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IL-17 Induces Osteoclastogenesis From Human Monocytes Alone in the Absence of Osteoblasts, Which Is Potently Inhibited by Anti-TNF-α Antibody: A Novel Mechanism of Osteoclastogenesis by IL-17

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ABSTRACT

IL-17 is a proinflammatory cytokine crucial for osteoclastic bone resorption in the presence of osteoblasts or synoviocytes in rheumatoid arthritis. However, the role of IL-17 in osteoclastogenesis from human monocytes alone remains unclear. Here, we investigated the role of IL-17 in osteoclastogenesis from human monocytes alone and the direct effect of infliximab on the osteoclastogenesis induced by IL-17. Human peripheral blood mononuclear cells (PBMC) were cultured for 3 days with M-CSF. After non-adherent cells were removed, IL-17 was added with either infliximab or osteoprotegerin (OPG). Seven days later, adherent cells were stained for vitronectin receptor. On the other hand, CD11b-positive monocytes purified from PBMC were also cultured and stained as described above. CD11b-positive cells were cultured with TNF-α and receptor activator of NF-κB ligand (RANKL). In the cultures of both adherent cells and CD11b-positive cells, IL-17 dose-dependently induced osteoclastogenesis in the absence of soluble-RANKL. OPG or infliximab inhibited IL-17-induced osteoclastogenesis. Interestingly, in the culture of CD11b-positive cells, the osteoclastogenesis was more potently inhibited by infliximab than by OPG. TNF-α and RANKL synergistically induced osteoclastogenesis. The present study clearly demonstrated the novel mechanism by which IL-17 directly induces osteoclastogenesis from human monocytes alone. In addition, infliximab potently inhibits the osteoclastogenesis directly induced by IL-17. J. Cell. Biochem. 108: 947–955, 2009.

KEY WORDS: INTERKEUKIN-17; INFLIXIMAB; OSTEOCLASTS; MONOCYTES

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of articular cartilage and bone [Wilder, 1993]. We and another group have detected osteoclasts in synovial tissues [Kotake et al., 1996] and eroded bone surfaces [Gravallese et al., 1998], suggesting that osteoclastic bone resorption is involved in the pathogenesis of RA. We and other groups also reported that RANKL is expressed in activated T cells as well as in osteoblastic cells and that concentrations of soluble-RANKL (sRANKL) in the synovial fluids are significantly higher in patients with RA than in patients with other arthropathies, while the level of OPG is decreased in synovial fluids from RA patients [Kong et al., 1999; Gravallese et al., 2000; Kotake et al., 2001]. These results suggest that both membrane-associated RANKL on activated T cells and osteoblasts, and sRANKL derived from activated T cells contribute to osteoclastic bone resorption in RA patients.

Furthermore, levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 are elevated in synovial fluid of RA patients [Steiner et al., 1999], and these cytokines promote bone resorption by inducing the differentiation or activation of osteoclasts [Kotake et al., 1996; Assuma et al., 1998]. Infliximab, a recombinant IgG1κ monoclonal antibody specific for TNF, has been proven to be effective at inhibiting structural damage, as assessed

Abbreviations used: BMM, bone marrow-derived macrophages; M-CSF, macrophage-colony stimulating factor; MEM, minimal essential medium; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; RANKL, receptor activator of NF-κB ligand; sRANKL, soluble-RANKL; TRAP, tartrate-resistant acid phosphatase.

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with radiographs [Lipsky et al., 2000]. Thus, attenuation of the activity of proinflammatory cytokines in RA patients inhibits bone resorption and destruction.

IL-17 is a proinflammatory cytokine that is mainly secreted by activated T cells [Broxmeyer, 1996]. IL-17 stimulates the production and expression of TNF-α and IL-1β by human macrophages [Jovanovic et al., 1998], and induces production of IL-1β in osteoblasts [Rifas and Avioli, 1999]. We have reported that IL-17 levels in synovial fluids are significantly higher in RA patients than in OA patients, and that IL-17 stimulates osteoclast differentiation by inducing the expression of RANKL via a mechanism involving the synthesis of PGE2 in osteoblasts in vitro [Kotake et al., 1999]. In addition, IL-23 exists upstream of IL-17 [Vanden Eijnden et al., 2005]; we also demonstrated that IL-17 is one of the factors associated with pathogenesis of RA as manifested IL-23-induced osteoclastogenesis [Yago et al., 2007]. Recently, it has been reported that IL-17 also plays important roles in joint destruction in animal models [Lubberts et al., 2005]. Furthermore, The existence of a third helper T cells (Th) population, a distinct subset of IL-17-producing T cells (Th17), was recently proposed, which may be the pathogenic population in autoimmune disease and bone destruction [Langrish et al., 2005; Sato et al., 2006]. Thus, IL-17 is a cytokine crucial for osteoclastic bone resorption in RA. However, the direct effect of IL-17 on purified human osteoclast precursors (monocytes) remains unclear.

In the present study, we examined the direct role of IL-17 in osteoclatogenesis using cultures of human CD11b-positive cells (monocytes) alone. IL-17 dose-dependently induced osteoclastogenesis, which was inhibited by adding OPG and infliximab, suggesting that TNF-α and RANKL were, at least in part, responsible for the IL-17-induced osteoclastogenesis. Furthermore, we also investigated whether IL-17 activates mature osteoclasts.

MATERIALS AND METHODS

REAGENTS

Recombinant human IL-17 (rhIL-17) was purchased from PeproTech EC Ltd. (London, United Kingdom). Recombinant human M-CSF (Leukoprol) was obtained from Yoshitomi Pharmaceutical (Osaka, Japan). The recombinant human sRANKL and human OPG were prepared as described previously [Kotake et al., 2001]. Recombinant murine TNF-α was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-human CD51/61 mAb was purchased from BD Bioscience Pharmigen (San Diego, CA). Infliximab was kindly provided by Centocor, Inc. (Pennsylvania, MA).

CULTURE SYSTEM FOR OSTEOCLASTOGENESIS IN THE ABSENCE OF OSTEOBLASTS

Human peripheral blood from the buffy coat of healthy volunteers was collected (Japanese Red Cross Society, Tokyo, Japan) (this study is approved by Institutional Review Board), then peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Histopaque 1077 (Sigma, St.Louis, MO) density gradients, washed, and resuspended at 1.3×10^6 cells/ml in α-minimal essential medium (α-MEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). CD11b-positive cells were separated from PBMC of the same volunteers by magnetic cell sorting (Miltenyi Biotec, Sunnyvale, CA) with positive selection using CD11b microbeads (Miltenyi-Biotec, Bergisch Gladbach, Germany) and cultured in the presence of M-CSF (100 ng/ml) for 3 days. PBMC or CD11b-positive cells were cultured for 3 days in 48-well plates (5×10^5 cells/0.3 ml/well; Corning, Inc., Corning, NY) in the presence of M-CSF (100 ng/ml). The adherent PBMC were used as monocytes in the culture system. Then non-adherent cells were removed before adding various concentrations of recombinant human IL-17 (rhIL-17) and M-CSF (100 ng/ml). The adherent PBMC, as described above, or CD11b-positive cells were cultured in the presence of M-CSF and rhIL-17 with various concentrations of infliximab or human OPG for 7 days. The culture medium was replaced every 3 days, with fresh medium supplemented with the agents described above. Osteoclast formation was evaluated by immunohistochemical staining for vitronectin receptors vitronectin receptor αvβ3 (CD51/61) [Udagawa et al., 1997] after culture for 10 days. CD51/61-positive cells containing more than three nuclei were counted as osteoclasts. Tartrate-resistant acid phosphatase (TRAP) activity was detected as described previously [Kotake et al., 1999].

In some experiments, after CD11b-positive cells were cultured in the presence of M-CSF (100 ng/ml) for 3 days, the cells were further cultured for 7 days with both sRANKL and human TNF-α to evaluate the synergistic effect of these cytokines.

CELL CULTURES FROM MURINE MACROPHAGES

BMM were prepared as osteoclast precursors as described previously [Li et al., 2003]. Briefly, bone marrow cells were obtained from tibiae of 5- to 8-week-old male ddY mice (Shizuoka Laboratories Animal Center, Shizuoka, Japan). All procedures for animal care were approved by the Animal Management Committee of Tokyo Women's Medical University. Bone marrow cells were suspended in α-MEM supplemented with 10% FBS on 60-mm diameter dishes for 16 h in the presence of M-CSF (100 ng/ml). Then, non-adherent cells were harvested and further cultured for 2 days with M-CSF (50 ng/ml). The adherent cells, most of which expressed macrophage-specific antigens such as Mac-1, Moma-2, and F4/80, were used as BMM. Primary osteoblasts were obtained from calvariae of newborn ddY mice, C57BL/6 (B6) mice [Itoh et al., 2006]. The calvarium from 2-day-old mice was cut into small pieces and embedded in type I collagen gel, according to the manufacturer's instructions (Cell matrix Type I-A; Nitta Gelatin Co., Osaka). After 3 days in culture, osteoblasts grown from the calvariae were collected by treating them with PBS containing 0.1% collagenase. Osteoclasts were generated in co-cultures of mouse primary osteoblasts and bone marrow cells in collagen gel-coated dishes as described previously [Itoh et al., 2003]. Primary osteoblasts and bone marrow cells were co-cultured in a-MEM supplemented with 10% FBS in 100-mm tissue culture dishes (Corning, Inc.) precoated with type I-collagen gel (Nitta Gelatin Co.) in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M). After the cells were cultured for 7 days, all cells were recovered from the dishes by treatment with 0.2% collagenase. The purity of osteoclasts in this preparation was about 5%. To purify osteoclasts, the crude osteoclast preparation was plated in 100-mm tissue culture dishes. After the cells were cultured for 6 h, osteoblasts were

removed by treatment of cells with PBS(−) containing 0.001% pronase E and 0.02% EDTA for 5 min. The purity of osteoclasts in this preparation was about 95%. The concentration of sRANKL was measured using an ELISA we devised [Kinpara et al., 2000]. OPG levels were also determined using a commercially available two-site sandwich ELISA kit (R&D Systems, Inc.). All samples and standards were assayed twice.

MEASUREMENT OF RANKL AND OPG ON MONOCYTES STIMULATED BY IL-17

CD11b-positive cells were cultured with various concentrations of rhIL-17 for 24 h. Then, the conditioned medium and monocytes were separated by centrifuge. The concentration of RANKL and OPG were measured by ELISA as described above.

ASSESSMENT OF ACTIN RING FORMATION

Osteoclasts were formed from human PBMC in the presence of M-CSF (100 ng/ml) and sRANKL (100 ng/ml) for 17 days as described above before rhIL-17 was added without M-CSF and sRANKL for 5 days. Actin rings were evaluated as previously described [Nakamura et al., 1996].

STATISTICAL ANALYSIS

The data were analyzed using the Mann–Whitney *U* test, and Student's *t*-test (Stat View; Abacus Concepts, Berkeley, CA). *P* values less than 0.01 or 0.05 were considered significant. All values are represented as the mean ± SEM.

RESULTS

IL-17 DIRECTLY INDUCED OSTEOCLASTOGENESIS IN CULTURES OF ADHERENT CELLS FROM PBMC

To investigate whether rhIL-17 induces osteoclastogenesis, PBMC were cultured with M-CSF for 3 days and with rhIL-17 for a further 7 days (Fig. 1). Compared with a negative (A) and positive control (B) stained by TRAP, multinuclear cells formed by rhIL-17 and M-CSF showed TRAP activity (C), CD51 expression (D). Therefore, the multinuclear cells exhibited the functions and properties of authentic osteoclasts.

Stimulation with rhIL-17 for 7 days increased the number of vitronectin receptor (CD51)-positive multinuclear cells without triggering phagocytosing activity (Fig. 2A). We investigated whether vitronectin receptor (CD51)-positive multinuclear cells had phagocytosing activity or not, because osteoclasts do not have phagocytosing activity. These multinucleated cells formed actin rings (Fig. 3A). Therefore, the multinuclear cells exhibited the functions and properties of authentic osteoclasts. As shown in Figure 2A, treatment with rhIL-17 for the entire 7 days dose-dependently increased the number of vitronectin receptor (CD51)-positive osteoclasts even in the absence of osteoblasts or exogenous sRANKL. The formation of osteoclasts induced by rhIL-17 was dose-dependently inhibited by adding OPG (100, 250, and 500 ng/ml) (Figs. 2A,B; the data of 100 and 250 ng/ml were not shown). Infliximab also inhibited rhIL-17-induced osteoclastogenesis dose-dependently as well as OPG (Figs. 2C,D). We used infliximab at 1,000 ng/ml, which was equal to the serum concentration of infliximab in RA patients [Maini et al., 2004].

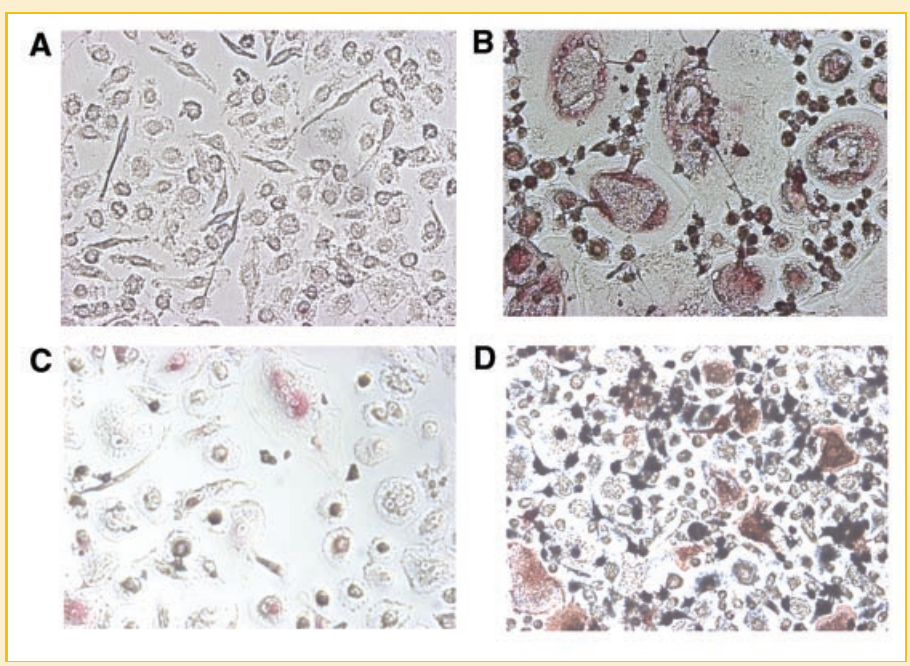

Fig. 1. Human osteoclasts induced from PBMC by rhIL-17 (1.0 ng/ml) and M-CSF. As negative and positive control, PBMC were cultured with M-CSF alone during the first 3 days, and then adherent cells were cultured M-CSF only (A; negative control) or M-CSF with sRANKL (100 ng/ml) (B; positive control) for the last 7days. Then osteoclasts were stained with Tartrate-resistant acid phosphatase (TRAP). Human osteoclasts induced from PBMC by rhIL-17 (1.0 ng/ml) and M-CSF were detected by TRAP staining (C), immunohistological staining by vitronectin receptor αvβ3 (CD51/61) (D). Original magnification, 100×.

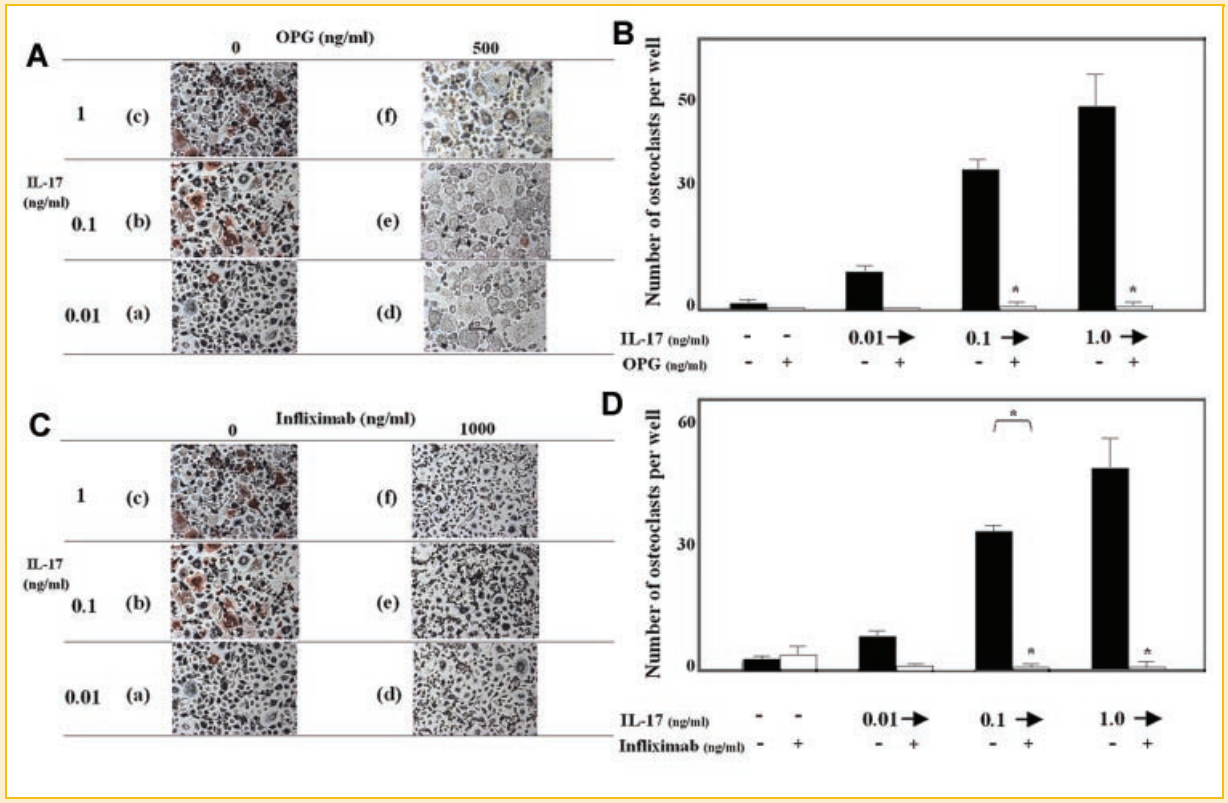

Fig. 2. A: IL-17-induced formation of osteoclasts from PBMC in cultures of adherent cells was inhibited by OPG. In the presence of M-CSF, adherent cells were cultured with rhIL-17 alone (0.01, 0.1, 1 ng/ml) (a–c), or with rhIL-17 (0.01, 0.1, 1 ng/ml) and OPG (500 ng/ml) (d–f). Original magnification, 100×. B: Effect of IL-17 and OPG on osteoclastogenesis in cultures of adherent cells obtained from PBMC. rhIL-17 (0.01, 0.1, 1 ng/ml) and OPG (500 ng/ml) were present during the last 7 days (4–10). After 10 days, osteoclasts positive for anti-vitronectin receptor Ab were counted. Data are expressed as the mean ± SEM for duplicate cultures. $^{*}P < 0.05$. Experiments were repeated three times with similar results. C: IL-17-induced formation of osteoclasts from PBMC in cultures of adherent cells was inhibited by infliximab. In the presence of M-CSF, adherent cells from PBMC were cultured with rhIL-17 alone (0.01, 0.1, 1 ng/ml) (a–c), or with rhIL-17 (0.1, 1.0, 1 ng/ml), and infliximab (1,000 ng/ml) (d–f). Original magnification, 100×. D: Effect of IL-17 and infliximab on osteoclastogenesis in cultures of adherent cells obtained from PBMC. rhIL-17 (0.01, 0.1, 1 ng/ml) and infliximab (1,000 ng/ml) were present during the last 7 days (4–10). After 10 days, osteoclasts positive for anti-vitronectin receptor Ab were counted. Data are expressed as the means ± SEM for duplicate cultures. $^{*}P < 0.05$. Experiments were repeated three times with similar results.

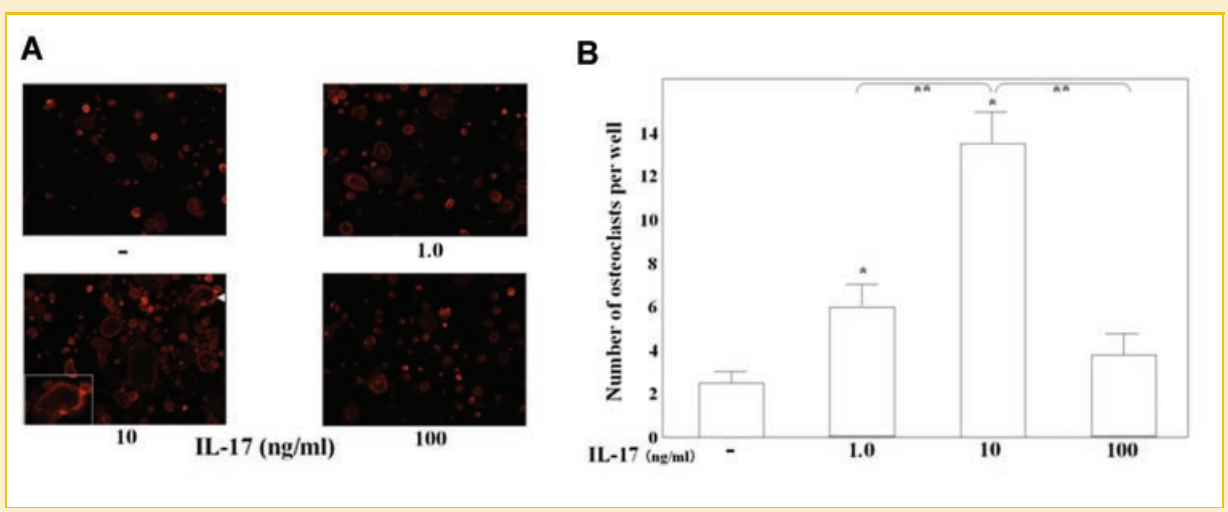

Fig. 3. A: IL-17 induced actin ring formation. Osteoclasts formed from human monocytes in the presence of M-CSF (100 ng/ml) and sRANKL (100 ng/ml) over 17 days. rhIL-17 was then added and the cells were incubated for 5 days. The cells were stained with rhodamine-conjugated phallodin solution and examined for actin rings. Original magnification, 40×. A panel inset is corresponding high magnification of an actin ring (arrowhead). B: Effect of IL-17 on actin ring formation in cultures of PBMC. rhIL-17 (1.0, 10, 100 ng/ml) was present during the last 5 days (18–22). After 22 days, the cells that had formed actin rings were counted. Data are expressed as the mean ± SEM (repeated eight times). $^{*}P < 0.05$ vs. IL-17 (−), $^{**}P < 0.01$ vs. 1.0 ng/ml IL-17 or 100 ng/ml of IL-17. Experiments were repeated three times with similar results.

IL-17 DIRECTLY INDUCED OSTEOCLASTOGENESIS IN THE CULTURE OF CD11b-POSITIVE CELLS

In the culture of adherent cells from PBMC, we could not exclude the possibility that IL-17-induced osteoclastogenesis was affected by membrane type RANKL on T cells during the first 3 days. Thus, we examined the issue whether rhIL-17 induces the osteoclastogenesis of purified monocytes. We confirmed by immunohistochemistry that purified CD11b-positive cells did not contain CD11b-negative cells (data not shown). In the culture of adherent cells from purified CD11b-positive cells, treatment with rhIL-17 for the entire 7 days dose-dependently increased the number of vitronectin receptor (CD51)-positive osteoclasts even in the absence of osteoblasts or exogenous sRANKL (Figs. 4A,C). OPG and infliximab inhibited the rhIL-17-induced osteoclastogenesis as well as in the culture of adherent cells from PBMC. Interestingly, infliximab, even at a low concentration of 10 ng/ml, more potently inhibited osteoclastogenesis than OPG (Figs. 4B,D). These findings suggested that IL-17, at least in part, induces osteoclastogenesis via a mechanism involving both RANKL and TNF-α.

TNF-α SYNERGIZED WITH sRANKL TO INDUCE OSTEOCLASTOGENESIS

We cultured CD11b-positive cells in various concentrations of both TNF-α and sRANKL as shown in Figure 5A. Each agent alone induced osteoclastogenesis in a dose-dependent manner. Most importantly, a synergistic, but not additive effect was observed. The synergistic effect was maxima at 1.0 ng/ml of RANKL and at 1.0 ng/ml of TNF-α (Fig. 5B). This marked synergistic effect indicated that TNF-α augments osteoclastogenesis in the presence of sRANKL. Furthermore, the minimal dose of TNF-α induced the differentiation of osteoclasts in the absence of sRANKL. A similar synergistic effect was observed using PBMC (data not shown).

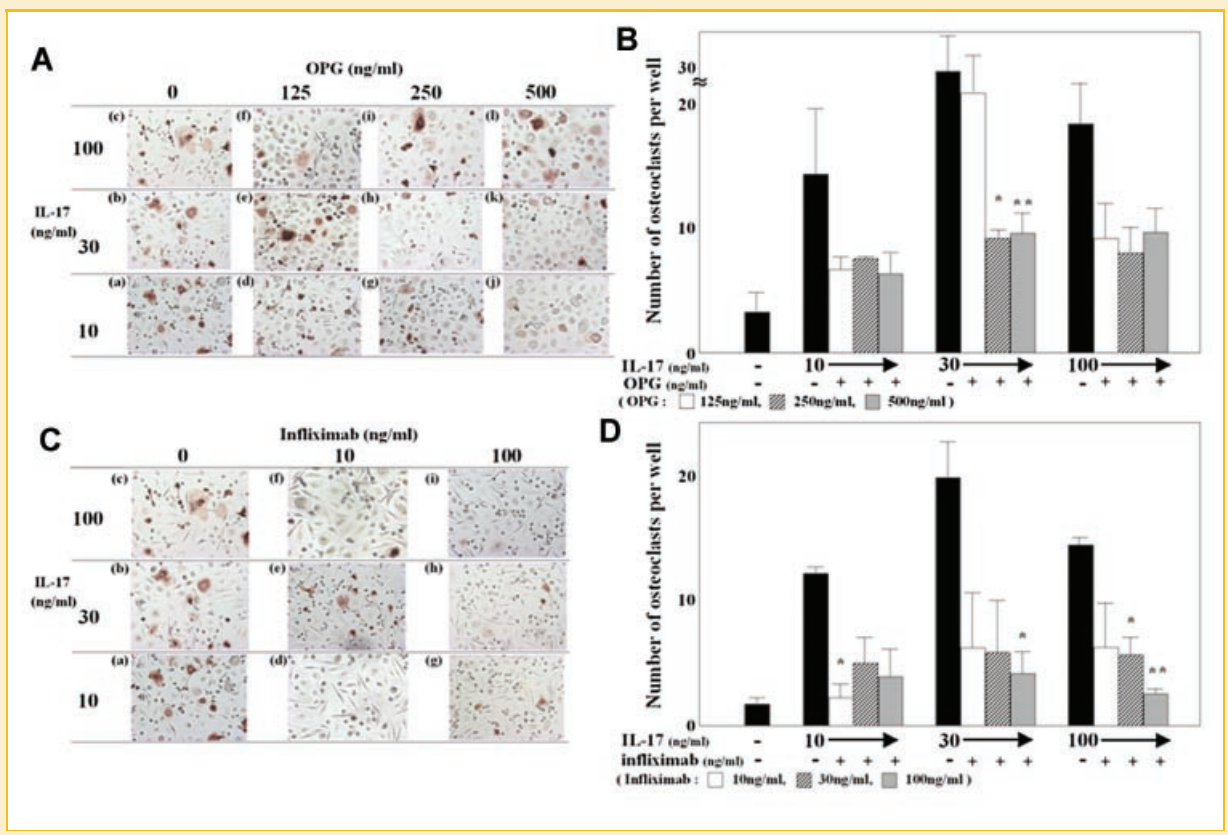

Fig. 4. A: IL-17-induced formation of osteoclasts in cultures of CD11b-positive cells was inhibited by OPG. In the presence of M-CSF, adherent CD11b-positive cells were cultured with rhIL-17 alone (10, 30, 100 ng/ml) (a–c), with rhIL-17 (10, 30, 100 ng/ml) and OPG (125 ng/ml) (d–f), with rhIL-17 (10, 30, 100 ng/ml) and OPG (250 ng/ml) (g–i), or with rhIL-17 (10, 30, 100 ng/ml) and OPG (500 ng/ml) (j–l). Original magnification, 100×. B: Effect of L-17 and OPG on osteoclastogenesis in cultures of CD11b-positive cells. rhIL-17 (10, 30, 100 ng/ml) and OPG (125, 250, 500 ng/ml) were present during the last 7 days (4–10). After 10 days, osteoclasts positive for anti-vitronectin receptor Ab were counted. Data are expressed as the mean ± SEM for duplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.05$ vs. OPG (−). Experiments were repeated three times with similar results. C: IL-17-induced formation of osteoclasts in cultures of CD11b-positive cells was inhibited by infliximab. In the presence of M-CSF, adherent CD11b-positive cells were cultured with rhIL-17 alone (10, 30, 100 ng/ml) (a–c), with rhIL-17 (10, 30, 100 ng/ml) and infliximab (10 ng/ml) (d–f), with rhIL-17 (10, 30, 100 ng/ml) and infliximab (30 ng/ml) (g–i), or with rhIL-17 (10, 30, 100 ng/ml) and infliximab (100 ng/ml) (j–l). Original magnification, 100×. D: Effect of IL-17 and infliximab on osteoclastogenesis in cultures of CD11b-positive cells. rhIL-17 (10, 30, 100 ng/ml) and infliximab (10, 30, 100 ng/ml) were present during the last 7 days (4–10). After culture for 10 days, osteoclasts positive for anti-vitronectin receptor Ab were counted. Data are expressed as the mean ± SEM for duplicate cultures. $^{*}P < 0.05$ vs. Infliximab (−). $^{**}P < 0.01$ vs. Infliximab (−). Experiments were repeated three times with similar results.

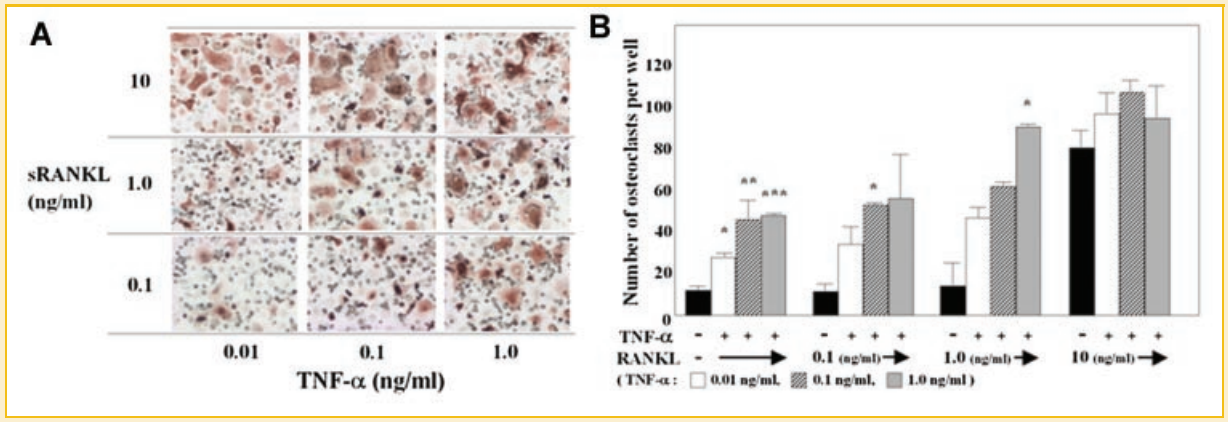

Fig. 5. A: TNF-α acted synergistically with sRANKL to induce osteoclastogenesis in cultures of CD11b-positive cells. In the presence of M-CSF, adherent cells from CD11b-positive cells were cultured with TNF-α (0.01, 0.1, 1 ng/ml), or with sRANKL (0.1, 1.0, 10 ng/ml). Original magnification, 100×. B: Effect of TNF-α and sRANKL on osteoclastogenesis in cultures of CD11b-positive cells. TNF-α (0.01, 0.1, 1.0 ng/ml) and sRANKL (0.1, 1.0, 10 ng/ml) were present during the last 7 days (4–10). After 10 days, osteoclasts positive for anti-vitronectin receptor Ab were counted. Data are expressed as the mean ± SEM for duplicate cultures. $^{*}P < 0.05$ vs. TNF-α (–), $^{**}P < 0.01$ vs. TNF-α (–), $^{***}P < 0.005$ vs. TNF-α (–). Experiments were repeated three times with similar results.

CONCENTRATION OF RANKL AND OPG ON MONOCYTES STIMULATED BY IL-17

To clarify whether purified macrophages as well as osteoblasts produce RANKL, we measured the concentration of murine RANKL in conditioned medium (Table I). When osteoblasts were stimulated with 1,25(OH)2D3 and PGE2, the concentration of RANKL was elevated from 5.5 ± 1.4 pg/ml to $3{,}671 \pm 238$ pg/ml ($P = 6.0 \times 10^{-15}$). When we used purified macrophages, the concentrations of RANKL in conditioned medium were 44.4 ± 4.4 pg/ml ($P = 2.5 \times 10^{-9}$).

Then, we measured the concentration of RANKL and OPG in conditioned medium and cell lysates of human monocytes stimulated with rhIL-17 for 24 h (Table II). When CD11b-positive cells were exposed to rhIL-17 at 0.0 and 0.1 ng/ml for 1 day, the concentration of RANKL in the cell lysates was 230.0, and 85.0 pg/ml, respectively. We did not detect RANKL when the rhIL-17 concentration was 1.0 ng/ml. Furthermore, stimulation with rhIL-17 for 24 h did not change significantly the concentration of OPG in the cell lysates. In conditioned medium, RANKL and OPG were not detected in the absence or presence of rhIL-17.

rhIL-17 CAUSED MATURE OSTEOCLASTS TO FORM ACTIN RINGS

To elucidate whether rhIL-17 has the potential to activate mature osteoclasts, we evaluated actin rings after adding rhIL-17 in cultures of mature osteoclasts formed from PBMC. Actin rings form rapidly when osteoclasts are activated. These distinctive rings represent the circumferential zone of polymerized actin at the lateral limit to the resorptive hemivacuole and are characteristic of osteoclasts that are actively resorbing bone [Lakkakorpi and Vaananen, 1991]. The formation of actin rings has been used as an indicator of the activation status of osteoclasts, because it has been reported that actin rings correlate strongly with resorptive activity [Lakkakorpi and Vaananen, 1991]. rhIL-17 induced the formation of actin rings of concentrations of 1.0 and 10 ng/ml in a dose-dependent manner (Figs. 3A,B).

TABLE I. Concentration of Murine RANKL in Culture Medium

	RANKL (pg/ml)
Purified macrophage	44.4 ± 4.4
Purified osteoblasts	5.5 ± 1.4
Purified osteoblasts + 1,25(OH)2D3 (10 nM) + PGE2 (100 nM)	$3671 \pm 238^{*}$

Murine macrophages and osteoblasts were cultured, and sRANKL concentration in culture medium were measured by ELISA. Data are expressed as the mean ± SD (N = 4).
$^{*}P = 2.5 \times 10^{-9}$, versus purified osteoblasts.

DISCUSSION

In the current study, we demonstrated that IL-17 directly induced osteoclastogenesis in cultures of CD11b-positive cells as purified monocytes, in the absence of osteoblasts or exogenous sRANKL; in contrast to our previous finding that rhIL-17 induces osteoclastogenesis in co-cultures of mouse hemopoietic cells and primary osteoblasts by stimulating RANKL expression in osteoblasts [Kotake et al., 1999]. In addition, the IL-17-induced osteoclastogenesis was completely inhibited not only by OPG, but also by infliximab. The

TABLE II. Effects of rhIL-17 on the Concentration of RANKL and OPG in Lysates of CD11b-Positive Cells or Conditioned Medium

	Cell lysate		Conditioned medium	
	RANKL (pg/ml)	OPG (pg/ml)	RANKL (pg/ml)	OPG (pg/ml)
Control	230.0	52.7	ND	ND
IL-17 (ng/ml): 0.1	85.0	55.3	ND	ND
1.0	ND	59.5	ND	ND

Separated CD11b-positive cells were cultured with rhIL-17 for 24 h. The concentrations of RANKL and OPG in cell lysates or conditioned medium were measured. ND, not detectable.

inhibition of osteoclastogenesis by adding only infliximab suggests that TNF-α, as well as RANKL, was also involved in the IL-17-mediated differentiation. Furthermore, it is noted that infliximab, even at a low concentration of 10 ng/ml, more potently inhibited osteoclastogenesis than OPG in the cultures of CD11b-positive cells alone. Thus, we speculated that TNF-α released from monocytes stimulated by IL-17 contributed markedly to IL-17-induced osteoclastogenesis.

First of all, we focused on the synergistic effect of both TNF-α and RANKL as an important mechanism or osteoclastogenesis in RA. To clarify further the mechanism of IL-17-induced osteoclastogenesis involving both TNF-α and RANKL, we demonstrated a synergistic effect on human osteoclastogenesis especially in cultures of CD11b-positive cells. The presence of both TNF-α and sRANKL markedly enhanced osteoclastogenesis, even when the level of either cytokine was low. Several reports have showed that the effect of TNF-α and RANKL in osteoclastogenesis is synergistic in animal models [Lam et al., 2000; Komine et al., 2001; Fuller et al., 2002]. O' Gradaigh et al. [2004] reported that TNF-α potently enhanced RANKL-mediated osteoclast activity. However, the present study is the first to demonstrate that the number of human osteoclasts formed from human CD11b-positive monocytes was increased by a synergistic effect of TNF-α and RANKL. Moreover, it has been reported that TNF-α accumulates in synovial fluids from RA patients [Saxne et al., 1988]. In addition, we and other groups have detected RANKL in synovial tissue and fluids from patients with RA [Kong et al., 1999; Gravallese et al., 2000; Kotake et al., 2001]. Taken together, our findings support the view that cooperation between TNF-α and RANKL plays an important role in the destruction of bone in RA patients.

We next investigated that whether monocytes/macrophages produced sRANKL both in murine and humans, because there are some differences in RANKL expression between murine and humans [Kotake et al., 2001]. We found that purified murine macrophages produced sRANKL (Table I). The findings strongly support that monocytes express RANKL and that interaction with RANK–RANKL system on monocytes themselves induces osteoclastogenesis, although RANKL production of purified monocytes were far less than those of stimulated osteoblasts. In contrast, when we used human CD11b-positive cells, RANKL was not detected in conditioned medium (Table II). Moreover, RANKL was detected in human cell lysates although rhIL-17 (0, 0.1, 1.0 ng/ml) dose-dependently decreased the concentrations of RANKL (Table II). The reason for a difference between murine and human monocytes remains unclear; however, we speculated that IL-17 does not significantly affect the level of RANKL on human monocytes in synovial fluid of RA patients because the concentrations of IL-17 in the synovial fluid and culture medium of synovial tissue from RA patients were less than 14 and 40 pg/ml, respectively, in our previous study [Kotake et al., 1999]. These results indicated that TNF-α is more important to IL-17-induced osteoclastogenesis than RANKL. Thus, suppression of TNF-α had a marked inhibiting effect on osteoclastogenesis induced by IL-17.

In the current study, rhIL-17 caused mature osteoclasts derived from PBMCs to form actin rings. Chabaud and Miossec [2001] reported that the blockade of TNF-α combined with the blockade of both IL-1 and IL-17 is more effective in controlling bone resorption ex vivo, indicating that IL-17, TNF-α, and IL-1 have independent effects on osteoclasts. Our results suggests that IL-17 directly activates mature osteoclasts, inducing activity for bone

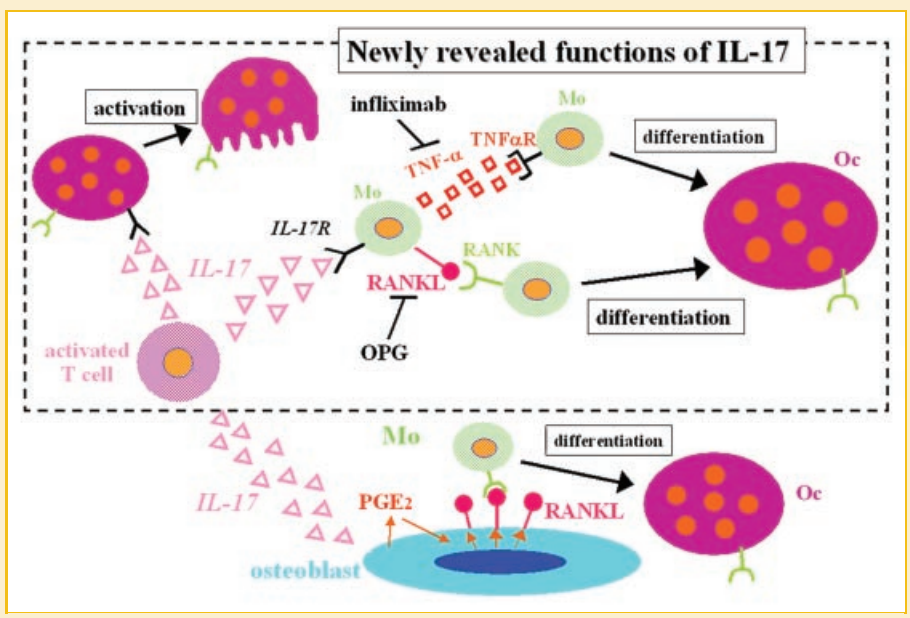

Fig. 6. Novel function of IL-17 in human monocytes. IL-17 acts on monocytes, resulting in TNF-α production, inducing the differentiation of monocytes into mature osteoclasts by cooperating with RANKL. In addition, RANKL on monocytes induces differentiation of osteoclasts by binding to RANK. Infliximab, anti-TNF-α antibody, potently inhibited IL-17-induced osteoclastogenesis even at a low concentration, indicating that anti-TNF-α therapy is effective for bone resorption. On the other hand, the osteoclastogenesis induced by IL-17 can be blocked by OPG, a decoy receptor of RANKL. IL-17 also promotes mature osteoclast activation.

resorption, which concurs with Chabaud's report [Chabaud and Miossec, 2001].

In summary, IL-17 directly induced osteoclastogenesis in cultures of human monocytes alone (Fig. 6). The TNF-α produced by monocytes stimulated with IL-17 cooperated with the RANKL expressed on the cells to have a marked synergistic effect on the differentiation of osteoclasts. Infliximab potently inhibited IL-17-induced osteoclastogenesis by blocking TNF-α, consistent with the clinical effect of anti-TNF-α antibody treatment in preventing bone destruction. The RANK–RANKL pathway was involved in IL-17-induced osteoclastogenesis as well. Moreover, IL-17 also promoted the formation of actin rings of mature osteoclasts. Thus, the combined effects of TNF-α, RANKL, and IL-17, levels of which are all elevated in RA patients, contribute to osteoclastic bone resorption in RA. Thus, the newly revealed mechanism of osteoclastogenesis beginning with an excess of IL-17 has a significant role in the destruction of bone in RA patients, suggesting that control of IL-17 production and function could be a strategy for the treatment of RA.

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