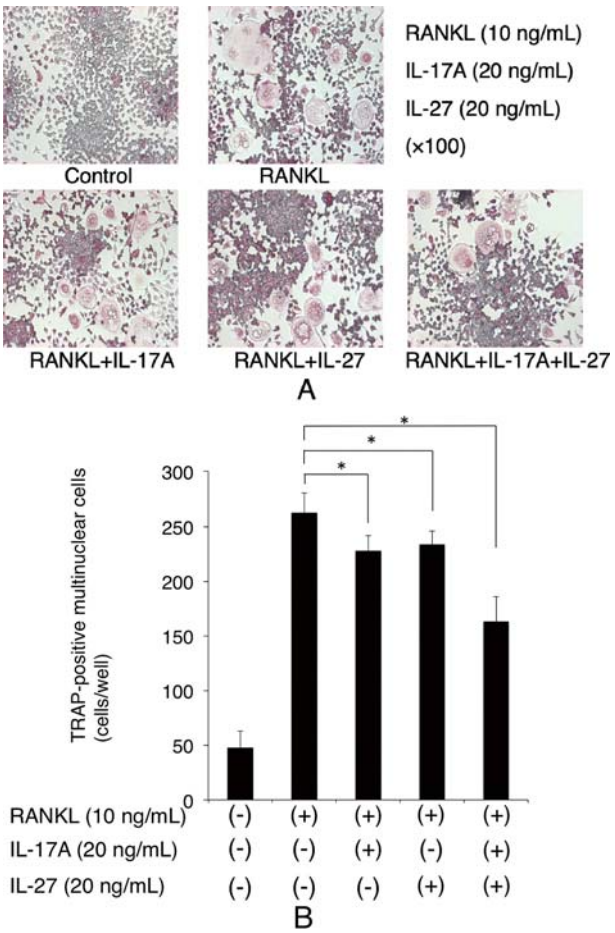

Fig. 2 Coordinated effect of IL-17A and IL-27 on osteoclast differentiation in RANKL-stimulated RAW264.7 cells. RAW264.7 cells were cultured at 3×10^3 cells/well with 10 ng/mL of RANKL at the indicated concentration of IL-17A and/or IL-27 for 72 h. After induction, the cells were fixed and subjected to TRAP staining (A). The TRAP-positive, multinucleated osteoclast cell number was counted (B). All the bars represent mean ± SD from five independent experiments. The significance was determined by Student's t-test (*$p < 0.05$ compared with the RANKL-stimulated group).

ls in the presence of RANKL was tested using cell proliferation reagent WST-1. We found that IL-17A and/or IL-27 did not mediate cytotoxicity or reduce growth on RAW264.7 cells at the concentrations that suppressed osteoclast formation (Fig. 3). Therefore, we used IL-17A and/or IL-27 at 20 ng/mL in the subsequent experiments.

Coordinated effect of IL-17A and IL-27 inhibited RANKL-induced phosphorylation of JNK in RAW 264.7 cells

RANKL is known to activate MAPKs (JNK, ERK and

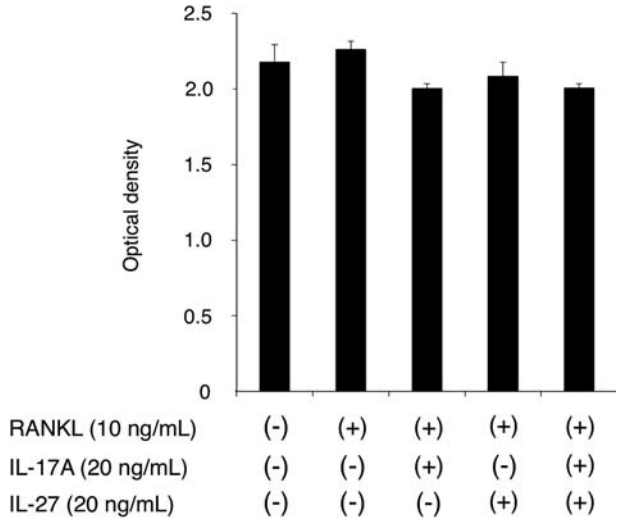

Fig. 3 Effect of IL-17A and IL-27 on proliferation of RAW264.7 cells. The cells were seeded at 3×10^3 cells/well on a 96-well plate and incubated for 72 h with 20 ng/mL of IL-17A and/or 20 ng/mL of IL-27 in the presence of 10 ng/mL of RANKL. Cell proliferation was assessed by measuring absorbency at 440/650 nm.

p38), which play important roles in the differentiation and formation of osteoclasts from osteoclast precursor cells. The NFATc1 expression by RANK stimulation is dependent on RANKL-induced activation of the MAPK pathways.[23, 24] Therefore, we studied whether the coordinated effect of IL-17A and IL-27 inhibits RANK signaling. To determine the intracellular mechanism by which osteoclast differentiation is inhibited by the coordinated effect of IL-17A and IL-27, we assessed the effects of IL-17A and/or IL-27 on RANKL-induced phosphorylation of JNK in RAW 264.7 cells using immunoblotting. The JNK activation states were determined by immunoblotting using antibodies specifically directed against the phosphorylated forms of JNK.

Consistent with previous results, we found that RANKL stimulation induced a marked phosphorylation of JNK compared with the situation of unstimulated RAW264.7 cells. When IL-17A or IL-27 was used alone in the culture with RANKL, it did not significantly decrease the RANKL-induced phosphorylation of JNK in RAW264.7 cells (Fig. 4 upper panel). But, the phosphorylation of JNK stimulated with RANKL was inhibited by the coordinated effect of IL-17A and IL-27 (Fig. 4 upper panel). To ensure that equal amounts of JNK were obtained from the lysates, the mem-

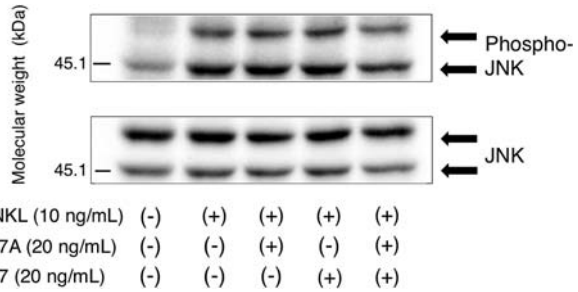

Fig. 4 Coordinated effect of IL-17A and IL-27 inhibited RANKL-induced phosphorylation of JNK in RAW264.7 cells. RAW264.7 cells were treated at 1×10^6 cells/sample with the indicated concentration of IL-17A and/or IL-27 in the presence of RANKL at 10 ng/mL for 15 min. Western blot analysis for phospho-JNK was performed. The phosphorylation of JNK stimulated with RANKL was inhibited by the coordinated effect of IL-17A and IL-27 (upper panel). To ensure that equal amounts of JNK were obtained from the lysates, the membranes were stripped and reprobed with anti-JNK antibody. Equal amounts of JNK were applied from lysates obtained from each sample (lower panel).

branes were stripped and reprobed with anti-JNK antibody. The results revealed that equal amounts of JNK were created by lysates obtained from each sample (Fig. 4 lower panel).

DISCUSSION

A goal of orthodontic treatment is to rapidly and safely move a tooth to the optimal position and finally to obtain optimal occlusion. Orthodontic tooth movement is based on the reaction of biological tissue to a mechanical force. The movement occurs as a result of alveolar bone remodeling through a long duration of the optimum force. During orthodontic tooth movement, bone resorption is induced at the compressed side and bone formation at the tensile side, moving the tooth gradually into the optimal position. Osteoclasts are formed on the compressed side of the orthodontic tooth, causing resorption of the alveolar bone.[25] Osteoclastogenesis is primarily regulated by the two cytokines RANKL and M-CSF. RANKL induces osteoclast differentiation from osteoclast precursors and stimulates their bone resorption activity. Thus, RANKL plays a key role in osteoclastogenesis and activation.

RANKL was detected in osteoblasts and periodontal ligament cells during experimental tooth movement. On both the compressed and tensile sides, stress is loaded on cells by means of physical stimulation, and these cells produce various cytokines that function as inflammatory factors during the process of adaptation to the stress and/or defense mechanisms against the stress.[25] Various cytokines are involved in the promotion or suppression of osteoclast formation. For example, IL-1, IL-6 and TNF-α act as a positive regulator of RANKL-mediated osteoclastogenesis, while IL-4, IL-10 and INF-γ act as negative regulators of RANKL-mediated osteoclastogenesis.[26] In this study, we investigated the coordinated effect of IL-17 A and IL-27 on RANKL-induced osteoclastogenesis, using RAW264.7 cells as osteoclast precursors. We first confirmed that high expression of the IL-17 R subunit IL-17RC and the IL-27 R subunit IL-27 Rα was detected on RAW264.7 cells. Our study indicated that IL-17A binds to IL-17RC and IL-27 binds to IL -27 Rα on RAW264.7 cells.

We examined the coordinated effect of IL-17A and IL-27 on the differentiation of osteoclast precursors in the presence of soluble RANKL using RAW264.7 cells. Adding soluble RANKL to the culture medium is indispensable for differentiation of osteoclasts on RAW264.7 cells.[27] In the present study, we found that the coordinated effect of IL-17A and IL-27 markedly suppressed the RANKL-induced osteoclast differentiation in RAW264.7 cells. Furthermore, we investigated the effect of RANKL on IL-17RC and IL-27 Rα expression in RAW264.7 cells. IL-17RC and IL-27 Rα expression in RANKL-stimulated RAW264.7 cells was not altered above that which occurred with the expression of non-stimulated RAW264.7 cells. Moreover, we found that IL-17A and/or IL-27 did not affect the proliferation of RAW264.7 cells. These results suggest that osteoclast differentiation suppressed by the coordinated effect of IL-17A and IL-27 was not based on the inhibition of cell proliferation and suppression of cytokine receptor (IL-17RC and IL-27 Rα) expression. Thus, we hypothesized that the coordinated effect of IL-17A and IL-27 inhibited the RANKL-induced osteoclast differentiation pathway.

MAPKs are serine/threonine kinases that activate an early intracellular response in many physiological processes. Three major subfamilies of MAPK have been identified : JNK, ERK and p38. It has been reported that MAPK is activated by RANKL and is asso-

ciated with osteoclastogenesis.[28] Previous studies indicated that JNK and p38 play an important role in osteoclast formation, because the treatment of cells with JNK or p38 specific inhibitors suppressed RANKL-induced osteoclastogenesis.[29] Moreover, dominant negative JNK abrogated RANKL-induced osteoclastogenesis.[29] In this study, we evaluated the coordinated effect of IL-17A and IL-27 on the activation of JNK and found that this coordinated effect inhibited the phosphorylation of JNK. These results demonstrated that phosphorylation of JNK may contribute to inhibiting osteoclastogenesis by the coordinated effect of IL-17A and IL-27 in RANKL-stimulated RAW 264.7 cells.

In summary, we demonstrated that the coordinated effect of IL-17A and IL-27 reduced the RANKL-induced osteoclast differentiation in RAW264.7 cells as osteoclast precursors. We also found molecular mechanisms for this reduction. The coordinated effect of IL-17A and IL-27 attenuated RANKL-induced phosphorylation of JNK. Our findings present new insights into mechanisms of the coordinated effect of IL-17A and IL-27 induced inhibition of osteoclastogenesis. Therefore, we suggest that this information may make it possible to control orthodontic tooth movement and may have other useful clinical applications.

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