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ORIGINAL ARTICLE

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Effects of IL-23 and IL-27 on osteoblasts and osteoclasts: inhibitory effects on osteoclast differentiation

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Abstract Interleukin (IL)-23 and IL-27 are IL-6/IL-12 family members that play a role in the regulation of T helper 1 cell differentiation. Cytokines are known to be involved in the bone remodeling process, although the effects of IL-23 and IL-27 have not been clarified. In this study, we examined the possible roles of these cytokines on osteoblast phenotypes and osteoclastogenesis. We found that IL-27 induced signal transducers and activators of transcription 3 activation in osteoblasts. However, neither IL-23 nor IL-27 showed any significant effects on alkaline phosphatase activity, receptor activator of nuclear factor κB ligand (RANKL) expression, mRNA expression such as alkaline phosphatase type I procollagen, or the proliferation of osteoblasts. Osteoclastogenesis from bone marrow cells induced by soluble RANKL was partially inhibited by IL-23 and IL-27 with reduced multinucleated cell numbers, but these interleukins did not affect the proliferation of osteoclast progenitor cells. These results indicate that IL-23 and IL-27 could partly modify cell fusion or the survival of multinucleated osteoclasts. On the other hand, partially purified T cells, which are activated by 2 μg/ml anti-CD3 antibody, completely inhibited osteoclastogenesis by M-CSF/RANKL. On using T cells activated with 0.2 μg/ml anti-CD3 antibody, in which osteoclastogenesis was partially inhibited, the interleukins had additive effects for inhibiting osteoclastogenesis. Although the consequences of phosphorylated signals in osteoblasts have not been identified, IL-23 and IL-27, partly and indirectly through activated T cells, inhibited osteoclastogenesis, indicating that these interleukins may protect against bone destructive autoimmune disorders.

Key words osteoblasts · osteoclasts · cytokines · interleukin-23 · interleukin-27

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Introduction

It is well recognized that the gp130 family of cytokines, including interleukin (IL)-6/soluble IL-6 receptor (sIL-6R), IL-11, leukemia inhibitory factor, and oncostatin M, uses the common receptor subunit gp130 to transduce signals, and that these cytokines play an important role in osteoclastogenesis through the action of osteoblasts [1,2]. An experiment using dominant–negative (dn)-gp130 and dn-signal transducers and activators of transcription (STAT) 3 revealed that activation of STAT 3 via gp130 in osteoblasts is required for induction of the receptor activator of nuclear factor κB ligand (RANKL) and osteoclastogenesis [3].

Recently, a novel member of the IL-6/IL-12 family of cytokines was identified and termed IL-27 [4]. IL-27 is a heterodimeric cytokine that consists of an IL-12 p40-related protein, Epstein–Barr virus-induced gene 3, and a newly discovered IL-12 p35-related protein, p28 [4]. IL-27 receptor (R) is composed of the orphan cytokine receptor WSX-1/T cell cytokine receptor, which is homologous to the IL-12R subunit, and gp130 [4,5]. IL-27 performs several functions: it activates Janus kinase (JAK) 1/2, tyrosine kinase (TYK) 2, STAT1/2/3/4, and STAT5; it induces proliferation of naive CD4+ T cells, expression of T-bet, a key molecule in T helper type 1 (Th1) cell lineage commitment, and IL-12Rβ2 subunits; and it synergizes with IL-12 in interferon (IFN)-γ production in CD4+ or CD8+ naive T cells [6–9]. We have recently demonstrated that IL-27 showed an antitumor activity with less adverse cytotoxicity than IL-12, which is mainly mediated by CD8+ T cells with enhanced cytotoxic T lymphocyte activity [9–11] and by antiangio-

genic activity [12]. Recently, IL-27 was reported to negatively regulate the development of IL-17-producing T helper (Th-17) cells [13,14]. IL-17 that induces RANKL expression via induction of prostaglandin E_2 production in osteoblasts has been shown to be released by IL-23 stimulation [15,16]. Thus, IL-27 contributes to the differentiation of CD4+ T helper type 1 (Th1) cells and Th-17 cells and modulation of CD8+ T cell function.

IL-23, which is also a member of the IL-6/IL-12 family of cytokines, consists of a heterodimer of an IL-12 p40 protein and a newly discovered IL-12 p35-related protein, p19 [17]. IL-23 activates JAK2, TYK2, STAT1/3/4, and STAT5, and induces both proliferation and IFN-γ production of memory Th1 cells via specific IL-23R heterodimers: the IL-12Rβ1 subunit and the IL-23R subunit [18].

Although the gp130 family of cytokines plays an important role in osteoclastogenesis, the effects of IL-27 and its structurally related protein IL-23 on bone remodeling have not been investigated. In this study, we examined the possible roles of these cytokines on osteoblast phenotypes and osteoclastogenesis induced by a co-culture system of osteoblasts and bone marrow cells. We further tested whether IL-23 and IL-27 affected osteoclastogenesis from bone marrow cells induced by monocyte–macrophage colony stimulating factor (M-CSF)/soluble RANKL (sRANKL). We found that IL-27 induced STAT1 and STAT3 activation in osteoblasts and bone-marrow-derived macrophage-like cells, although the consequences of phosphorylated signals in osteoblasts have not been identified. We also found that both IL-23 and IL-27 inhibited osteoclastogenesis from bone marrow cells induced by M-CSF/sRANKL in the presence or absence of activated T cell fractions.

Materials and methods

Animals and reagents

The ddY mice were obtained from Japan SLC Co., Shizuoka, Japan. All procedures for animal care were approved by the Animal Management Committee of Josai International University (approval number 6). Recombinant murine single-chain IL-23 and IL-27 were prepared from plasmid constructs as described previously [19]. Recombinant murine single-chain IL-6/sIL-6R was also prepared as described previously [19]. 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] was purchased from Wako Pure Chemical Industries, Osaka, Japan. Rabbit antimouse phosphotyrosine-STAT1 (Tyr701) and STAT3 (Tyr705) antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA, and antimouse STAT1, STAT3, gp130, and IL-12 receptor β1 chain antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Rabbit antimouse TCCR (WSX-1) was purchased from Abcam, Cambridge, UK. Goat antimouse IL-23 receptor antibody and IFN-γ ELISA kit was purchased from R&D Systems, Minneapolis, MN, USA. Specific polymerase chain reaction (PCR) primers for mouse alkaline phosphatase (ALP) and type I procollagen were synthesized by Invitrogen, Carlsbad, CA, USA. Recombinant human M-CSF was given by Morinaga Milk Industry, Tokyo, Japan, and mouse sRANKL was given by Sankyo, Tokyo, Japan. Other chemicals and reagents were of analytical grade.

Cell culture

Murine calvarial cells were obtained from neonatal (0- to 2-day-old) ddY mice, as previously described, with a slight modification [20]. Briefly, calvaria were incubated in phosphate-buffered saline (PBS) containing 0.1% collagenase and 0.2% pronase for 5 min. Calvaria digestion was repeated 5 times with fresh solutions. Cells released during the first digestion were discarded. Cells prepared from the subsequent digestions were combined and used as primary osteoblasts. Bone marrow cells were obtained from the tibia and femora of 5- to 10-week-old male ddY mice. Osteoblasts and bone marrow cells were cultured in minimum essential medium eagle, alpha modification (αMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37°C.

Western blotting

For the detection of phosphorylated STATs, osteoblasts plated on 6-well plates, or bone-marrow-derived macrophage-like cells (BMMs) derived from bone marrow cells by culturing in media containing 15 ng/ml M-CSF in 6-well plates for 3 days, were preincubated in the fresh culture media containing 10% FCS for 60 min, stimulated with IL-23 (20 ng/ml) or IL-27 (10 ng/ml) for 20 min, and then harvested. IL-6 (3 ng/ml) was also used as a positive control. Cells lysed in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer were separated by SDS–PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was then blocked, probed with anti-pYSTAT1, -STAT1, -pYSTAT3, or -STAT3 antibody, probed with an appropriate secondary antibody conjugated to horseradish peroxidase, and visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. For IL-23R and IL-27R subunit detection, cell lysates of osteoblasts and BMMs were separated by SDS–PAGE, transferred to a membrane. The membrane was treated with anti-IL-23R or IL-27R subunit antibodies, and then visualized as described above.

Alkaline phosphatase activity

Osteoblasts in the culture media were inoculated into 96-well plates at a density of 5×10^3 cells/well and then cultured to spread overnight. The media were removed, replaced with fresh media containing 10% FCS, 50 mg/ml ascorbic acid, and 10 mM β-glycerophosphate, with or without stimu-

lants, and cultured for 8 days. The media were replaced every 3 days. Alkaline phosphatase activity in the cell lysates was measured using *p*-nitrophenylphosphate as a substrate. Briefly, the conditioned media were discarded and replaced with substrate buffer (100 µl/well) containing 0.1% Triton X-100, 50 mM Tris-HCl, 4 mg/ml *p*-nitrophenyl phosphate, 2 mM $MgCl_2$, and 0.1 M 2-amino-2-methyl-1-propanol. After 40 min, the reaction was stopped by adding 0.5 M NaOH (20 µl/well). The production of *p*-nitrophenol was determined by measuring the absorbance at 405 nm. A relative cell number was concomitantly assayed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The data were expressed as µM of *p*-nitrophenol (p-np) per 40 min per cell.

Total RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted from osteoblasts using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcriptase–polymerase chain reaction (RT–PCR) was carried out using MuLV reverse transcriptase (Applied Biosystems, Foster, CA, USA) according to the manufacturer's protocol. Aliquots of 1 µg total RNA were reverse-transcribed into cDNA at 42°C for 20 min in a thermal cycler using a random hexamer (Applied Biosystems). The reverse-transcribed cDNA products were amplified using a PCR technique with the following primer sets: WSX-1 sense primer, 5′-CAAGAAGAGGTCCCGT GCTG-3′; WSX-1 antisense primer, 5′-TTGAGCCCAGTC CACCACAT-3′; gp130 sense primer, 5′-AGTCTGGGTG GAAGCAGAGA-3′; gp130 antisense primer, 5′-CTTG GTGGTCTGGATGGTCT-3′; IL-23R sense primer, 5′-GCACTGCCGACCAAGGAATC-3′; IL-23R antisense primer, 5′-GAGTTCTCCATGCCTAGGGA-3′; IL-12Rβ1 sense primer, 5′-TGAGTGCTCCTGGCAGTATG-3′; IL-12Rβ1 antisense primer, 5′-CAAATGTCACCAAGCA CACC-3′; ALP sense primer, 5′-ATTGCCCTGAAACTC CAAAACC-3′; ALP antisense primer, 5′-CCTCTGGT GGC-ATCTCGTTATC-3′; type I procollagen sense primer, 5′-GCAATCGGGATCAGTACGAA-3′; type I procollagen antisense primer, 5′-ACACGGTGTCACT GCGCTGAAGA-3′; β-actin sense primer, 5′-CAGCTT-CTTTGCAGCTCCTT-3′; β-actin antisense primer, 5′-TCACCCACATAGGAGTCCTT-3′. The cycle conditions were 94°C for 30 s, 58°C for 20 s, and 72°C for 30 s. The PCR products were subjected to electrophoresis on 2% agarose gels, and visualized by ethidium bromide staining with ultraviolet light illumination.

Evaluation of RANKL expression on osteoblasts

Osteoblasts in the 6-well plates were cultured in the presence or absence of IL-6 (5 ng/nl), IL-23 (20 ng/ml), or IL-27 (10 ng/ml) for 3 days. The cells were harvested using 2 mM ethylenediaminetetraacetic acid (EDTA)/PBS, and then treated with a 0.5 µg/ml biotin-conjugated antimouse TRANCE/RANKL antibody (eBioscience, San Diego, CA, USA) at 4°C for 20 min in 100 µl PBS containing 2% FCS and 0.1% sodium azide, followed by avidin–phycoerythrin. These cells were analyzed for RANKL expression on cell surfaces using a fluorescence-activated cell sorter (FACS) (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Assessment of in vitro osteoclast development

Co-culture system of bone marrow cells and osteoblasts. Bone marrow cells (2×10^5 cells/500 µl/well) were co-cultured with osteoblasts (10^4 cells/500 µl/well) in 48-well plates for 7 days in αMEM containing 10% FCS and 10^{-8} M 1,25$(OH)_2$ D_3 in the presence of IL-23 or IL-27. All cultures were incubated in quadruplicate, and culture media were replaced with fresh media every 3 days. The cells were fixed with 10% formalin in PBS and stained for tartrate-resistant acid phosphatase (TRAP) as described previously [21]. TRAP-positive multinucleated (≥3 nuclei) cells were counted as osteoclasts under a light microscope.

M-CSF/sRANKL-induced osteoclastogenesis. Bone marrow cells (1.5×10^6 cells/500 µl/well) in culture media containing 15 ng/ml M-CSF were cultured in 48-well plates for 3 days to induce bone marrow-derived macrophage-like cells (BMMs), followed by M-CSF and 100 ng/ml sRANKL in the presence or absence of IL-23 or IL-27 for 4 more days. Culture media were replaced with fresh media every 3 days. The cells were fixed and stained for TRAP as described above.

Co-culture system of BMMs and activated T cells. Mouse T cells were purified from splenocytes, as described previously [22]. Briefly, splenocytes derived from ddY mice were applied to a nylon wool column and incubated at 37 °C for 45 min. Unbound cells were washed out and used as a T cell fraction, which consists of about 60% CD4+ and 10% CD8+ T cells, 15% Mac-1+ monocytes/macrophages, 3% B220+ B cells, and 1% Gr-1+ granulocytes by FACS analysis. These cells in RPMI medium, supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, were stimulated by plate-immobilized anti-CD3 antibodies (0.2 or 2 µg/ml, 37°C, 2 h) for 2 days, and then subjected to experiments as an activated T cell fraction. The BMMs derived from bone marrow cells, as described above, were co-cultured with the activated T cell fraction in the presence of 15 ng/ml M-CSF, 100 ng/ml soluble RANKL, and indicated concentrations of soluble anti-CD3 antibody for 5 more days. The cells were fixed and stained for TRAP as described above.

Statistical analysis

The results are expressed as the mean ± SD of 4 or 6 cultures. Comparisons between multiple groups were performed using one-way analysis of variance followed by Fisher's protected least significant difference test.

Results

IL-23 and Il-27 receptor expression and activation of STAT1 and STAT3 by IL-23 or IL-27 in osteoblasts

Using primary osteoblasts, we first evaluated the mRNA expression of IL-23R subunits IL-23R and IL-12Rβ1, and IL-27R subunits WSX-1 and gp130, by RT–PCR. As shown in Fig. 1A, the mRNA of both IL-23R and IL-27R subunits was clearly identified in osteoblasts. Protein levels of IL-27R subunits, but not IL-23R subunits, were also detected by Western blotting (Fig. 1B). To explore the roles of ligands, the effects of IL-23 or IL-27 on STAT1 and STAT3 activation was tested by the cells; it has been shown that both IL-27 and IL-23 activate STAT1 and STAT3 molecules in naive and memory primary CD4+ T cells [8,17]. STAT3 activation in stromal/osteoblastic cells is also important for the induction of RANKL [3]. Primary osteoblasts were stimulated with IL-23 (20 ng/ml) or IL-27 (10 ng/ml) for 20 min. Total cell lysates were prepared and subjected to Western blotting using antiphosphotyrosine STAT1 (pY701) and antiphosphotyrosine STAT3 (pY705) antibodies. As shown in Fig. 1C, stimulation by IL-27 increased the relative amounts of phosphorylated STAT1 and STAT3 in osteoblasts, although no significant changes by IL-23 on these molecules were observed. IL-6, hereafter used as a positive control, also activated STAT1 and STAT3, as described in previous reports [22]. These results demonstrated the absence of IL-23R on osteoblasts.

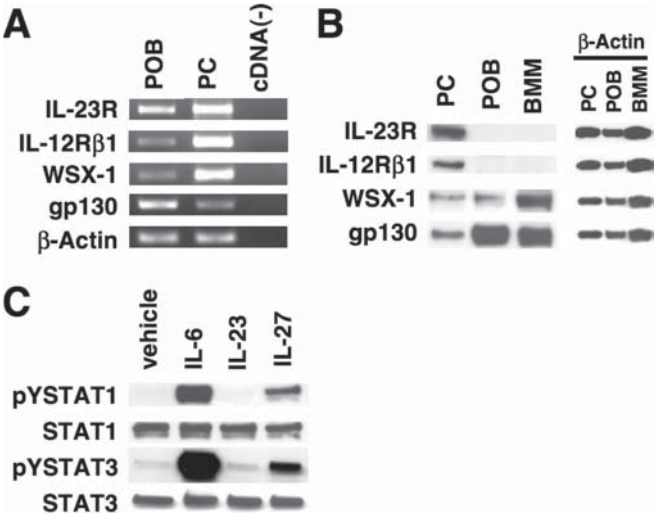

Fig. 1. IL-23 and IL-27 receptor expression in primary mouse calvarial osteoblasts. **A** Total RNA was prepared from neonatal mouse calvarial primary osteoblasts (*POB*), reverse transcribed with a random hexamer, and subjected to amplification by polymerase chain reaction for IL-23 receptor (*R*) subunits, IL-12Rβ1 and IL-23R, and IL-27R subunits, WSX-1 and gp130, with specific primer sets as described in Materials and Methods. Amplification products were resolved on 2.0% agarose gels containing ethidium bromide. A positive control (*PC*) was prepared from activated T cells stimulated by anti-CD3 antibody for 3 days, as described previously [8]. **B** Cell lysates of mouse calvarial osteoblasts and bone-marrow-derived macrophages (*BMMs*) were subjected to Western blot analysis using antibody against IL-23R and IL27R subunits. Positive controls for IL-23R and IL-27R were prepared from Th-17 cells induced as described previously [13] and splenocytes, respectively. **C** Osteoblasts plated onto 6-well plates were stimulated with IL-6 (3 ng/ml) used as a positive control, IL-23 (20 ng/ml), or IL-27 (10 ng/ml) for 20 min, and cells were harvested and analyzed for STAT1 and STAT3 tyrosine phosphorylation by Western blotting using anti-pYSTAT1 and pYSTAT3 antibody and anti-STAT1 and STAT3 antibody as described in Materials and Methods. Representative results of two independent experiments are shown

Evaluation of IL-23 or IL-27 on several phenotypes of osteoblasts

We next examined the effects of IL-23 or IL-27 on various aspects of osteoblastic phenotypes. First, ALP activity in osteoblasts was examined in the presence of IL-23 or IL-27 for 8 days. Treatment with these interleukins had no significant effects on ALP activity as compared with vehicle treatment (Fig. 2), whereas IL-6 augmented ALP activity ($P < 0.01$), as reported previously [23]. Second, to study the effects on osteoblasts of these interleukins, we examined the mRNA expression of ALP and type I procollagen in osteoblasts. These mRNA expressions could not be modified by treatment with IL-23 or IL-27 (Fig. 3). Osteocalcin and Runx2 mRNA expressions were also not affected (data not shown). In contrast, IL-6 enhanced ALP mRNA expression. Furthermore, the number of osteoblasts was assessed in the presence of IL-23 or IL-27 up to 48 h. Treatment with these ligands, along with IL-6, did not significantly change the number of osteoblasts (data not shown).

Effects of IL-23 or IL-27 on osteoclastogenesis in co-cultures of osteoblasts and bone marrow cells

Because IL-6 has been reported to induce RANKL expression on osteoblasts [1,3], we tested whether IL-23 or IL-27 could modulate RANKL expression in primary osteoblasts. Using the FACS procedure, osteoblasts treated with IL-6, IL-23, or IL-27 for 3 days were evaluated for the expression of RANKL. Although IL-6 induced RANKL expression, as

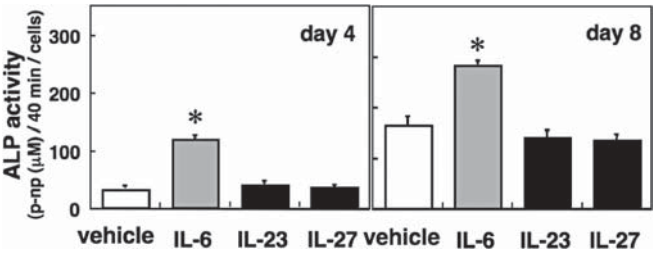

Fig. 2. Effects of IL-23 and IL-27 on alkaline phosphatase (*ALP*) activity in osteoblasts. Osteoblasts in culture media containing β-glycerophosphate and ascorbic acid were cultured in the presence of IL-6 (3 ng/ml), IL-23 (20 ng/ml), IL-27 (10 ng/ml), or vehicle for 4 (*left panel*) or 8 (*right panel*) days. Alkaline phosphatase activities were measured as described in Materials and Methods. Concomitantly, relative cell numbers were measured as described in Materials and Methods. The data are shown as *p*-nitro-phenol (p-np) μM/40 min/cells of quadruplicate cultures (mean±SD). The results were reproduced in two independent experiments

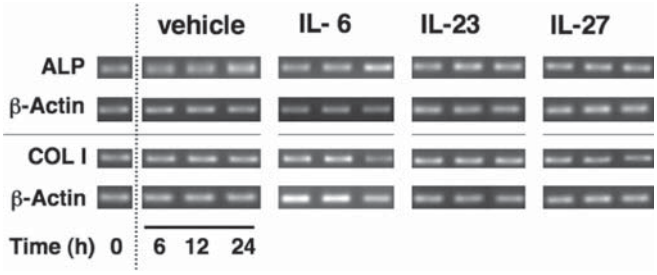

Fig. 3. Effects of IL-23 and IL-27 on mRNA expression in osteoblasts. Osteoblasts in culture media containing β-glycerophosphate and ascorbic acid were cultured in the presence of IL-6 (3 ng/ml), IL-23 (20 ng/ml), IL-27 (10 ng/ml), or vehicle for 6, 12, and 24 h. Total RNA was extracted from osteoblast layers, and then semiquantative reverse transcriptase–polymerase chain reaction (RT–PCR) was carried out using specific primers as described in Materials and Methods. The amplification products were resolved in 2% agarose gels containing ethidium bromide. Similar results were obtained from two independent experiments

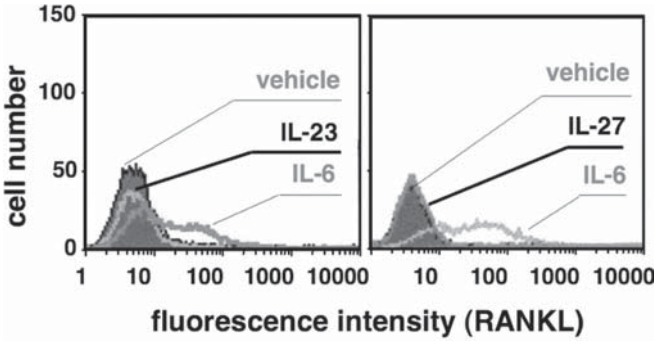

Fig. 4. Effects of IL-23 and IL-27 on RANKL expression on osteoblasts. Osteoblasts in culture media were cultured with IL-6 (5 ng/ml), IL-23 (20 ng/ml), IL-27 (10 ng/ml), or vehicle for 3 days. Osteoblasts were harvested using 2 mM EDTA/PBS, treated with a biotin-conjugated anti-RANKL antibody, followed by an avidin-phycoerythrin reagent, and then FACS analysis was performed. Representative result of two independent experiments are shown

shown previously, neither IL-23 nor IL-27 significantly modified RANKL expression in osteoblasts (Fig. 4). Furthermore, no significant effect of IL-23 and IL-27 were found on 1,25$(OH)_2$ D_3-induced osteoclast development (co-culture system), as shown in Fig. 5.

Effects of IL-23 and IL-27 on osteoclastogenesis induced by M-CSF/sRANKL

To examine the effects of IL-23 and IL-27 on osteoclastogenesis, we used bone-marrow-derived macrophage-like cells (BMMs) with more precise stimulation of M-CSF/sRANKL. When we examined the mRNA and protein levels of IL-23R and IL-27R in BMMs by RT–PCR and Western blot, respectively, both IL-23R and IL-27R subunit mRNAs were clearly identified (Fig. 6A), whereas protein levels of IL-27R subunits were detected, but not those of IL-23R subunits (Fig. 1B). Phosphorylated STAT1 and STAT3 in BMMs stimulated by IL-23 or IL-27 were then evaluated. IL-27, but not IL-23, phosphorylated STAT1

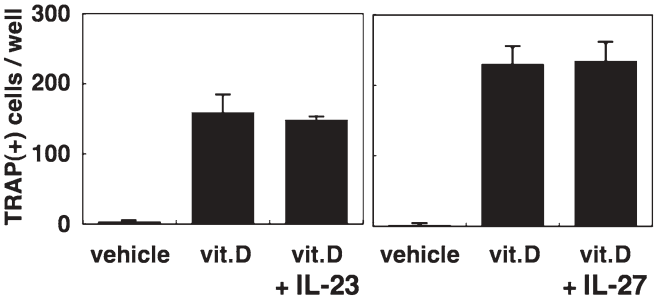

Fig. 5. Effects of IL-23 and IL-27 on osteoclastogenesis induced by 1,25 $(OH)_2$ vitamin D_3 in an osteoblasts and bone marrow cells co-culture system. Osteoblasts and bone marrow cells in culture media containing 1,25 $(OH)_2$ vitamin D_3 were co-cultured in the presence of IL-23 (20 ng/ml), IL-27 (10 ng/ml), or vehicle for 7 days. The cells were fixed and stained for tartrate-resistant acid phosphatase (*TRAP*) as described in Materials and Methods. The number of cells (TRAP-positive, ≥3 nuclei) was indicated as TRAP(+) cells/well of quadruplicate cultures (mean±SD). The results were reproduced in two independent experiments

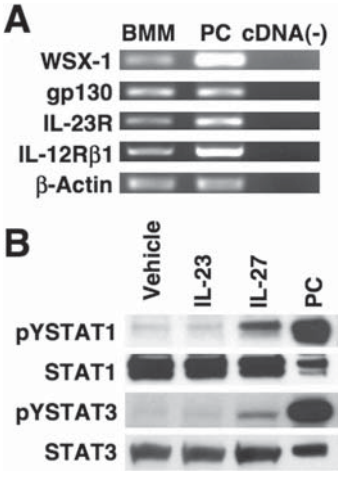

Fig. 6. IL-23 and IL-27 receptor expression in bone marrow cell-derived macrophage-like cells. **A** Total RNA was prepared from bone marrow cell-derived macrophage-like cells (*BMMs*) that were induced by the treatment of bone marrow cells with M-CSF for 3 days. RT–PCR for IL-23R and IL-27R subunits was performed as described in Fig. 1. A positive control (*PC*) was prepared from activated T cells stimulated by anti-CD3 antibody for 3 days, as described previously [8]. **B** BMMs plated onto 6-well plates were stimulated with IL-6 (3 ng/ml), IL-23 (20 ng/ml), or IL-27 (10 ng/ml) for 20 min, and cells were harvested and analyzed for STAT1 and STAT3 tyrosine phosphorylation by Western blotting as described in Fig. 1. Representative results of two independent experiments are shown

and STAT3 (Fig. 6B). These results demonstrated the absence of IL-23R on BMMs. We then tested whether IL-23 and IL-27 affected osteoclastogenesis in this BMM and M-CSF/sRANKL system. The BMMs were treated with IL-23 or IL-27 for the last 4 days. Osteoclastogenesis was reduced to about 70% of controls by either 2 ng/ml IL-23 or IL-27 (Fig. 7). Similar results were obtained at 20 ng/ml of these interleukins. These results suggest that either IL-23 or IL-27 may partially inhibit osteoclastogenesis in this system. To examine inhibitory mechanisms by IL-23 and IL-27 on osteoclast formation, we examined the effects on

proliferation and IFN-γ production of BMMs. Treatment with 30 ng/ml IL-23 or IL-27 for 3 days did not affect the number of BMMs between treated and control cells, and IFN-γ was not detected in the culture supernatants of BMMs treated with or without these interleukins for 3–5 days (data not shown).

Indirect effects of IL-23 and IL-27 on osteoclastogenesis by way of T cells

We investigated the indirect effects of the cytokines on osteoclastogenesis by way of a T cell fraction which was partially purified from splenocytes with a purity of about 70% for T cells, and which was activated with anti-CD3 antibody. As a result, the T cell fraction stimulated by 2 μg/ml anti-CD3 antibody almost completely inhibited osteoclastogenesis (Fig. 8A, upper panel), as previously reported [24]. The effects of IL-23 and IL-27 on osteoclastogenesis were tested in the presence of 0.2 μg/ml anti-CD3 antibody, by which osteoclastogenesis was partially inhibited. Both IL-23 and IL-27 additively inhibited osteoclastogenesis (Fig. 8B,C, upper panels). The concentration of IFN-γ in the supernatants of the co-culture was simultaneously measured using an enzyme-linked immunosorbent assay. An anti-CD3 antibody stimulation dose-dependently increased IFN-γ production (Fig. 8A, lower panel). IL-27, but not IL-23, augmented IFN-γ production in the presence of 0.2 μg/ml anti-CD3 antibody (Fig. 8B,C, lower panels). These results suggested that IL-23 and IL-27 have indirect effects on osteoclastogenesis through activated T cells, although more precise studies would be needed to clarify these mechanisms.

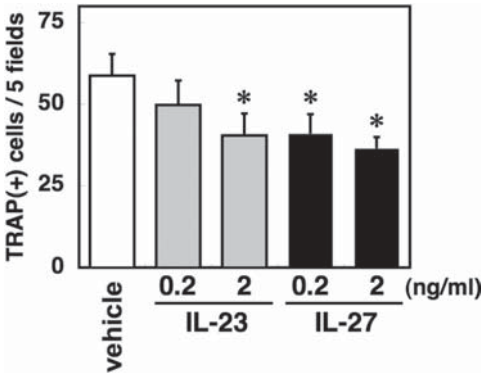

Fig. 7. Effects of IL-23 and IL-27 on osteoclastogenesis induced by M-CSF/sRANKL. BMMs were cultured with M-CSF/RANKL in the presence of IL-23 or IL-27 at the concentrations indicated for 4 days. The cells were fixed and stained for TRAP as described in Materials and Methods. The number of cells (TRAP-positive, ≥3 nuclei) was indicated as TRAP(+) cells/5 fields of each well (mean±SD, $n = 6$). The results were reproduced in two independent experiments. Asterisks indicate a significant difference ($P < 0.01$) compared with cultures without interleukins

Discussion

Cytokines are recognized to be involved in the bone remodeling process. The gp130 family of cytokines, including IL-6, has been identified for its significant role in osteoclast formation. Because IL-27 has been reported to transduce its signals via IL-27R, WSX-1 and gp130 [5], to induce expression of RANKL mRNA in human mast cells [5], and to have similar functions to IFN-γ, which inhibits osteoclast formation [24], in inducing T-bet, IL-12Rβ2, MHC class I expression in T cells, and IgG2a class switching in B cells [8,25–28], we studied the effects of IL-27 and its structurally related molecule, IL-23, on osteoblasts and osteoclasts. In this study, treatment with IL-27 but not IL-23 activated STAT1 and STAT3 in osteoblasts. However, these interleukins did not show any significant effects on ALP activity,

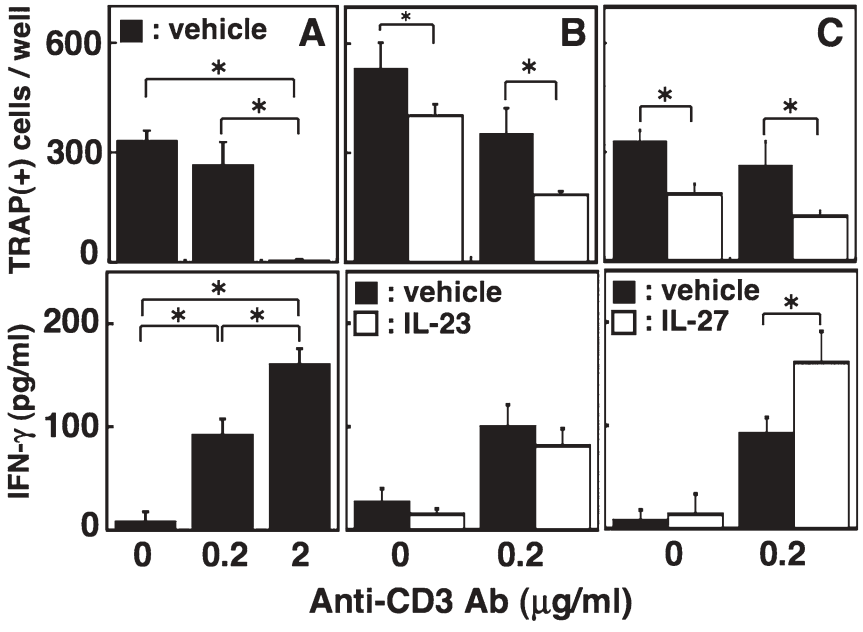

Fig. 8. Indirect effects of IL-23 and IL-27 on osteoclastogenesis through T cells. BMMs and T cell fraction, stimulated by anti-CD3 antibody at the concentration indicated, were cocultured with M-CSF/RANKL in the presence of (**A**) vehicle, (**B**) IL-23 (20 ng/ml), and (**C**) IL-27 (10 ng/ml) for 4 days. The number of cells (TRAP-positive, ≥3 nuclei) was indicated as TRAP(+) cells/well (mean ± SD, $n = 4$) (*upper panels*). The concentrations of IFN-γ in the culture supernatants were simultaneously measured by enzyme-linked immunosorbent assay (*lower panels*). Representative results of two independent experiments are shown. Asterisks indicate a significant difference ($P < 0.01$) compared with cultures without interleukins

mRNA expressions of ALP, type I procollagen, osteocalcin, Runx2, and proliferation of osteoblasts. Furthermore, we could not find any actions on RANKL expression and osteoclastogenesis induced by $1,25(OH)_2D_3$ in the co-culture system. In contrast, consistent with previous reports [1,3,23], IL-6 induced STAT1 and STAT3 activation and increased ALP activity and RANKL expression in osteoblasts. Although we found rapid and significant phosphorylation of STAT1 and STAT3 in osteoblasts, the consequences of activated signals by IL-27 could not be identified. One reason for the different consequences between IL-6 and IL-27 stimulation may be that the amount of phosphorylated STAT1 and STAT3 in osteoblasts stimulated by IL-27 was very small as compared with that by IL-6. The difference in consequences could depend on a balance between the amount and combination of phosphorylated STATs and the extracellular environment. For example, IL-6 is reported to induce Th-17 cell differentiation from naïve CD4+ T cells in the presence of TGF-β [13,14], whereas IL-27 induces Th1 cell differentiation from naïve CD4+ T cells. On the other hand, it is thought that IL-27 inhibits Th-17 cell differentiation through inducing a suppressor of cytokine signaling-3, which inhibits STAT3 signaling via a feedback mechanism [14,29], suggesting that IL-27 might have the potential to inhibit RANKL expression on osteoblasts stimulated by IL-6. It has been reported that a number of cytokines directly act on osteoclast progenitors and consequently affect the proliferation of matured or multinucleated osteoclasts [24,30–33]. We therefore examined the effects of IL-23 and IL-27 on osteoclast formation using a BMM and M-CSF/sRANKL system. When BMMs were treated with IL-23 or IL-27, osteoclast formation was reduced to about 70% of control, and proliferation was not affected. Since protein levels of IL-23R were not found in BMMs (Figs. 1B), it is conceivable that IL-23 may act indirectly on BMMs through contaminated cells in the culture. IL-23R, however, does not seems to be completely absent because of its mRNA expression. To confirm this, it would be necessary to elucidate the expression pattern of IL-23R at the protein level during osteoclast differentiation. On the other hand, IL-27, like IFN-γ, might directly act on BMMs through STAT1 signaling, which contributes to the inhibition of osteoclastogenesis, although the detailed mechanisms remain to be determined. Because we found mRNA expression of both IL-23R and IL-27R subunits, the direct effects on BMM were tested as cellular proliferation. Although there were no significant changes in total M-CSF-dependent proliferative cell number, we found changes in the nuclear number or maturation of osteoclasts, as shown in Table 1. Furthermore, there was no difference in proliferation between controls and IL-23- or IL-27-treated BMMs (data not shown). These results indicate that IL-23 or IL-27 could partly modify cell fusion or the survival of multinucleated osteoclasts. The effects of IL-23 and IL-27 on the production of factors that inhibit osteoclastogenesis, i.e., IL-10 or GM-CSF induced by osteoclast progenitors, remain to be elucidated.

There is a discrepancy between $1,25(OH)_2D_3$-induced osteoclastogenesis in the presence of osteoblasts and M-CSF/sRANKL-induced osteoclastogenesis. The former is a system in which stromal cells or osteoblasts support osteoclastogenesis. In addition to RANKL signaling, there are co-stimulatory signals in the co-culture system, such as ITAM and ICAM signals [34,35], which are also important in osteoclastogenesis. These signals are recognized to facilitate osteoclastogenesis rather than RANKL alone. Therefore, the weak inhibitory effects of IL-23 and IL-27 on osteoclastogenesis might be inhibited in the co-culture system.

Table 1. Effects of IL-23 and IL-27 on multinucleated osteoclast differentiation

N	Vehicle	IL-23	IL-27
1, 2	298 ± 31	253 ± 46	299 ± 43
3–5	46 ± 11	35 ± 3*	30 ± 7**
≥6	9 ± 4	7 ± 4	6 ± 2

Osteoclasts were induced by M-CSF/sRANKL with or without ILs. TRAP(+) cells which have 1–2, 3–5, or ≥6 nuclei were counted. Data are mean±SD ($n = 6$). *$P < 0.05$, **$P < 0.01$ as compared with vehicle. *N*, nuclear number

IL-23 induces a proliferation of memory CD4+ T cells, and subsequently accelerates production of IL-17 and IFN-γ [16,17]. IL-17 has been reported to induce RANKL expression on osteoblasts [15], and IFN-γ inhibits osteoclast formation from precursor macrophages [24]. IL-27 also has a synergistic action with IL-12 on IFN-γ production of naive CD4+ T cells [4]. These findings suggest that either IL-23 or IL-27 could modify osteoclasts or their progenitors with the action of T cells. We therefore investigated the effects of IL-23 and IL-27 on osteoclastogenesis by way of T cells. Both IL-23 and IL-27 additively inhibited osteoclastogenesis with activated T cells. IL-27 probably inhibited through inducing IFN-γ production of T cells, whereas IL-23 might interfere via other factors. Additional studies, such as confirmation of the inhibitory effects of these interleukins by using anti-IFN-γ antibody, determination of cytokines, and specification of T cell subsets which affect osteoclastogenesis, are needed to clarify inhibitory mechanisms by IL-23 and IL-27.

In summary, we found several actions of novel members of the IL-6/IL-12 family cytokines, i.e., IL-23 and IL-27, in mouse osteoblasts and osteoclasts. Although the consequences of activated signals by IL-27 in osteoblasts were not identified, this finding, along with the apparent expression of WSX-1 and gp130, would indicate that IL-27 affects osteoblast responses in some circumstances. It is also intriguing that both IL-27 and IL-23 partially attenuated the number of multinucleated osteoclasts, and that both cytokines additively inhibited osteoclastogenesis with activated T cells. Although we could not find any very significant changes of cytokines in BMMs and T cells for osteoclast formation, it is possible that IL-23 and IL-27 interact together in autoimmune-related skeletal disorders.

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