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IL-1α Stimulation of Osteoclast Survival through the PI 3-Kinase/Akt and ERK Pathways[1]

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Osteoclasts, cells that resorb bone, die once fully differentiated. Several factors including interleukin-1 (IL-1) have been shown to regulate the survival of mature osteoclasts. However, information on the mechanism underlying the regulation of osteoclast survival has been limited. In this study, we investigated the mechanism for the IL-1-stimulated survival of osteoclasts. Treatment of purified osteoclasts with IL-1α led to activation of the serine-threonine kinases Akt and ERK. Blocking the activation of Akt with LY294002, a specific inhibitor of the Akt up-stream molecule PI 3-kinase, or with an adenoviral vector for a dominant-negative form of Akt prevented the stimulation of osteoclast survival by IL-1α. PD98059, a specific inhibitor of the ERK-activating kinase MEK1, also abolished the effects of IL-1α on ERK activation and osteoclast survival. IL-1α reduced the apoptosis of osteoclasts by reducing caspase 3 activity. The IL-1α-mediated suppression of apoptosis was abolished by the PI 3-kinase/Akt or MEK1/ERK pathway inhibitor. These findings implicate the PI 3-kinase/Akt and ERK signaling pathways in the promotion of osteoclast survival by IL-1α.

Key words: Akt, ERK, interleukin-1, osteoclast, survival.

Interleukin 1 (IL-1), a cytokine produced by monocytes-macrophages and bone marrow stromal cells, promotes the formation and bone-resorbing activity of osteoclasts (*1, 2*). The osteotropic effect of IL-1 has been ascribed to an indirect action on osteoblasts/stromal cells, i.e. the induction of the expression of osteoclast differentiation factor (ODF), a key osteoclastogenic molecule that directly binds to its cognate receptor, receptor activator of NF-κB (RANK), on osteoclast precursor cells (*3*). However, evidence of a direct action of IL-1 on osteoclasts has also been reported. Jimi *et al.* (*4*) showed that IL-1 stimulated the formation of mature multinucleated osteoclasts and the bone-resorbing function of osteoclasts in the absence of osteoblasts/stromal cells. In addition to its stimulatory effect on the differentiation and activity of osteoclasts, IL-1 was shown to support the survival of both prefusion mononuclear and mature multinuclear osteoclasts (*4, 5*). The combination of these effects of IL-1 may account for the strong *in vivo* bone-resorbing activity of this cytokine (*6*).

IL-1 has been shown to stimulate the transcription factors activating protein–1 and NF-κB through the activation of c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) (*7, 8*). The physiological significance of IL-1 activation of NF-κB was supported by a study involving mice deficient in both the p50 and p52 subunits of NF-κB, which exhibited a lack of osteoclastogenesis and consequent osteopetrosis (*9*). On the other hand, accumulating evidence has implicated NF-κB activation in cell survival (*10*). Accordingly, the IL-1–promoted survival of osteoclasts was shown to be suppressed by antisense oligomers to the p50 and p65 subunits of NF-κB (*11*). However, a recent study involving an adenoviral gene transduction system of dominant negative and constitutively active forms of signaling molecules demonstrated that the extracellular signal–regulated kinase (ERK) rather than the NF-κB pathway is involved in the survival of osteoclasts, NF-κB rather playing a role in the activation of osteoclasts, raising a question as to the implication of NF-κB in osteoclast survival (*12*).

Besides NF-κB activation, other mechanisms have been reported to operate to support cell survival by suppressing apoptosis. The Ras/ERK pathway has been shown to be an important component for the survival of neuronal and hematopoietic cells (*13–15*). In addition, the PI 3-kinase/Akt signaling pathway has been demonstrated to stimulate the survival of various cell types. PI 3-kinase–dependent activation of Akt leads to phosphorylation and inactivation of BAD, which subsequently prevents apoptotic cell death by blocking the formation of a complex of BAD with the apoptotic proteins Bcl-2 and Bcl-xL (*16*).

In this study, we investigated whether or not the PI 3-

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Abbreviations: IL-1, interleukin-1; RANK, receptor activator of NF-κB; ERK, extracellular signal–regulated kinase; PI, phosphatidylinositol; ODF, osteoclast differentiation factor; TRAP, tartrate-resistant acid phosphatase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase

kinase/Akt and ERK signaling pathways are involved in the IL-1–stimulated survival of osteoclasts. IL-1 promoted the survival of mature osteoclasts by reducing apoptotic cell death. This effect was attenuated when the activation of Akt and ERK was blocked with specific inhibitors. Furthermore, IL-1 induced the activation of Akt and ERK in mature osteolcasts, which was abolished by the inhibitors.

MATERIALS AND METHODS

Osteoclast Culture—Mature osteoclasts were obtained from cocultures of mouse bone marrow cells and primary osteoblastic cells as previously described (*17*). Briefly, 1×10^7 bone marrow cells prepared from the tibiae of 6–8-week-old ICR mice, and 1×10^6 calvarial osteoblasts were seeded on to a collagen gel–coated 90-mm dish and cultured in the presence of 10^{-8} M 1α,25-dihydroxyvitamin D3 (VtD_3) and 10^{-6} M prostaglandin E2 (PGE_2). The complete culture medium was changed every three days. After 6 days culture, the cells were harvested by treatment with 0.2% collagenase (Gibco BRL, Gaithersburg, MD, USA) at 37°C for 10 min, replated on a plain 6- or 48-well culture plate, and then cultured for another day. Osteoblasts were then removed by treating the plate with 0.1% collagenase at 37°C for 30 min followed by rigorous pipetting. The remaining cells were more than 95% pure osteoclasts, as determined by tartrate-resistant acid phosphatase (TRAP) cytochemistry as described below.

Osteoclast Survival Assay—After removal of osteoblasts as above, the remaining cells were incubated in the presence or absence of IL-1α (R&D Systems, Minneapolis, MN, USA) for 24 h. Detached cells were removed by washing twice with medium and the remaining cells were stained for TRAP enzyme with a Leukocyte Acid Phosphatase Assay Kit (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. TRAP-positive cells with five or more nuclei were counted. The survival rate was calculated as the percentage of TRAP-positive multinuclear cells remaining after 24-h treatment compared to that at the beginning of the treatment.

Adenovirus Infection—Recombinant adenoviruses carrying the dominant-negative Akt and β-galactosidase have been described (*18*). For the infection of osteoclasts with adenoviruses, mouse bone marrow cells were cocultured with calvarial osteoblasts for 5 days as above. After removing the medium, the cells were incubated with a small amount of medium containing the recombinant adenovirus carrying either a dominant-negative form of Akt or β-galactosidase at a multiplicity of infection (MOI) of 100 for 1 h at 37°C. The cells were then washed with α-MEM, and incubated in α-MEM/10% FBS containing 10^{-8} M VtD_3, and 10^{-6} M PGE_2 for 4 h at 37°C. The plates were then treated with collagenase as described above, and the cells were replated on 48-well plates and cultured for 24 h. After removing the osteoblasts, 20 ng/ml IL-1α was added and osteoclast survival assays were performed as described above.

Caspase Assay—Purified mature osteoclasts were treated with or without IL-1α for 7 h. After brief rinsing in PBS, the cells were lysed in the Cell Lysis Buffer included in the Caspase 3 Colorimetric Assay Kit (R&D Systems). The cell lysates were concentrated with Centricons (mol. wt. cut-off 3,000; Millipore) to obtain protein concentrations of 2–4 mg/ml. The enzymatic reaction for caspase 3 activity was carried out with the *p*-nitroanilide conjugated DEVD peptide (DEVD-pNA) substrate as described by the manufacturer.

Western Blotting—Cell lysates were prepared in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors, and Western blotting analyses were performed as described (*19*). 10–20 μg of cellular proteins was resolved by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-phospho Akt (New England Biolabs, Beverly, MA, USA) or anti-phospho ERK (New England Biolabs). The same membrane was stripped and reprobed with anti-Akt (New England Biolabs) or anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the detection of caspase 3 activation, membranes were probed with a caspase 3 antibody that recognizes 17- and 19-kDa fragments of the cleaved caspase 3 protein (New England Biolabs #9661).

Apoptosis Analysis—Purified osteoclasts were incubated in the presence or absence of IL-1α for 9 h. After rinsing once in PBS, the cells were fixed with 10% formaldehyde for 5 min at 20°C and then incubated with 4,6-diamidino-2-phenylindole (DAPI) 1 μg/ml in PBS for 20 min. Cells showing condensed chromatin and fragmented nuclei were scored as being apoptotic.

RESULTS AND DISCUSSION

Direct Stimulation of the Survival of Mature Osteoclasts by IL-1—To determine whether IL-1 promotes osteoclast survival through a direct action on osteoclasts or through an indirect effect, *i.e.* the stimulation of the expression of a osteoclast survival factor by the supporting cells, we incubated purified osteoclasts (see "MATERIALS AND METHODS") in the presence or absence of IL-1α and then assessed the survival rate. Within 24 h, more than 90% of the osteoclasts died, losing contact with the culture plates in the absence of IL-1α (Fig. 1, A and B). Treatment with IL-1α significantly increased the survival rate of osteoclasts in a dose-dependent manner (Fig. 1B). IL-1 has been shown to stimulate osteoblasts/stromal cells to express ODF, which promotes the survival as well as the differentiation and activation of osteoclasts (*20, 21*). Thus, we determined whether or not the IL-1α–induced survival was mediated by ODF that might have been expressed on osteoblasts, even though the purity of the osteoclasts in our culture was determined to be more than 95%. The addition of osteoprotogerin (OPG), the decoy receptor for ODF that blocks the binding of ODF to its receptor, did not have a significant effect on the IL-1 stimulation of osteoclast survival (Fig. 1B). These results suggest that IL-1α promotes osteoclast survival, probably by acting directly on osteoclasts.

Reduction in Caspase 3 Activity upon IL-1 Treatment—The morphology of dying osteoclasts showed apoptotic characteristics including chromosomal condensation and nuclear fragmentation (data not shown). Apoptosis is processed by caspases, a family of cysteine proteases. Activation of caspase 3 frequently occurs in various cell types in response to diverse apoptotic stimuli. By cleaving ICAD (inhibitor of caspase-activated DNase), activated caspase 3 allows the release of CAD from the inhibitory activity of ICAD and the subsequent chromatin DNA fragmentation (*22*). We investigated the effect of IL-1α on caspase 3 activ-

ity in our osteoclast cultures. When purified osteoclasts were incubated for 7 h in the absence of any survival factor,

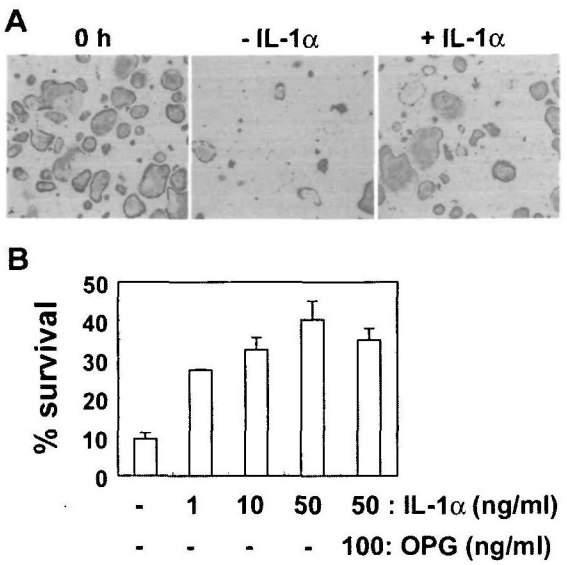

Fig. 1. **IL-1–stimulated survival of mature osteoclasts.** (A) Differentiated osteoclasts were purified from cocultures of mouse bone marrow cells and osteoblasts as described under "MATERIALS AND METHODS." Cells were incubated in the presence (+ IL-1) or absence (– IL-1) of 20 ng/ml IL-1α for 24 h. Cells before (0 h) and after the 24-h incubation were stained for TRAP. (B) Purified osteoclasts were incubated with the indicated concentrations of IL-1α and OPG for 24 h, and then TRAP-stained. The percentage of starting TRAP-positive multinuclear osteoclasts remaining after the incubation was determined. The data for triplicate samples are shown as means ± SD. Representative results of three experiments are presented.

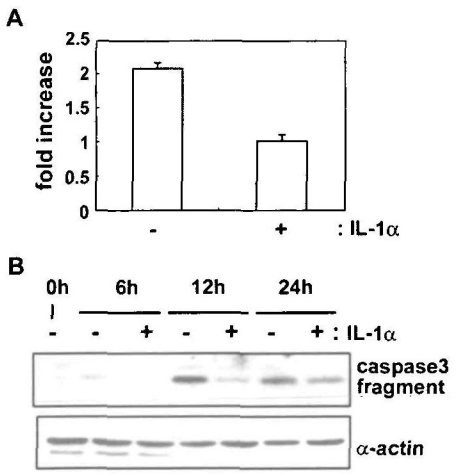

Fig. 2. **Suppression of caspase 3 activity by IL-1 in osteoclasts.** (A) Osteoclasts prepared as described under "MATERIALS AND METHODS" were treated with or without 20 ng/ml IL-1α for 7 h, and then the cells were lysed. Caspase 3 activity in lysates was measured using the DEVD-pNA substrate. The values are means ± SD for triplicate samples. (B) Purified osteoclasts were treated with 20 ng/ml IL-1α for the indicated times. Cell lysates were prepared and Western blotting was performed with an antibody for cleaved caspase 3 fragments (top panel). The same membrane was stripped and reprobed with anti–α-actin (bottom panel). Similar results were obtained in a separate experiment.

strong stimulation of caspase 3 activity was observed in a catalytic activity assay (Fig. 2A). The addition of IL-1α during the incubation period significantly attenuated the caspase 3 activation (Fig. 2A). As caspase 3 becomes activated upon cleavage by upstream caspases, Western blotting of the cleaved fragments of caspase 3 can also be used to assess the extent of caspase 3 activation. In the purified osteoclasts, caspase 3 fragments were clearly detected after 6 h incubation and the level of the fragments had further increased by 12 h (Fig. 2B). The addition of IL-1α greatly reduced the caspase 3 fragmentation (Fig. 2B).

Role of PI 3-Kinase/Akt in IL-1α–Induced Osteoclast Survival—The PI 3-kinase/Akt signaling pathway has been demonstrated to have an antiapoptotic function in response to various stimuli (*23*). Therefore, we examined whether or not this pathway is involved in the IL-1α–mediated osteoclast survival. Mature osteoclasts were stimulated with IL-1α in the presence or absence of a specific PI 3-kinase inhibitor, LY294002, and then the survival rate was assessed. LY294002 greatly inhibited the effect of IL-1α on the survival of mature osteoclasts (Fig. 3A). Generation of the 3-phosphoinositide lipids by activated PI 3-kinase leads to the recruitment and activation of phosphoinositide-dependent kinase–1 (PDK1), which phosphorylates Akt at Thr 308 in the activation loop. Thus, the activation of Akt is often assessed by means of Western blotting with a phopho-Akt specific antibody. We examined the effect of LY294002

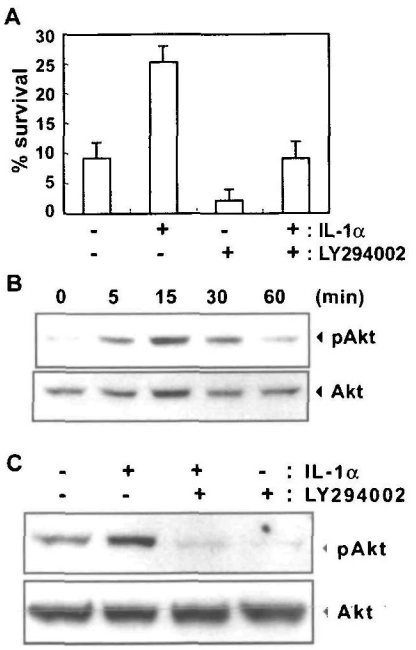

Fig. 3. **Involvement of PI 3-kinase/Akt in IL-1–stimulated osteoclast survival.** (A) Purified osteoclasts were incubated with or without 20 ng/ml IL-1α in the presence or absence of 400 nM LY294002 for 24 h. The survival of TRAP-positive multinuclear cells is shown as the mean ± SD. (B) Purified osteoclasts were incubated in the medium containing 0.5% FBS for 5 h and the stimulated with 20 ng/ml IL-1α for the indicated times. Cell lysates were prepared and subjected to Western blotting with anti-phospho-Akt (top panel). The membrane was reprobed with anti-Akt (bottom panel). (C) Cells were pretreated with or without 20 μM LY294002, and then stimulated with 20 ng/ml IL-1α for 30 min. Western blotting analyses with anti-phospho-Akt (top panel) and anti-Akt (bottom panel) were performed. Similar results were observed in two other experiments.

on the extent of Akt phosphorylation. IL-1α induced the phosphorylation of Akt by 5 min, which had decreased to the basal level by 60 min (Fig. 3B). This IL-1α–induced Akt phosphorylation was completely blocked by treatment with LY294002 (Fig. 3C).

To more directly examine the role of Akt in IL-1α–induced osteoclast survival, we utilized a dominant-negative form of Akt. Replication-defective adenoviruses carrying a dominant-negative mutant Akt (T308A, S473A) (*18*) were used to infect differentiated osteoclasts and then the extent of IL-1α–dependent survival was assessed. The recombinant adenovirus carrying β-galactosidase was used as a control. Adenovirus infection itself seemed to have no deleterious effect on osteoclasts (data not shown). Infection with the dominant-negative Akt adenoviruses reduced the survival effect of IL-1α by ~70% compared to that in the case of the control β-galactosidase viruses (Fig. 4).

Involvement of the ERK Pathway in Osteoclast Survival Promoted by IL-1α—ERK is another signaling component implicated in the survival of certain cell types (*13–15*). Therefore, we examined whether or not the ERK signaling pathway plays a role in the IL-1α–mediated survival of mature osteoclasts. PD98059, which is known to specifically inhibit the ERK-activating kinase MEK1, has been widely used to assess the effects of dampening ERK activation. When we included this inhibitor in the culture, the effect of IL-1α on osteoclast survival was abolished (Fig. 5A). The activation of ERK was then determined by Western blotting with an antibody specific for the phosphorylated form of ERK1 and ERK2. IL-1α induced transient phosphorylation of both ERK1 and ERK2, which reached the peak at 15 min and had returned to the basal level by 30 min (Fig. 5B). The treatment with PD98059 abrogated the increase in ERK phosphorylation induced by IL-1α (Fig. 5C).

Induction of Apoptosis by PI 3-kinase and ERK Inhibition in Osteoclasts—We next examined the effects of the PI 3-kinase and ERK pathway inhibitors on the apoptosis of osteoclasts. The cells that showed condensed and fragmented nuclei on DAPI staining were considered to be apoptotic. As shown in Fig. 6, treatment with IL-1α significantly reduced the percentage of apoptotic osteoclasts. This antiapoptotic effect of IL-1α was blocked by treatment with LY294002 (lane 3) or PD98059 (lane 5). PD98059 not only blocked the IL-1–dependent reduction of apoptosis but also increased the percentage of apoptotic cells compared to the control (lane 1 *vs.* 6), suggesting that blocking of the ERK pathway accelerated the apoptosis of mature osteoclasts in the absence of any survival factor.

In this study, we identified new mechanisms by which IL-1α stimulates the survival of differentiated osteoclasts. The PI 3-kinase/Akt and ERK signaling pathways were found to mediate the IL-1α–induced osteoclast survival by suppressing apoptosis. As the IL-1α–promoted survival was observed with highly purified cultures of osteoclasts and the effect of IL-1α was not affected by OPG (Fig. 1), IL-1α was likely to act directly on osteoclasts rather than exerting its effect by inducing the expression of ODF, which also stimulates the survival of osteoclasts (*20, 21*). In accordance with this notion, we could not detect any ODF expression in either the unstimulated or IL-1α–stimulated purified osteoclasts used in our study by RT-PCR analyses under the conditions that permitted a clear detection of IL-1α–stimulated ODF expression in osteoblasts (data not

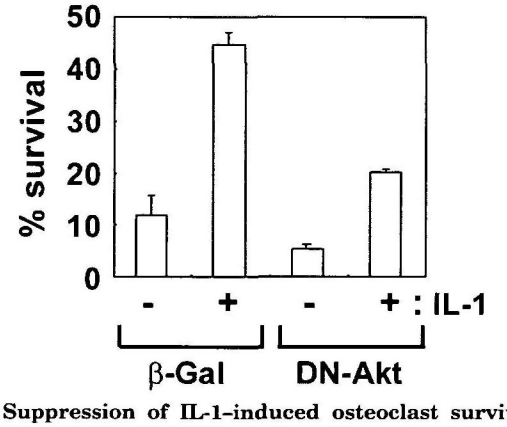

Fig. 4. **Suppression of IL-1–induced osteoclast survival by dominant-negative Akt.** Osteoclast cocultures were infected with recombinant adenoviruses for a dominant-negative form of Akt or β-galactosidase and then replated in 48-well plates as described under "MATERIALS AND METHODS." After removing osteoblasts, cells were treated with or without 20 ng/ml IL-1α for 24 h, and then the percentages of surviving cells were determined. Similar observations were made in another expeiment.

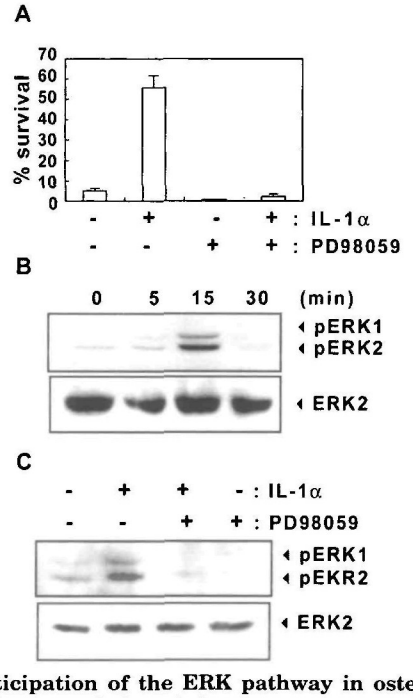

Fig. 5. **Participation of the ERK pathway in osteoclast survival.** (A) The survival of purified osteoclasts was determined as described under "MATERIALS AND METHODS" after 24-h incubation with 20 ng/ml IL-1α in the presence or absence of 40 μM PD98059. The percentage of surviving TRAP-positive multinuclear cells is shown as the mean ± SD. (B) Cells were serum-deprived in the medium containing 0.5% FBS for 5 h and then stimulated with 20 ng/ml IL-1α for the indicated times. Western blotting analyses with anti–phospho-ERK (top panel) and then with anti-ERK2 (bottom panel) were performed. (C) Osteoclasts were pretreated with 40 μM PD98059 for 15 min, and then cultured in the presence or absence of 20 ng/ml IL-1α for 15 min and Western blotted with anti–phospho-ERK (top panel). The same membrane was reprobed with anti-ERK2 (bottom panel). Representative results of three experiments are shown.

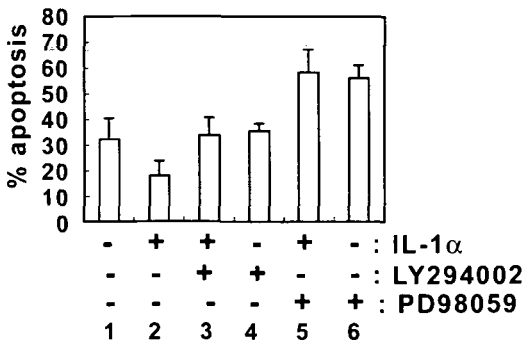

Fig. 6. **Effects of IL-1, LY294002, and PD98059 on the apoptosis of osteoclasts.** Purified osteoclasts were incubated for 9 h with the indicated combinations of 20 ng/ml IL-1α, 400 nM LY294002, and 10 μM PD98059. Cells were then stained with DAPI as described under "MATERIALS AND METHODS." Cells with apoptotic nuclei were scored. Data for an experiment performed in triplicate are presented as means ± SD. Similar results were obtained in another experiment.

shown). Osteoclasts have been shown to express IL-1 receptor 1 (IL-1R1) (*11*). Binding of IL-1 to IL-1R1 induces aggregation of the receptor and the receptor-associated protein IL-1RAcP (IL-1R accessory protein), which recruits MyD88 and IRAK (IL-1R–associated kinase) (*24*). Then the activated IRAK forms a complex with TRAF6 (tumor necrosis factor receptor–associated factor 6), leaving the IL-1 receptor complex (*25*). Unlike IL-1R, TRAF6 can directly bind RANK, a TNF receptor family member important for the differentiation, activation, and survival of osteoclasts (*20, 26*). The RANK-TRAF6 interaction has been suggested to induce the PI 3-kinase/Akt activation through Src family kinases in response to ODF (*27*). Whether or not the involvement of PI 3-kinase/Akt in the IL-1 stimulation of osteoclast survival is mediated by TRAF6 and Src family kinases warrants further investigation. The signal for ERK activation in IL-1 stimulated osteoclasts may also be transmitted through TRAF6. TRAF6 has been reported to activate ERK through a Ras-independent pathway in CD40 signaling (*28*). Lastly, TRAF6 is a strong activator of NF-κB, which impedes apoptosis by inducing the expression of antiapoptotic molecules such as inhibitor-of-apoptosis proteins (IAP) (*29*). Upon IL-1 stimulation, the PI 3-kinase/Akt and ERK pathways may operate rapidly to prevent the initiation of apoptosis, whereas the NF-κB pathway may have effects at a later time point to sustain the survival of osteoclasts.

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