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J Immunol 2010; 185:7460-7466; Prepublished online 10 November 2010;
doi: 10.4049/jimmunol.1000885
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Pim-1 Regulates RANKL-Induced Osteoclastogenesis via NF-κB Activation and NFATc1 Induction

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Pim kinases are emerging as important mediators of cytokine signaling pathways in hematopoietic cells. In this study, we demonstrate that Pim-1 positively regulates RANKL-induced osteoclastogenesis and that Pim-1 expression can be upregulated by RANKL signaling during osteoclast differentiation. The silencing of Pim-1 by RNA interference or overexpression of a dominant negative form of Pim-1 (Pim-1 DN) in bone marrow-derived macrophage cells attenuates RANKL-induced osteoclast formation. Overexpression of Pim-1 DN blocks RANKL-induced activation of TGF-β-activated kinase 1 (TAK1) and NF-κB as well as expression of NFATc1 during osteoclastogenesis. However, we found that overexpression of TAK1 in the presence of Pim-1 DN rescues NF-κB activation. Additionally, Pim-1 interacts with RANKL as well as TAK1, indicating that Pim-1 is involved in RANKL-induced NF-κB activation via TAK1. Furthermore, we demonstrate that Pim-1 also regulates NFATc1 transcription activity and subsequently induces osteoclast-associated receptor expression, an osteoclast-specific gene. Taken together, our results reveal that Pim-1 positively regulates RANKL-induced osteoclastogenesis.

expression during osteoclastogenesis. Moreover, we show that Pim-1 is involved in RANKL-induced NF-κB activation through TAK1. Taken together, our results suggest that Pim-1 acts as a positive modulator for RANKL-induced signaling pathways during osteoclastogenesis.

**Materials and Methods**

**Constructs**

Pim-1, Pim-2, Pim-3, and RANK were generated by RT-PCR using RNA from bone marrow-derived macrophages (BMMs) and cloned into pMX-IRES-EGFP, pcDNA3.1, or pIRES-hrGFP-2a. The deletion mutant forms of Pim kinase members, that is, Pim-1 DN (aa 81–313), Pim-2 DN (aa 74–311), and Pim-3 DN (aa 84–326), were constructed by deleting the N-terminal region of each Pim gene, which contains a kinase domain (15–17). A point mutant form of Pim-1, Pim-1 K67M (14), was generated by the QuickChange method of site-directed mutagenesis (Stratagene, La Jolla, CA). CMV-HA-TAK1 wild-type (WT) and CMV-HA-TAK1 K63W were provided by Dr. S. Y. Lee (Ewha Womans University, Seoul, Korea) and have been described previously (18).

**Osteoclast formation and TRAP staining**

Femurs were aseptically removed from 6- to 8-wk-old ICR mice, and bone marrow cells were flushed out with a sterile 21-gauge syringe. The cells were cultured in αMEM (HyClone Laboratories, Walhtham, MA) containing 10% FBS (HyClone Laboratories) with M-CSF (30 ng/ml) for 3 d. Floating cells were removed and adherent cells (BMMs) were used as osteoclast precursors. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 d. Floating cells were removed and adherent cells (BMMs) were used as osteoclast precursors. To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 d. Cultured cells were fixed and stained for TRAP. TRAP+ multinuclear cells (MNCs) containing more than three nuclei were counted as osteoclasts.

**Retroviral gene transduction**

To generate retroviral stock, recombinant plasmids and the parental pMX vector were transfected into the packaging cell line Plat-E using FuGENE 6 (Roche Applied Sciences, Indianapolis, IN). Plat-E cells were maintained in DMEM supplemented with 10% FBS and 2 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). After removing the viral supernatant, BMMs were further cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 d.

**Semiquantitative RT-PCR and real-time PCR**

Semiquantitative RT-PCR and real-time PCR analyses were performed as previously described (19, 20).

**In vitro kinase assay**

For the TAK1 kinase assay, BMMs were transduced with either a control or Pim-1 DN retrovirus and subsequently stimulated for the times indicated in Fig. 5C. Whole cell lysates were harvested from cells and immunoprecipitated with an anti-TAK1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were mixed with 30 μl kinase buffer (20 mM Pipes [pH 7.0], 5 mM MnCl₂, 7 mM 2-ME, 0.25 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 0.4 mM spermine, 50 μM ATP, and 5 μCi [γ-³²P] ATP). GST-IKKβ (1.5 mg) (Millipore, Billerica, MA) recombinant protein was added to the kinase buffer as a substrate. After incubation at 30°C for 30 min, reaction mixtures were resolved by SDS-PAGE followed by autoradiography. To detect Pim-1 phosphorylation by RANKL stimulation, BMMs were stimulated with RANKL (100 ng/ml) for the times indicated in Fig. 4A. A kinase assay was performed using whole cell lysates from samples as described above. GST or GST-Pim-1 recombinant purified proteins were used as substrates.

**Western blot analysis**

Cells from transfected 293T, transduced 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, and protease inhibitors). Cell lysates were subjected to SDS-PAGE and Western blotting. Primary Abs used included NFATc1 (Santa Cruz Biotechnology), OSCAR (21), c-Fos (Calbiochem, San Diego, CA), IκB, phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK (Cell Signaling Technology, Danvers, MA), Pim-1, Pim-2, Pim-3 (Santa Cruz Biotechnology), actin (Sigma-Aldrich), Flag (Sigma-Aldrich), and hemagglutinin (HA) (Roche Applied Sciences). HRP-conjugated secondary Abs (Amersham Biosciences, Piscataway, NJ) were probed and developed with ECL solution (Millipore). Signals were detected and analyzed by an LAS3000 luminescent image analyzer (Fuji, Tokyo, Japan).

**FIGURE 1.** Expression of Pim family members during RANKL-mediated osteoclastogenesis. BMMs were cultured with M-CSF and RANKL for the indicated times. A, Whole cell lysates were harvested from cultured cells and analyzed by Western blot using Abs specific for Pim-1, Pim-2, Pim-3, c-Fos, NFATc1, OSCAR, and actin (control). B, Relative amounts of each protein are shown as indicated.
Transfection and luciferase assay

RAW264.7 cells were plated on 24-well plates at a density of $7 \times 10^5$ cells/well 1 d before transfection. Plasmid DNA was mixed with Lipofectamine reagent and Lipofectamine Plus reagent in serum-free DMEM and transfected into the cells per the manufacturer’s protocol. After 20 h of transfection, cells were treated with RANKL (100 ng/ml) for 12–16 h. Cells were washed twice with PBS and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured with a dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

Small interfering RNA preparation and transfection

Predesigned mouse Pim-1 small interfering RNAs (siRNAs) (ID nos. 63532 and 150114), Pim-2 siRNAs (ID nos. 88062 and 88132), and Pim3 siRNAs (ID nos. 89580 and 169806) were purchased from Ambion (Austin, TX). Among siRNAs, ID no. 150114 (for Pim-1), ID no. 88062 (for Pim-2), and ID no. 89580 (for Pim-3) demonstrated a significant inhibitory effect on Pim expression in BMMs. Therefore, we used GFP siRNA as a control, no. 150114 as Pim-1 siRNA, no. 88062 as Pim-2 siRNA, and no. 89580 as Pim-3 siRNA. siRNAs were transfected into BMMs using X-tremeGENE siRNA transfection reagent (Roche Applied Sciences). After 72 h, cells were used for osteoclastogenesis and other analyses as indicated.

Results

Pim-1 expression is upregulated by RANKL during osteoclast differentiation

To examine the expression patterns of Pim family members, we performed Western blot analysis during osteoclast differentiation. Osteoclast differentiation was induced by culturing BMMs in the presence of M-CSF and RANKL (Fig. 1A). RANKL stimulation increased the expression of c-Fos and NFATc1, which are key transcription factors in osteoclastogenesis. The induction of NFATc1 gene expression was followed by the expression of OSCAR, which is an osteoclast-specific gene (21). Among Pim kinases, Pim-1 and Pim-2 expression was readily detectable during osteoclast differentiation, whereas expression of Pim-3 was only faintly detected. Pim-1 protein levels peaked at day 2 and subsequently decreased, whereas Pim-2 expression remained unchanged during RANKL-induced osteoclastogenesis (Fig. 1).

Pim-1 positively regulates RANKL-induced osteoclastogenesis

Given our observation that Pim family members are expressed in osteoclast lineage cells, we investigated whether Pim kinases are required for efficient osteoclast differentiation. To examine the effect of Pim family members on osteoclast differentiation, WT or DN forms of each Pim kinase were overexpressed in BMMs. Overexpression of Pim-1 DN in BMMs inhibits osteoclastogenesis. BMMs were transduced with pMX-IRE-EGFP empty vector (control) or pMX-IRE-EGFP with Pim-1 WT, Pim-1 DN, a kinase-dead mutant form of Pim-1 (Pim-1 K67M), Pim-2 WT, Pim-2 DN, Pim-3 WT, or Pim-3 DN, and cultured for 3 d with M-CSF alone or M-CSF and various concentrations of RANKL as indicated. As shown in Fig. 2A, siRNA-transfected BMMs increased the number of TRAP$^+$ MNCs in a dose-dependent manner (Fig. 3B, 3C). Compared with the control siRNA, the silencing of Pim-1 in BMMs resulted in a significant decrease in the formation of TRAP$^+$ MNCs mediated by RANKL. Similar to the results described for Pim-2 DN and Pim-3 DN above, the siRNAs specific for Pim-2 and Pim-3 did not affect RANKL-mediated osteoclastogenesis (Fig. 3B, 3C). Taken together, these results suggest that Pim-1 may play a role in RANKL-induced osteoclastogenesis.

Activation of Pim-1 plays a role in RANKL-induced NF-$\kappa$B activation

To investigate the mechanism by which Pim-1 modulates RANKL-induced osteoclastogenesis, we determined whether RANKL could activate Pim-1 by an in vitro kinase assay with GST or GST-Pim-1 recombinant proteins using whole cell lysates from unstimulated BMMs and from BMMs cultured in the presence of RANKL. As shown in Fig. 4A, RANKL stimulation resulted in the phosphory-
Downregulation of Pim-1 in BMMs attenuates RANKL-induced osteoclastogenesis. BMMs were transfected with GFP siRNA (control) or siRNA specific for Pim-1, Pim-2, or Pim-3. A, Transfected BMMs were cultured for 2 d with M-CSF. Whole cell lysates were harvested from cultured cells and analyzed by Western blot using Abs specific for Pim-1, Pim-2, Pim-3, and actin (control). B, Transfected BMMs were cultured for 4 d with M-CSF alone or M-CSF and various concentrations of RANKL as indicated. Cultured cells were fixed and stained for TRAP. C, Numbers of TRAP+ MNCs were counted. Data represent means ± SD of triplicate samples. **p < 0.01; #p < 0.001 versus GFP siRNA. Results are representative of at least three independent sets of similar experiments.

Next, we examined the role of Pim-1 in osteoclastogenesis downstream of RANK by transducing BMMs with control or Pim-1 DN retroviruses followed by RANKL stimulation. Consistent with previous results (22, 23), RANKL induced degradation of IkB and activation of ERK, p38 MAPK, and JNK in control vector-infected BMMs. However, RANKL-induced IkB degradation was blocked by overexpression of Pim-1 DN, while other signaling pathways, including activation of ERK, JNK, and p38 MAPK, were not significantly affected (Fig. 4B).

To examine the role of Pim-1 in RANKL-induced NF-kB activation, we used a reporter assay involving transient transfection into RAW264.7 cells, which is a monocytic/macrophage cell line capable of differentiating into multinuclear osteoclasts. An NF-kB luciferase reporter plasmid was cotransfected with Pim-1 WT or Pim-1 DN in the presence or absence of RANKL. RANKL stimulation induced NF-kB transcriptional activity, and this induction was further potentiated by coexpression of Pim-1 WT. In contrast, overexpression of Pim-1 DN inhibited the effects of RANKL on NF-kB transcriptional activity (Fig. 4C). Taken together, these results suggest that RANKL activates Pim-1, and that activation of Pim-1 plays a role in RANKL-induced NF-kB activation.

Pim-1 regulates RANKL-induced NF-kB activation through TAK1

TAK1 has been shown to play a role in IL-1– and RANKL-mediated NF-kB activation (5, 6, 24). Given our data indicating that Pim-1 modulates RANKL-induced NF-kB activation, we examined whether TAK1 is involved in the RANKL/Pim-1/NF-kB signaling pathway. Overexpression of Pim-1 WT induced RANKL-mediated NF-kB activation (Fig. 5A). However, the induction of luciferase activity by RANKL/Pim-1 was significantly reduced by overexpression of TAK1 K63W (a DN TAK1), indicating that TAK1 might be a downstream target of RANKL/Pim-1 in NF-kB activation. To test this hypothesis, we examined whether overexpression of TAK1 WT could override the inhibitory effects of Pim-1 DN on
activation of NF-κB by RANKL in RAW264.7 cells. As shown in Fig. 5B, TAK1 WT rescued activation of NF-κB by RANKL in the presence of Pim-1 DN.

Next, we examined whether Pim-1 kinase is involved in RANKL-induced TAK1 activation via an in vitro kinase assay. BMMs were transduced with pMX-IRES-EGFP (control) or Pim-1 DN retrovirus and cultured for 2 d with M-CSF and RANKL. Cell lysates from preosteoclasts were immunoprecipitated with anti-TAK1 Ab, and immunoprecipitates were subjected to an in vitro kinase assay with GST-IKKβ as a substrate (upper panel) and Western blot analysis with anti-TAK1 Ab (middle panel). Whole cell extracts were probed with anti-HA (for Pim-1 DN) or anti-actin (control) Abs (lower panel). The results are representative of two independent sets of similar experiments.

Pim-1 interacts with RANK and TAK1

Given our data indicating that Pim-1 participates in RANKL-induced NF-κB activation, we examined whether Pim-1 interacts with RANK. 293T cells were cotransfected with RANK and Pim-1, and cell lysates were immunoprecipitated with anti-Flag (Fig. 6A) or anti-HA (Fig. 6B) Abs. Immunoprecipitated samples were subjected to SDS-PAGE and Western blotting by anti-Flag or anti-HA Abs. As shown in Fig. 6A and 6B, we confirmed that RANK interacts with Pim-1. Consistent with previous results (5), RANK also interacts with TAK1 (Fig. 6C, 6D). When Pim-1 and TAK1 were overexpressed in 293T cells, the interaction between Pim-1 and TAK1 was observed by immunoprecipitation assay (Fig. 6E). This interaction between TAK1 and both Pim-1 and RANK was also observed endogenously in preosteoclast cells (Fig. 6F). These data suggest that the formation of a complex containing RANK, Pim-1, and TAK1 might play a role in RANKL-mediated signaling cascades.

Pim-1 regulates RANKL-mediated induction of NFATc1 and OSCAR during osteoclastogenesis

Because Pim-1 modulates RANKL-induced osteoclast differentiation, we investigated whether overexpression of Pim-1 DN could affect the expression of other genes known to play a role in osteoclastogenesis. Consistent with previous results (25–27), RANKL induced the expression of c-Fos, NFATc1, OSCAR, and TRAP during osteoclast differentiation (Fig. 7A). Compared with samples transduced with a control vector, overexpression of Pim-1 DN in BMMs inhibited the induction of NFATc1, OSCAR, and TRAP during RANKL-mediated osteoclastogenesis (Fig. 7A). We confirmed these results by Western blot (Fig. 7B), demonstrating that all downstream molecules were affected at both the mRNA and protein level by overexpression of Pim-1 DN during osteoclastogenesis, with the exception of c-Fos (Fig. 7A–C).

To investigate whether Pim-1 directly regulates NFATc1 transactivation, we used an OSCAR luciferase reporter assay. When an OSCAR luciferase reporter plasmid was cotransfected with NFATc1, NFATc1 increased OSCAR luciferase activity (Fig. 7D). NFATc1-induced transcriptional activity was further enhanced by Pim-1 WT, whereas Pim-1 DN significantly reduced NFATc1 transactivation (Fig. 7D). Taken together, our results indicate that Pim-1 is involved in NFATc1 induction as well as NFATc1 transcriptional activity during RANKL-mediated osteoclastogenesis.

Discussion

The Pim kinase family consists of three members: Pim-1, Pim-2, and Pim-3. Because of sequence similarity between these Pim kinases, overlapping functions have been inferred. Although Pim kinases are widely expressed, patterns of expression of each Pim kinase are somewhat different: the highest Pim-1 mRNA levels...
are found in the thymus and testis (11, 28), whereas Pim-2 mRNA expression is highest in the brain and thymus (11, 29), and Pim-3 mRNA is most abundant in the kidney (11, 30). Additionally, induction of Pim kinase expression by a wide range of growth factors and cytokines differs between the different Pim family members. For example, IL-3 stimulation of bone marrow-derived mast cells strongly induces Pim-1 and Pim-2 expression, but only weakly induces expression of Pim-3 (11). Therefore, it seems that the spatial and temporal expression of each Pim kinase might be important for cell growth and differentiation upon engagement with various cytokines. Of note, our data demonstrate a previously undescribed pattern of expression during RANKL-induced osteoclastogenesis with significant upregulation of Pim-1 expression and unchanged Pim-2 and Pim-3 expression. Additionally, our results from overexpression of DN forms of each Pim kinase by siRNA demonstrate that only Pim-1 affects RANKL-induced osteoclastogenesis. Furthermore, unlike Pim-1 DN, overexpression of Pim-2 DN or Pim-3 DN in BMMs did not block RANKL-mediated degradation of IkB and induction of NFATc1 expression during osteoclastogenesis (data not shown). Thus, our results indicate that Pim-1 acts as a major modulator of RANKL-mediated osteoclastogenesis.

The downstream events of multiple signal-transduction pathways used by RANK include activation of transcription factors such as NF-κB, induction and activation of NFATc1, and stimulation of three MAPK families: ERK, JNK, and p38 (1, 2). We show that Pim-1 regulates RANKL-induced NF-κB activation among RANK-proximal signaling pathways. Similar to our results, Zemskova et al. (31) reported that Pim-1 mediates docetaxel-induced activation of NF-κB transcriptional activity in prostate cancer cells, suggesting that Pim-1 may play a role in NF-κB activation. Of note, although NF-κB activation appears to be the major RANKL-induced proximal signaling pathway regulated by Pim-1, in some experimental replicates we did observe a slight effect of Pim-1 on p38 activation as well. Thus, we cannot rule out the possibility that Pim-1 might have an effect on RANKL-induced p38 activation.

RANKL activates TAK1 through a signaling complex containing RANK, TAB2, and TRAF6 (5). It has been reported that TAK1 and MKK6 are required for RANKL-mediated NFATc1 induction and NF-κB transactivation during osteoclast differentiation (32). Our data demonstrate that the Pim-1/TAK1 signaling cascade plays a role in RANKL-induced NF-κB activation during osteoclastogenesis. First, we demonstrated that TAK1 DN blocked RANKL/Pim-1–induced NF-κB transactivation. Second, we showed that TAK1 WT rescued the inhibitory effect of Pim-1 DN on RANKL-induced NF-κB transactivation. Third, we found that Pim-1 DN blocked RANKL-induced activation of TAK1, suggesting that TAK1 may be a downstream target of RANKL/Pim-1 in NF-κB activation. Fourth, we observed the formation of a RANK-Pim-1-TAK1 complex. Thus, our data demonstrate that the RANKL/Pim-1/TAK1 signaling cascade is potentially important for NF-κB activation during osteoclastogenesis.
RANKL/RANK signaling increases expression levels of NFATc1, a key modulator in osteoclastogenesis. Takatsuna et al. (33) showed that an NF-κB inhibitor can suppress RANKL-induced osteoclastogenesis by downregulation of NFATc1. Additionally, chromatin immunoprecipitation experiments have revealed that the NF-κB components p50 and p65 are recruited to the NFATc1 promoter by RANKL stimulation (34). Consistent with this, NFATc1 promoter regions contain NF-κB binding sites and NF-κB activates NFATc1, as shown by a luciferase reporter gene assay (7, 34). These data indicate that NFATc1 expression is regulated by RANKL stimulation (34). Consistent with this, NF-kB chromatin immunoprecipitation experiments have revealed that the osteoclastogenesis by downregulation of NFATc1. Additionally, k

References

Disclosures