The Mechanism of Osteoclast Differentiation Induced by IL-1

Jung Ha Kim, Hye Mi Jin, Kabsun Kim, Insun Song, Bang Ung Youn, Koichi Matsuo and Nacksung Kim

*J Immunol* 2009; 183:1862-1870; Prepublished online 8 July 2009;
doi: 10.4049/jimmunol.0803007

http://www.jimmunol.org/content/183/3/1862

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/07/07/jimmunol.0803007/7.DC1

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article *cites 43 articles*, 20 of which you can access for free at:

http://www.jimmunol.org/content/183/3/1862.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Mechanism of Osteoclast Differentiation Induced by IL-1

Jung Ha Kim,* Hye Mi Jin,* Kabsun Kim,* Insun Song,* Bang Ung Youn,* Koichi Matsuo,† and Nacksung Kim2*

IL-1 is a potent cytokine that can induce bone erosion in inflammatory sites such as rheumatoid joint regions via activation of osteoclasts. Not only is IL-1 capable of activating osteoclasts, but it is also a key cytokine involved in the differentiation, multinucleation, and survival of osteoclasts. Herein, we show that IL-1 has the potential to drive osteoclast differentiation via a receptor activator of NF-κB ligand (RANKL)/RANK-independent mechanism. Although IL-1 has a synergistic effect on RANKL-induced osteoclast formation, IL-1 alone cannot induce osteoclast differentiation from osteoclast precursors (bone marrow-derived macrophages (BMMs)) due to a lack of IL-1 signaling potential in these cells. However, we demonstrate that overexpression of the IL-1RI receptor in BMMs or induction of IL-1RI by c-Fos overexpression enables IL-1 alone to induce the formation of authentic osteoclasts by a RANKL/RANK-independent mechanism. The expression of IL-1RI is up-regulated by RANKL via c-Fos and NFATc1. Furthermore, the addition of IL-1 to IL-1RI overexpressing BMMs (IL-1/IL-1RI) strongly activates NF-κB, JNK, p38, and ERK which is a hallmark gene activation profile of osteoclastogenesis. Interestingly, IL-1/IL-1RI does not induce expression of c-Fos or NFATc1 during osteoclast differentiation, although basal levels of c-Fos and NFATc1 seem to be required. Rather, IL-1/IL-1RI strongly activates MITF, which subsequently induces osteoclast-specific genes such as osteoclast-associated receptor and tartrate-resistant acid phosphatase. Together, these results reveal that IL-1 has the potential to induce osteoclast differentiation via activation of microphthalmia transcription factor under specific microenvironmental conditions. The Journal of Immunology, 2009, 183: 1862–1870.

Osteoclasts are responsible for bone resorption, a process which is regulated by various factors and is delicately balanced with bone formation (1, 2). A disrupted balance between bone resorption and formation can lead to bone diseases such as osteoporosis. Osteoclasts are formed from hematopoietic monocyte/macrophage cells in response to macrophage CSF (M-CSF)3 and receptor activator of NF-κB ligand (RANKL) (1, 3–7). RANKL, a TNF family member, is expressed on the surface of osteoblasts and is essential for osteoclast differentiation (5–9). Binding of RANKL to its receptor, RANK, activates a cascade of transcription factors including c-Fos, microphthalmia transcrip- tion factor (MITF), PU.1, and NF of activated T cells (NFAT) c1, all factors that are known to be important for osteoclastogenesis (1, 3, 10).

RANK, similar to other TNF receptor family members, interacts with TNF receptor-associated factors (TRAFs), which in turn act as adaptors to downstream signaling pathways (11). Of the six known TRAFs, RANK interacts with TRAFs 1, 2, 3, and 5 in a membrane-distal region of the cytoplasmic tail, and with TRAF6 at a distinct membrane-proximal binding motif (12). TRAF6 appears to be the most crucial adapter for RANK signaling during osteoclastogenesis in vivo and in vitro, indicating that RANKL-induced signaling is predominantly mediated by TRAF6 during osteoclastogenesis (13–15).

The proinflammatory cytokine IL-1 binds two surface IL-1 receptors, type I (IL-1RI) and type II (IL-1RII). Both receptors have a single transmembrane domain and an IgG-like extracellular domain. IL-1RI has a conserved region of 212 amino acids in the cytoplasmic tail, which is known as the Toll/IL-1R domain. IL-1RII contains a signaling-incompetent cytoplasmic domain of only 29 amino acids and acts as a negative regulator of IL-1 signaling by serving as a docking site for IL-1, thereby preventing its interaction with IL-1RI. Upon IL-1 binding, IL-1RI homodimerizes and undergoes a conformational change required for the recruitment of downstream signaling molecules including MyD88, IRAKs, TAK1, TAB1, TAB2, and TRAF6 (16).

Even though IL-1RI uses TRAF6 as a signaling adaptor molecule, it has been shown that IL-1 does not directly induce osteoclast formation (17, 18) It is worth noting, though, that the detailed mechanisms of IL-1-mediated downstream signaling pathways have yet to be revealed. Herein, we demonstrate that IL-1 can directly induce osteoclast formation if IL-1RI is overexpressed in osteoclast precursor cells. Unlike RANKL, IL-1 does not induce the expression of c-Fos and NFATc1, although basal levels of both genes seem to be required. Furthermore, IL-1 strongly induces expression of osteoclast-specific genes such as

*National Research Laboratory for Regulation of Bone Metabolism and Disease, Medical Research Center for Gene Regulation, Brain Korea 21, Chonnam National University Medical School, Gwangju, Korea; and †Department of Microbiology and Immunology, School of Medicine, Keio University, Tokyo, Japan

Received for publication September 10, 2008. Accepted for publication May 26, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work is supported in part by the Korea Science and Engineering Foundation (KOSEF) National Research Laboratory (NRL) Program Grant funded by the Korean government (MEST) (ROA-2007–000-20025–0); Grant R13–2002-013–03001–0 from the Korea Science and Engineering foundation through the Medical Research Center for Gene Regulation at Chonnam National University; the Korea Health 21 R&D Project (A060164) from Ministry of Health and Welfare. J.H.K. and K.K. were supported by the Brain Korea 21 Project.

Address correspondence and reprint requests to Dr. Nacksung Kim, Department of Pharmacology, Chonnam National University Medical School, Hak-Dong 5, Dong-Ku, Gwangju, Korea. E-mail address: nacksung@chonnam.ac.kr

Abbreviations used in this paper in this paper: M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; MITF, microphthalmia transcription factor; NFAT, NF of activated T cell; TRAF, TNF receptor-associated factor; IL-1RI, IL-1 receptor; BMM, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; OSCAR, osteoclast-associated receptor; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-related kinase; EMSA, electrophoretic mobility shift assay.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/52.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803007
tartrate-resistant acid phosphatase (TRAP) and osteoclast-associated receptor (OSCAR) via activation of MITF. Thus, our data indicate that IL-1 has the potential to directly induce osteoclast formation in the context of suitable microenvironmental conditions.

Materials and Methods

Reagents
All cell culture medium and supplements were obtained from Invitrogen. Soluble recombinant mouse RANKL and RANK-Fc were purified from insect cells as described previously (19). M-CSF, TGF-β, and IL-1α were purchased from R&D Systems. Cyclosporine A was purchased from Calbiochem. Texas red-X phalloidin was obtained from Invitrogen.

 Constructs
The promoter regions of human IL-1RI (2 Kb) were amplified by PCR using human genomic DNA. The amplified PCR fragments were cloned into the pGL3 basic luciferase vector (Promega). Murine IL-1RI or c-Fos was amplified by RT-PCR and cloned into pMX-IRES-EGFP vector. The oligonucleotide sequences for retroviral and promoter luciferase constructs are provided in supplementary Table I.4 A retroviral vector containing constitutively active NFATc1 was previously described (20).

Osteoclast formation
Murine osteoclasts were prepared from bone marrow cells or splenocytes as previously described (21, 22). Spleen cells from c-Fos-deficient mice and littermates were provided by Dr. Koichi Matsuo (Keio University, Japan) (23). In brief, cells from bone marrow or spleens were cultured in α-MEM containing 10% FBS with M-CSF (30 ng/ml) alone or with M-CSF (30 ng/ml) plus TGF-β (1 ng/ml). After 3 days of culture, floating cells were removed, and the attached cells, bone marrow-derived macrophages (BMMs), were used as osteoclast precursors. To generate osteoclasts, BMMs were further cultured with various combinations of M-CSF (30 ng/ml), RANKL (100 ng/ml), and IL-1α (10 ng/ml), as indicated in the figures. When osteoclasts were derived by M-CSF and IL-1α from BMMs, BMMs were prepared by treatment with M-CSF and TGF-β. After an additional 3–6 days of culture, cells were fixed and stained for TRAP as previously described (19). TRAP-positive multinucleated cells containing more than three nuclei were considered TRAP+ multinuclear osteoclasts (TRAP+ MNCs). MNCs were inoculated at all times. In some experiments, RANK-Fc (2 μg/ml) was added to the cultures.

Pit formation assay and F-actin ring staining
Bone marrow cells were incubated for 3 days with M-CSF (30 ng/ml) alone or with M-CSF (30 ng/ml) plus TGF-β (1 ng/ml) to generate BMMs. BMMs were plated on dentine slices or calcium plates (Oscotec) and were cultured for an additional 3–6 days with various combinations of M-CSF (30 ng/ml), RANKL, and IL-1α (10 ng/ml), as indicated in the figures. In some experiments (shown in Fig. 4B) BMMs were infected with control or c-Fos retrovirus and were cultured with an additional 3–6 days with various combinations of M-CSF (30 ng/ml), RANKL (100 ng/ml), IL-1 (10 ng/ml), and RANK-Fc (2 μg/ml), as indicated. The slices were then recovered, cleaned by ultrasonication in 0.5 M H9262 acid to remove adherent cells, and stained with toluidine blue to visualize resorption pits. For F-actin ring staining, BMMs were prepared with M-CSF and TGF-β and were infected with control or IL-1RI retrovirus, and further cultured for 3–6 days with various combinations of M-CSF (30 ng/ml), RANKL (100 ng/ml), IL-1 (10 ng/ml), and RANK-Fc (2 μg/ml), as indicated in Fig. 2C. Cultured cells were fixed and stained with Texas red-X phalloidin.

Retroviral infection
Transduction with retrovirus was performed as previously described (24, 25). In brief, Plat-E cells were transfected with pMX-IRES-EGFP (control) or pMX-IRES-EGFP vector containing IL-1RI or c-Fos. Viral supernatant was collected from cultured medium 24–48 h after transfection. BMMs were incubated with viral supernatant for 8 h in the presence of polybrene (10 μg/ml). After removing viral supernatant, BMMs were cultured for an additional 3–6 days with various combinations of M-CSF (30 ng/ml), RANKL (100 ng/ml), IL-1 (10 ng/ml), and RANK-Fc (2 μg/ml), as indicated in the figures.

Semiquantitative RT-PCR
RT-PCR analysis was performed using cDNA from wild-type or c-Fos-deficient osteoclasts as previously described (26, 27). Primer sequences are provided in supplementary Table II.

Western blot analysis
Cells from BMMs or osteoclasts were harvested after washing with ice-cold PBS and then lysed in extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1% protease inhibitor mixture). Forty micrograms of whole cell lysates were resolved by 10% SDS-PAGE and analyzed by Western blotting. Primary Abs used at 1/1,000 dilution included IκB, p-ERK, ERK, p-JNK, JNK, p-p38, p38 (Cell Signaling Technology), actin, and Flag (Sigma-Aldrich). HRP-conjugated secondary Abs (Amersham Biosciences) were probed at 1/5,000 dilution and developed with ECL solution (Amersham Biosciences). Signals were detected and analyzed by LAS3000 luminescent image analyzer (Fuji).

Electrophoretic mobility shift assay (EMSA)
Osteoclast nuclear extracts were made as previously described (22). The double strand oligonucleotide probes containing the E-box site of the TRAP and OSCAR promoter regions were labeled using T4 polynucleotide kinase (New England Biolabs). In brief, 50,000 cpm-labeled probes were incubated with 5 μg of nuclear extract and 1 μg of poly (dl-dc) in 20 μl reaction buffer consisting of 10 mM Tris (pH 7.4), 0.2 mM DTT, 0.1 M KCl, and 5% glycerol. Samples were incubated at room temperature for 30 min and resolved on a 4% native polyacrylamide gel in 0.5× TBE buffer. Primer sequences for the EMSA assay are provided in supplementary Table III.

Transfection and luciferase assay
For transfection of reporter plasmids, 293T cells were plated into 24-well plates (2 × 105 cells/well) 24 h before transfection. Plasmid DNA was mixed with FuGENE6 (Roche Applied Sciences) and transfected into the cells following the manufacturer’s protocol. After 48 h of transfection, the cells were washed twice with PBS and then lysed in passive lysis buffer (Promega). Luciferase activity was measured using a dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

Chromatin immunoprecipitation (ChIP) assay
A ChIP assay was performed with a ChIP kit (Upstate Biotechnologies) according to the manufacturer’s instructions, using Abs against MITF (Calbiochem) or IgG (control). The precipitated DNA was subjected to PCR amplification with primers specific for the promoter regions of genes containing MITF-binding sites. The primer sequences are provided in supplementary Table IV.

Results

IL-1 has the potential to replace RANKL during the late stage of osteoclast differentiation

Although RANKL and IL-1 share important signaling components for osteoclastogenesis, it has been shown that IL-1 cannot directly induce osteoclast differentiation (17, 28, 29). To investigate the different abilities of both cytokines to induce osteoclastogenesis, we first confirmed that IL-1 alone cannot generate osteoclasts in our system. Consistent with previous results (17), whereas RANKL can induce formation of TRAP-positive multinuclear osteoclasts (TRAP+ MNCs) from BMMs, IL-1 alone cannot (Fig. 1A). However, IL-1 can have a synergistic effect on RANKL-induced osteoclast differentiation as well as bone resorption (Fig. 1A). To further investigate the role of IL-1 in RANKL-induced osteoclastogenesis, we added IL-1 to our cultures at various time points during osteoclastogenesis mediated by M-CSF and RANKL. The synergistic effect of IL-1 on RANKL-induced formation of TRAP+ MNCs was readily apparent when IL-1 was added in the later stage of osteoclastogenesis (Fig. 1C). Next, BMMs were incubated with RANKL for 1, 2, or 3 days, washed with PBS, and further cultured with IL-1 and RANK-Fc until day 4. In the presence of IL-1, cells that had been cultured for 2 or 3 days

4 The online version of this article contains supplemental material.
TRAP-positive multinucleated cells were counted. The replacement effect of IL-1 on RANKL-induced osteoclast differentiation one day with RANKL did not demonstrate the same potential (Fig. 1). Cultured cells were fixed and stained for TRAP. Numbers of TRAP-positive multinucleated cells were counted in at least two independent sets of similar experiments.

**FIGURE 1.** IL-1 enhances late phase osteoclast differentiation and bone resorption. A, BMMs were cultured for 3 days with various combinations of M-CSF (M, 30 ng/ml), RANKL (R, 20 ng/ml), and IL-1 (10 ng/ml) as shown. Left, Cultured cells were fixed and stained for TRAP. Right, Numbers of TRAP-positive multinucleated cells were counted (*, p < 0.005). B, BMMs were cultured for 3 days with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the absence or presence of IL-1 (10 ng/ml) on dentine slices. Left, Dentine slices were stained for resorption pits. Right, Numbers of pits were counted (*, p < 0.05). C, BMMs were cultured for 4 days with M-CSF (30 ng/ml) and RANKL (30 ng/ml). IL-1 (10 ng/ml) was added for various time periods as indicated (arrow). D, BMMs were cultured for various times (dot) with M-CSF (30 ng/ml) and RANKL (30 ng/ml). Cells were washed and further cultured until day 4 with M-CSF (30 ng/ml) alone or M-CSF (30 ng/ml), IL-1 (10 ng/ml), and RANK-Fc (2 μg/ml) (dashed). C and D, Cultured cells were fixed and stained for TRAP. Numbers of TRAP-positive multinucleated cells were counted (*, p < 0.0001; n.s., not significant). The results are representative of at least two independent sets of similar experiments.

days with RANKL-formed TRAP MNCs, but cells treated for one day with RANKL did not demonstrate the same potential (Fig. 1D). The replacement effect of IL-1 on RANKL-induced osteoclastogenesis took effect only at the later stage of osteoclastogenesis. These data indicate that IL-1 has the potential to replace RANKL during the late stage of osteoclast differentiation although IL-1 alone cannot initiate the process.

**Overexpression of IL-1RI in BMMs enables IL-1 to induce osteoclastogenesis**

To address the reason that IL-1 alone cannot induce osteoclast differentiation but that IL-1 has the strong effect on the late stage of osteoclastogenesis, we examined the expression of IL-1 receptors during RANKL-induced osteoclastogenesis. Interestingly, IL-1RI, which is sufficient for the transmission of IL-1 signals across the plasma membrane, was weakly expressed in osteoclast precursors (BMMs) and strongly up-regulated by RANKL during osteoclast differentiation. Conversely, the expression of IL-1RI, a decoy receptor for IL-1 with a short cytoplasmic tail which cannot transduce downstream signals, was abundantly expressed in BMMs and gradually reduced in the presence of RANKL stimulation (Fig. 2A). These results indicate that the inability of IL-1 to induce early stage osteoclastogenesis could in part be due to the relatively low levels of IL-1RI expression and high levels of IL-1RI expression in osteoclast precursors.

From these results, we postulated that if sufficient levels of IL-1RI were expressed in osteoclast precursors, IL-1 alone could induce osteoclast differentiation. To test this possibility, we retrovirally overexpressed IL-1RI in BMMs and cultured cells for 6 days in the absence or presence of IL-1. When IL-1RI was overexpressed in BMMs, IL-1 could indeed induce the formation of TRAP MNCs even in the presence of RANK-Fc (Fig. 2B), suggesting that IL-1 can induce osteoclast differentiation independent of RANKL. Furthermore, the IL-1-induced mature TRAP MNCs formed F-actin rings and resorbed bone independent manner of RANKL (Fig. 2C). Together, these data indicate that inability of IL-1 to induce early osteoclastogenesis is due to the expression patterns of IL-1 receptors in the precursor cells and if these cells express sufficient amounts of the signaling-competent IL-1RI receptor, then IL-1 alone can drive early stage osteoclastogenesis.

c-Fos and NFATc1 are involved in RANKL-mediated IL-1RI induction

Given that IL-1RI expression is up-regulated during RANKL-induced osteoclastogenesis (Fig. 2A) and that RANKL induces c-Fos expression at the early stage of osteoclastogenesis, we examined whether c-Fos is requisite for IL-1RI expression in the presence of RANKL. When c-Fos was retrovirally overexpressed in BMMs, IL-1RI expression was markedly increased even in the absence of RANKL (Fig. 3A). Consistent with this result, IL-1RI induction by RANKL stimulation was attenuated in c-Fos knockout cells as compared with wild-type (Fig. 3B). These data indicate that c-Fos is involved in RANKL-mediated IL-1RI induction during osteoclastogenesis. Next, we examined whether NFATc1, a downstream target gene of c-Fos, is involved in RANKL-mediated IL-1RI receptor expression and we demonstrate that overexpression of a constitutively active form of NFATc1 can lead to IL-1RI expression in the presence of RANKL. When c-Fos was overexpressed in BMMs, IL-1RI expression was markedly increased even in the absence of RANKL (Fig. 3A). Consistent with this result, IL-1RI induction by RANKL stimulation was attenuated in c-Fos knockout cells as compared with wild-type (Fig. 3B). These data indicate that c-Fos is involved in RANKL-mediated IL-1RI induction during osteoclastogenesis. Next, we examined whether NFATc1, a downstream target gene of c-Fos, is involved in RANKL-mediated IL-1RI receptor expression and we demonstrate that overexpression of a constitutively active form of NFATc1 can lead to IL-1RI expression in BMMs (Fig. 3C). To investigate whether down-regulation of NFATc1 expression by cyclosporine A would affect the expression levels of IL-1RI, we performed RT-PCR on samples prepared in the absence or presence of cyclosporine A. Compared with the control, cyclosporine A inhibited the expression of NFATc1 as well as that of IL-1RI, which we have demonstrated is induced by RANKL stimulation (Fig. 3D). To examine whether c-Fos and NFATc1 could directly regulate IL-1RI expression in osteoclasts, we used a reporter assay involving transient transfections into highly transfectable 293 human embryonic kidney cells.
The 2.0-Kb human IL-1RI promoter region, including the transcription initiation site, was constructed in the pGL3 basic luciferase vector. This pGL3 IL-1RI 2.0 plasmid was cotransfected with combinations of the c-Fos and NFATc1 plasmids. As shown in Fig. 3E, c-Fos and NFATc1 alone increased the luciferase activity, but the greatest increase was observed when both c-Fos and NFATc1 were expressed. These data indicate that c-Fos and NFATc1 could play a key role in RANKL-mediated IL-1RI induction during osteoclastogenesis.

Overexpression of c-Fos alone in BMMs leads to the expression of IL-1RI resulting in IL-1-driven early osteoclastogenesis

Because IL-1 alone can induce osteoclast differentiation if the early stage of osteoclastogenesis is initiated by RANKL (Fig. 1D) and c-Fos can induce IL-1RI expression (Fig. 3A), we hypothesized that the early stage of signaling may compensate the inability of IL-1 on osteoclastogenesis. Therefore, we examined whether overexpression of c-Fos can rescue the inability of IL-1 on osteoclast differentiation. Consistent with previous results (30), the combination of c-Fos overexpression and IL-1 treatment resulted in the formation of TRAP/MNCs, whereas retroviral overexpression of c-Fos alone induced a small number of TRAP/MNCs formation (Fig. 4A). Mature osteoclasts derived by c-Fos overexpression and IL-1 stimulation resorbed bone even in the presence of RANK-Fc, which can block RANKL signaling (Fig. 4B). These data indicate that if proper signaling pathways are activated during the early stages of osteoclastogenesis, IL-1 can induce osteoclast formation independent of RANKL.

IL-1 activates key osteoclastogenesis signaling transduction pathways

RANKL, a critical osteoclastogenic factor, activates NF-κB, JNK, p38 MAPK, extracellular signal-related kinase (ERK), and Akt (1,
3). To examine whether IL-1 can activate a similar signaling profile, BMMs were infected with control or IL-1RI retrovirus and stimulated with IL-1. IL-1 weakly activated p38, JNK, and ERK in control vector-infected BMMs, whereas IL-1 strongly activated p38, JNK, ERK, and NF-κB when IL-1RI was overexpressed (Fig. 5). The level of gene activations by IL-1 in IL-1RI overexpressing BMMs (IL-1/IL-1RI) was comparable to the pattern of expression induced by RANKL in BMMs (Fig. 5).
IL-1 induces osteoclast-specific gene expression by means of MITF

To elucidate the requisite signaling molecules for IL-1-mediated osteoclastogenesis, we examined the expression pattern of multiple genes known to play key roles in osteoclast differentiation. In control vector-infected BMMs, IL-1 did not induce expression of key transcription factors including c-Fos and NFATc1, nor did we observe detectable up-regulation of other osteoclast-specific genes such as TRAP, cathepsin K, or OSCAR (Fig. 6A). However, when we examined gene expression in IL-1RI-overexpressing BMMs, we found that the addition of IL-1 up-regulated expression of TRAP, cathepsin K, and OSCAR, although c-Fos and NFATc1 expression was not affected. To investigate the necessity of c-Fos and NFATc1 activation in IL-1-induced osteoclastogenesis, we tested the capacity of IL-1 to induce osteoclast formation from spleen cells derived from c-Fos knockout mice in conjunction with spleenocytes from wild-type control littermates. In the c-Fos-deficient cells, IL-1 could not induce osteoclastogenesis, whereas IL-1-mediated osteoclast differentiation was readily observable in the wild-type control cultures (Fig. 6B). Furthermore, we found that addition of cyclosporine A, a calcineurin inhibitor, also inhibited IL-1-mediated osteoclast formation (Fig. 6D). When we examined expression profiles of various genes during IL-1-induced osteoclastogenesis in wild-type and c-Fos-deficient cells, IL-1 did not induce any osteoclast-specific genes such as TRAP, cathepsin K, and OSCAR in c-Fos-deficient cells (Fig. 6C). Together, these data indicate that IL-1-induced signaling does not up-regulate the expression of c-Fos and NFATc1. However, basal levels of both c-Fos and NFATc1 are required for IL-1-induced osteoclastogenesis.

It has been shown that MITF, an important transcription factor in osteoclast differentiation, can synergistically induce expression of target genes such as TRAP and OSCAR in the combination with PU.1 and NFATc1 (20, 22, 31, 32). Therefore, we investigated whether MITF is involved in IL-1-induced osteoclastogenesis. Of note, we observed that expression levels of MITF were not significantly altered during IL-1-induced osteoclast differentiation (data not shown). To further investigate whether IL-1 might activate MITF to induce osteoclast-specific genes, we performed an EMSA assay using probes containing the E-box region of the TRAP and OSCAR gene promoter regions. Endogenous MITF derived from IL-1-treated osteoclast nuclear extracts (NE2) were found to bind the E-box regions of TRAP and OSCAR (Fig. 6E). The intensity of the shifted band, the complex of MITF and probe, from IL-1-treated osteoclast nuclear extracts (lane 3) is relatively stronger in intensity than that of RANKL-induced osteoclast nuclear extracts (lane 4). The specificity of this binding was confirmed by competition studies using cold wild- and mutant-type competitor probes (lanes 5 and 6). These data suggest that IL-1 could induce osteoclast differentiation via activation of MITF.

To determine whether MITF binds to the promoter regions of TRAP and OSCAR endogenously in IL-1-treated osteoclasts, we performed a ChIP assay. Osteoclasts were derived from control vector- or IL-1RI-infected BMMs following treatment with IL-1. The cross-linked protein/chromatin complex from control vector- or IL-1RI-infected osteoclasts was precipitated with anti-MITF or control Ab, respectively. The precipitated DNA was subjected to PCR amplification with primers specific for promoter regions of genes containing MITF-binding sites. A single PCR product of TRAP and OSCAR was obtained using the DNA from osteoclasts following immunoprecipitation with an anti-MITF Ab, whereas no PCR product was observed using DNA immunoprecipitated by control Ab (Fig. 6F). The relative intensity of the amplified PCR product in osteoclasts derived from IL-1RI-infected BMMs was much stronger than in that from control vector-infected BMMs. These results provide direct evidence that MITF forms a complex with the TRAP and OSCAR promoter regions in vivo. Taken together, our results clearly demonstrate that IL-1 induces osteoclast-specific gene expression, in part, through MITF activation.

Discussion

IL-1 is a proinflammatory cytokine that acts as an important mediator of the peripheral immune response during infection and inflammation (33). It is also known that IL-1 can induce...
bone destruction in a variety of diseases such as osteoporosis, rheumatoid arthritis, and periodontal disease (34, 35). IL-1 stimulates osteoclast differentiation, fusion, and activation (35). In this paper, we examined the direct effect of IL-1 on osteoclast precursors which led to the elucidation of a previously unknown mechanism of downstream signaling pathways during IL-1-induced osteoclastogenesis.

Although TNF-α and IL-1 can activate early signaling pathways including NF-κB, JNK, and p38, which are important for RANKL-induced osteoclast differentiation, TNF-α alone has been shown to induce osteoclast differentiation in vitro (21, 36). IL-1 activates mature osteoclasts, thereby enhancing bone resorption (35), but our data along with previous studies (35, 36) demonstrate that IL-1 alone is insufficient to induce osteoclast differentiation from precursors. Our data reveal that the induction of IL-1RI expression and c-Fos expression by RANKL or TNF-α can lead to IL-1-induced osteoclast formation. These results explain the synergistic effect of IL-1 on RANKL- or TNF-induced osteoclastogenesis (36). In pathological conditions such as rheumatoid arthritis and periodontal disease, various inflammatory factors and cytokines, including TNF-α and IL-6, which are secreted by inflammatory cells (33, 34), could possibly enable IL-1 to induce osteoclastogenesis thereby leading to more severe disease.

IL-1 is an important proresorptive cytokine which can stimulate RANKL-induced osteoclast differentiation and function (35). Peripheral administration of IL-1 receptor antagonist decreases osteoclast formation and bone resorption in ovariectomized mice which are a model for bone loss associated with menopause (37). However, mice lacking a functional IL-1 receptor, IL-1RI, do not lose bone mass after ovariectomy (38). These data suggest that IL-1 is an important mediator of bone loss induced by gonadal hormone depletion. However, studies to examine the role of IL-1 in bone metabolism in vivo using IL-1RI-deficient mice have yielded conflicting results (39, 40). Vargas et al. (40) showed that calvariae and humeri of IL-1RI-deficient mice are normal with respect to trabecular bone volume, osteoclast number, osteoclast

FIGURE 6. IL-1 induces osteoclast-specific gene expression through MITF activation. A. BMMs were transduced with control (pMX-IRES-EGFP) or IL-1RI retrovirus and cultured with IL-1 (10 ng/ml) for the indicated times in the presence of M-CSF (30 ng/ml). RT-PCR was performed to access the expression of the indicated genes. B and C. Osteoclast precursors were prepared with M-CSF and TGF-β using spleen cells from c-Fos-deficient mice and its wild-type littermates. Osteoclast precursors were transduced with control (pMX-IRES-EGFP) or IL-1RI retrovirus and cultured with IL-1 (10 ng/ml) for 6 days (B) for the indicated times (C) in the presence of M-CSF (30 ng/ml). B. Left, Cultured cells were fixed and stained for TRAP. Right, Numbers of TRAP-positive multinucleated cells were counted. C. RT-PCR was performed to access the expression of the indicated genes. D. Osteoclast precursors were transduced with control (pMX-IRES-EGFP) or IL-1RI retrovirus and cultured with IL-1 (10 ng/ml) for 6 days in the absence or presence of cyclosporine A (5 μg/ml). Numbers of TRAP-positive multinucleated cells were counted. E. Nucleic extracts were prepared from osteoclast cells treated with vehicle (NE1), IL-1 (NE2), or RANKL (NE3). DNA fragments spanning E-box in OSCAR and TRAP promoter regions were used as probes for EMSA. Specific binding was determined by cold competition using unlabeled wild- or mutant-type probes at 50-fold molar excess concentrations. F. ChIP assay of MITF binding to the promoter regions of the indicated genes. BMMs were transduced with control (pMX) or IL-1RI retrovirus and cultured for 4 days before cross-linking. Samples were immunoprecipitated with control IgG or anti-MITF Ab and subjected to PCR amplification with primers specific for MITF-binding sites in the promoter regions of TRAP and OSCAR. The results are representative of at least two independent sets of similar experiments.
surface, growth plate widths, and cortical thickness. However, Bajayo et al. reported a low bone mass phenotype, including impairment of bone growth, in IL-1R1-deficient mice and mice with central nerve system-targeted IL-1 receptor antagonist overexpression (39), suggesting that central IL-1 receptor signaling is also a potent regulator of bone growth, bone remodeling, and bone mass. Further studies elucidating IL-1R1 signaling in bone remodeling are needed to confirm that IL-1 is indeed requisite for bone metabolism.

RANKL induces the expression of c-Fos during early osteoclastogenesis. RANKL accelerates the binding of c-Fos to the NFATc1 promoter region, thereby inducing NFATc1 gene expression. Later, expression of NFATc1 is strongly up-regulated by autoamplification (41, 42). NFATc1 synergistically induces the expression of osteoclast-specific genes such as TRAP, cathepsin K, and OSCAR in the combination with MITF and PU.1 (20, 32). It appears that the coordinated action of various transcription factors, including c-Fos, NFATc1, MITF, and PU.1, plays an important role in RANKL-induced osteoclastogenesis. We demonstrate that IL-1 stimulation of IL-1R1 overexpressing BMMS (IL-1/IL1R1) leads to osteoclast formation without induction of c-Fos and NFATc1, although basal levels of c-Fos and NFATc1 expression are required. These results suggest that IL-1 and RANKL operate using separate signaling pathways and rely upon different combinations of transcription factors. Recently, Sharma et al. (43) showed that RANKL promotes the recruitment of activated MITF, p38, and SWI/SNF chromatin-remodeling complexes to their target promoters like cathepsin K and TRAP. NFATc1 is subsequently recruited to promoter complexes during the terminal stages of osteoclastogenesis. From these results, the authors suggest that MITF and PU.1 are responsible for initiating induction of target genes during RANKL-induced osteoclastogenesis, whereas NFATc1 may play a role in maintaining target gene expression in mature osteoclasts. Indeed, we show herein that IL-1/IL-1R1 strongly led to the recruitment of MITF to the promoter regions of both TRAP and OSCAR in vitro and in vivo, suggesting that IL-1/IL-1R1 signaling may induce osteoclast differentiation through activation of MITF. However, it is worth noting that fewer TRAP+ MNCs formed from IL-1-IL1R1-treated cells than in RANKL-treated cultures. In addition, the relative intensity of IL-1/IL-1R1-induced resorption pits on dentine slices is weaker than those of RANKL-induced pits. These data suggest that although IL-1/IL-1R1 can induce osteoclast differentiation and activation without induction of c-Fos and NFATc1, induction of both gene pathways plays an important role in osteoclast formation and function.

In this study, we provide compelling evidence that IL-1 has the potential to induce osteoclast differentiation independent of RANKL/RANK interaction. IL-1 can directly induce osteoclastogenesis through the IL-1R1 receptor, whose expression is in turn up-regulated by osteoclast-inducing factors such as RANKL and TNF-α via c-Fos and NFATc1. In addition, IL-1 activates osteoclast-specific genes including TRAP and OSCAR, in part, via the MITF pathway. Thus, our study elucidates a direct effect of IL-1 on osteoclast formation as well as previous IL-1 mediated mechanisms during osteoclastogenesis.

Acknowledgments

We thank Aeran Ko for assistance.

Disclosures

The authors have no financial conflict of interest.

References

5. Bandhun, M., L. Duplomb, C. Ruiz Velasco, Y. Fortun, D. Heymann, and M. Palacio. 2007. Key roles of the OPG-RANK-RANKL system in bone onco-

Based on the provided text, the natural representation of the document is as follows:


