The Tec family tyrosine kinase Btk regulates RANKL-induced osteoclast maturation*

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A spontaneous mutation in Bruton’s tyrosine kinase (Btk) induces a defect in B-cell development, which results in the immunodeficiency diseases X-linked agammaglobulinemia in humans and X-linked immunodeficiency (Xid) in mice. Here we show an unexpected role of Btk in osteoclast formation. When bone marrow cells derived from Xid mice were stimulated with receptor activator of NF-κB ligand, an osteoclast differentiation factor, they did not completely differentiate into mature multinucleated osteoclasts. Moreover, we found that the defects appeared to occur at the stage in which mononuclear preosteoclasts fuse to generate multinucleated cells. Supporting this notion, macrophages from Xid mice also failed to form multinucleated foreign body giant cells. The fusion defect of the Xid mutant osteoclasts was caused by decreased expression of NFATc1, a master regulator of osteoclast differentiation, as well as reduced expression of various osteoclast fusion-related molecules, such as the d2 isoform of vacuolar H+-ATPase V0 domain and the dendritic cell-specific transmembrane protein. This deficiency was completely rescued by the introduction of a constitutively active form of NFATc1 into bone marrow-derived macrophages. Our data provide strong evidence that Btk plays a critical role in osteoclast multinucleation by modulating the activity of NFATc1.

The processes underlying bone remodeling, which are dependent on a balance between osteoblast-driven bone formation and osteoclast-mediated bone resorption, control homeostasis in the skeletal system (1,2). For efficient bone resorption, the fusion of mononuclear preosteoclasts from the myeloid lineage is critical for the formation of functional multinucleated osteoclasts (2-5). Recently, it was reported that mice genetically deficient for dendritic cell-specific transmembrane protein (DC-STAMP) or the d2 isoform of the vacuolar H+-ATPase V0 domain (Atp6v0d2) exhibit osteopetrotic phenotypes due to defects in osteoclast fusion during osteoclastogenesis (4,5). Interestingly, these studies also showed that cell-cell fusion is essential for the formation of giant macrophages (4,5).

In concert with macrophage-colony stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL), a member of the TNF superfamily, regulates the differentiation of osteoclasts as well as cell survival, fusion, and activation (3,6,7). The binding of RANKL to receptor activator of NF-κB (RANK) induces the expression of transcription factors, including NFATc1, NF-κB, c-Fos, Mitf, and PU.1; these transcription factors have been shown to be important for osteoclastogenesis in vitro and in vivo (2,3,6). For example, NFATc1-null cells do not differentiate into osteoclasts and overexpression of intact NFATc1 stimulates osteoclast development from monocyte lineage precursors in a RANKL-independent manner (8). In addition to its important role in osteoclast differentiation, NFATc1 appears to be involved in bone resorption by osteoclasts (9). Recently, we reported a novel role for NFATc1 as a positive regulator of RANKL-mediated osteoclast fusion; this effect occurs as a result of the direct upregulation of the expression of the fusion-regulating molecules Atp6v0d2 and DC-STAMP (10). Therefore, NFATc1 is crucial for fusion of osteoclasts, as well as their differentiation and...
The Tec family of tyrosine kinases are predominantly expressed in hematopoietic cells, where they are involved in signal transduction pathways that respond to extracellular stimuli (11). A broad range of mutations in one member of the Tec tyrosine kinase family, Bruton’s tyrosine kinase (Btk), have been shown to cause X-linked agammaglobulinemia (XLA) in humans (12,13). This disorder is characterized by defects in B-lymphocyte development and function, resulting in immunodeficiency and a severe reduction in serum immunoglobulin (Ig) levels (14,15). Moreover, X-linked immunodeficiency (Xid), a similar, although less severe syndrome in mice, is also caused by a point mutation in the \( Btk \) gene (16,17).

Receptor signaling pathways in most immune-related cells, in particular T cells, B cells, and mast cells, share similar signal transduction schemes that include members of the Tec kinase family (18,19). The B cell receptor (BCR), T cell receptor (TCR), and mast cell receptor (Fc\(\varepsilon\)RI) complexes include ITAM-containing transmembrane adaptors (the Ig\(\alpha/\beta\), CD3\(\varepsilon\), and Fc\(\varepsilon\)RI chains, respectively). Following receptor engagement, tyrosines within the ITAM adaptors are phosphorylated by Src-family kinases (Src, Lyn, or Fyn), leading to the recruitment and activation of Syk family kinases (Syk or ZAP-70). Activated Tec family kinases are then recruited to the receptor signaling complexes through interactions with adapter proteins, such as LAT, SLP-76, and BLNK, which recruit downstream effectors, including phospholipase C\(\gamma\) (PLC\(\gamma\)). PLC\(\gamma\) activation induces calcium mobilization and mitogen-activated protein kinase (MAPK) activation, resulting in the activation of nuclear factor of activated T cells (NFAT) and other transcription factors (20,21). Recent evidence also suggests that components of these ITAM-mediated signaling pathways also participate in immune cell processes that are unrelated to adaptive immune responses, including collagen-induced activation of platelets (22), NK cells, and myeloid phagocytes (18,19). Although the roles of the Tec tyrosine kinase family in cells of hematopoietic origin are generally well-characterized, how Tec tyrosine kinases function in ITAM-mediated costimulatory signaling in osteoclasts has not yet been elucidated.

Here we describe a previously unknown role for Btk in osteoclast multinucleation. Preosteoclasts from Xid mice, which possess a natural mutation in the \( Btk \) gene, exhibit a deficiency in cell-cell fusion and decreased NFATc1 expression during RANKL-induced osteoclastogenesis. Moreover, cytokine-induced multinucleation of macrophages was also impaired in the absence of Btk. The introduction of NFATc1 induced cell-cell fusion between preosteoclasts derived from Xid mice. Thus, this study demonstrates that Btk plays a critical role in the processes underlying osteoclast multinucleation.

**EXPERIMENTAL PROCEDURES**

**Mice** - CBA/CaJ (wild-type) and CBA/CaHN-Btk\(^{Xid}/J\) (Xid) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and used to generate osteoclasts. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and used to produce calvarial osteoblasts. All mice used in these experiments were 6-8 weeks old; they were maintained with sterilized food, water, and bedding at the animal facility of the University of Pennsylvania School of Medicine. All experiments were approved by the Animal Studies Committee of the University of Pennsylvania.

**Reagents** - All cell culture media and supplements were purchased from Invitrogen. Soluble recombinant mouse RANKL was purified from insect cells as described (34), and recombinant human M-CSF was a gift from Daved H. Fremont (Washington University, St. Louis, MO). Cyclosporin A (CsA) was purchased from Calbiochem (San Diego, CA).

**Osteoclast formation, F-actin staining, and pit formation** - Murine osteoclasts were prepared from bone marrow cells using two standard methods as previously described (4). In brief, bone marrow cells were obtained by flushing femurs and tibias from wild-type or Xid mice. For cocultures with osteoblasts, bone marrow cells (1 x 10\(^5\) cells/well in 96-well plates) were cocultured with calvarial osteoblasts (1 x 10\(^4\) cells/well) in \(\alpha\)-MEM containing 10% fetal bovine serum (FBS) and supplemented with 20 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\) and 1 \(\mu\)M PGE\(_2\) for 9 days. For stromal cell-free cultures, bone marrow cells were cultured with M-CSF (30 ng/ml) for 3 days in \(\alpha\)-MEM containing 10% FBS and attached cells were used as osteoclast precursors (BMMs).
BMMs were subsequently differentiated into osteoclasts with M-CSF (30 ng/ml) and RANKL (100 ng/ml, unless otherwise indicated) for 4 days. Fresh media containing M-CSF and RANKL were supplied on day 3. Cells were then fixed with 10% formalin and stained with rhodamine-phalloidin (Molecular Probes) to label the F-actin ring, followed by TRAP staining. TRAP(+) MNCs containing more than three nuclei or those that contained more than three nuclei and larger than 100 μm in diameter were counted as osteoclasts. In some experiments, TRAP activity was measured at an absorbance of 405 nm as described previously (4). In addition, bone resorption assays were performed as described (4,35). Briefly, BMMs were loaded onto dentine slices and cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days. To visualize resorption pits, bone slices were stained with 0.5% toluidine blue (Sigma).

**Fusion assays to identify osteoclasts and giant macrophages**

To generate mononuclear preosteoclasts and subsequently induce fusion, bone marrow cells (2 x 10^7 cells/100-mm dish) were cultured with calvarial osteoblasts (2 x 10^6 cells/dish) for 6 days in α-MEM supplemented with 10 nM 1α,25(OH)2D3 and 1 μM PGE2 as described (4). After floating cells were removed, mononuclear cells were harvested from the attached cells by gentle pipetting. TRAP(+) mononuclear preosteoclasts constituted more than 60% of the total harvested cells, and no multinucleated cells were detected. To induce fusion, purified TRAP(+) mononuclear preosteoclasts (1 x 10^5 cells in 200 μl/well in 96-well plates) were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 24 hours. After TRAP staining, TRAP(+) MNCs containing more than three nuclei or those that contained more than three nuclei and larger than 100 μm in diameter were counted as osteoclasts. To assess the fusion efficiency, cells were fixed with 10% formalin, and stained with DAPI (1 μg/ml; Roche) to detect the nuclei. Cells were examined using a Zeiss Axioplan II fluorescence microscope (Zeiss, Thornwood, NY). Total nuclei and TRAP(+) MNCs were counted in the same fields. The relative fusion efficiency was expressed as the ratio of nuclei/osteoclast number for Xid osteoclasts compared with that observed with wild-type osteoclasts. To induce giant macrophage cell formation, BMMs (2 x 10^7 cells/well in 96-well plates) derived from wild-type and Xid mice were cultured in the presence of 100 ng/ml IL-3 and 100 ng/ml IL-4 (R&D systems) for 4-5 days as described previously (5). Multinucleated giant macrophages were stained with May-Grünwald-Giemsa stain according to the manufacturer’s protocol (Sigma).

**Real-time quantitative PCR**

BMMs from wild-type and Xid mice were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days as described above. Total RNA was extracted from cultured cells using TRizol (Invitrogen) on the indicated days. First strand cDNA was transcribed from 1 μg of RNA using Superscript II reverse transcriptase (Invitrogen) following the protocol provided by the supplier. Five microliters of cDNA template (1/20 dilution) was used for real-time PCRs. Real-time PCRs were performed using the TaqMan universal PCR master mix and an ABI Prism 7000 sequence detection system (Applied Biosystems) as described previously (4). TaqMan primers for the indicated genes were purchased from Applied Biosystems.

**Fractionation and Western blot analysis**

BMMs from wild-type or Xid mice were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for the indicated periods of time and preosteoclasts prepared using a coculture system were incubated with M-CSF (30 ng/ml) alone or M-CSF plus RANKL (100 ng/ml) in the absence or presence of CsA (1 μg/ml) for 12 hours to induce fusion. Total lysates were prepared from cultured cells using a lysis buffer (20 mM HEPES, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 0.5 mM EDTA, protease inhibitor cocktail [Roche], 2 mM NaN3VO4, 10 mM NaF, and 1 mM PMSF). After centrifugation, the resulting supernatant was collected as the total lysates. To prepare cytosolic and nuclear extracts, cultured cells were fractionated using a Nuclear and Cytoplasmic Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. Total lysates or fractionated extracts were subjected to SDS-PAGE and Western blotting using antibodies specific for Btk (Santa Cruz), NFA1c1 (Santa Cruz), phospho-PLCγ2 (Cell Signaling), PLCγ2 (Santa Cruz), HA (Roche), actin (Sigma), and histone H3 (Santa Cruz). The degree to which NFA1c1 expression was induced was determined by normalizing the specific signal to that of actin or histone 3 using NIH Image 1.62.
as described (4).

Retrovirus preparation and infection - Retroviral vector encoding a constitutively active form of NFATc1 (MSCV-Ca-NFATc1) was kindly provided by Dr. N. A. Clipstone (Northwestern University) (36). A DNA fragment from MSCV-CaNFATc1 encoding HA-tagged Ca-NFATc1 (the XhoI/Klenow-EcoRI fragment) was subcloned into the pMX-puro vector (BamHI/Klenow-EcoRI) with puromycin as the selective marker (pMX-Ca-NFATc1). To prepare retroviral stocks, control and pMX-Ca-NFATc1 retroviral vectors were introduced into PLAT-E cells using Lipofectamine 2000 (Invitrogen) and the medium was harvested after 2 days. BMMs (1 x 10^7 cells/100-mm dish) were transfected with retroviral supernatant for 6 hours in the presence of M-CSF (120 ng/ml) and polybrene (5 μg/ml) as described (4). After washing, BMMs were cultured overnight, detached with Trypsin/EDTA, and further cultured with M-CSF (120 ng/ml) and puromycin (2 μg/ml) for 2 days. Puromycin-resistant BMMs were allowed to differentiate into osteoclasts for an additional 4-5 days in the presence of 30 ng/ml M-CSF and RANKL at the indicated concentrations.

RESULTS

Mutation of Btk in Xid mice results in decreased osteoclast formation.

To test the potential involvement of Btk in the regulation of osteoclast differentiation, we first examined Btk expression in osteoclast-lineage cells. After bone marrow derived-macrophages (BMMs) from wild-type mice were prepared and stimulated with RANKL, Btk expression was detected using Western blot analysis. The expression of Btk, which was detected in osteoclasts (denoted as day 0 in Figure 1A), increased as the osteoclasts matured (Figure 1A). To determine whether Btk participates in the differentiation of osteoclasts, bone marrow cells prepared from wild-type or Xid mice were cocultured with wild-type calvarial osteoblasts in the presence of 1α,25(OH)₂D₃ and prostaglandin E₂ (PGE₂). In these coculture assays, the differentiation of bone marrow cells derived from Xid mice into tartrate-resistant acid phosphatase-positive multinucleated cells (TRAP(+) MNCs; ≥ 3 nuclei) and large TRAP(+) MNCs (≥ 3 nuclei and ≥ 100 μm in diameter) was significantly diminished compared to the results observed for wild-type cells (Figure 1B and C). In contrast, total TRAP activity, including that in both mononuclear and multinucleated osteoclasts, was similar in the wild-type and mutant osteoclasts (Figure 1D). This result was confirmed by differentiating BMMs into osteoclasts using stromal cell-free BMM cultures in the presence of RANKL and M-CSF (Figure 2A-C). In addition, Btk mutant cells exhibited decreased pit formation on dentine slices due to defects in the maturation of the osteoclasts (Figure 2D). These data implicate Btk in RANKL-induced osteoclastogenesis.

Mutation of Btk causes impaired cell-cell fusion of preosteoclasts and macrophages.

Osteoclast formation involves a series of cell-adhesion, migration, and fusion processes, which results in the formation multinucleated giant cells. Cell-cell fusion of TRAP(+) mononuclear preosteoclasts during the late stages of osteoclastogenesis is a critical step for osteoclast maturation (2,5). As shown in Figures 1 and 2, total TRAP activity in osteoclasts from Xid mice is similar to that in wild-type cells, although the Btk mutant cells were defective for the formation of large TRAP(+) MNCs. Hence, the early steps in osteoclast differentiation do not appear affected by the Btk mutation. We then determined whether or not the efficiency of TRAP(+) mononuclear preosteoclast fusion was affected by the Btk mutation. There were no significant differences between wild-type and Xid preosteoclast formation (Figure 3A). On the other hand, the subsequent cell-cell fusion of preosteoclasts was markedly affected by the Btk mutation (Figure 3B). In addition, when the efficiency of preosteoclast fusion was determined by measuring the average number of nuclei per TRAP(+) MNC, there was a significant reduction in the relative fusion efficiency in Btk-deficient cells compared to that observed in wild-type cells (Figure 3C).

It has been reported that multinucleated giant macrophages are generated from BMMs by cell-cell fusion in the presence of IL-3 and IL-4 (5). To examine the role of Btk in the fusion of a different type of cell, we generated foreign body giant cells (FBGCs) from BMMs. Similar to the pre-osteoclast fusion, cytokine-induced fusion of macrophages was severely impaired in Btk mutant
Impaired osteoclast fusion in Xid mutants is due to defective induction of NFATc1 expression and nuclear translocation.

To investigate how a mutation in Btk induced the observed deficiency in cell fusion, we tested whether Btk regulated downstream effectors of RANKL-mediated signaling, including MAPKs (ERK, JNK, and p38) and transcription factors (NF-κB and NFATc1), which are essential for osteoclastogenesis (3). No significant differences between the Btk mutant and wild-type cells were observed for the activation of the MAPKs and NF-κB by RANKL (data not shown). Interestingly, we found decreased levels of NFATc1 mRNA in the Btk mutant cells during RANKL-induced osteoclast differentiation and preosteoclast fusion (Figure 4A and B). Consistent with the mRNA expression analysis, NFATc1 protein expression in Btk mutant cells was decreased compared to the levels detected in wild-type samples (Figure 4C). Thus, Btk seems to regulate the expression of NFATc1.

In addition, Btk-deficient cells showed reduced nuclear translocation of NFATc1 (Figure 4D). These results suggest that Btk is required for the activation of calcium signaling during osteoclast differentiation, and they are consistent with the previously defined functions of Tec family kinases in other cell types. Activation of calcium signaling in osteoclasts is mediated by ITAM-bearing receptors (Fcγ and DNA-activating protein [DAP] 12) via PLCγ. In osteoclasts, PLCγ2 appears to be critical for the activation of calcium signaling, because NFATc1 activation was impaired in the absence of PLCγ2 (20,21,23). Therefore, we tested whether PLCγ2 activation measured using its phosphorylation status depends on Btk in osteoclasts, and showed that PLCγ2 activation was significantly reduced in the absence of Btk (Figure 4E).

The initial induction of NFATc1 mRNA expression during RANKL-induced osteoclast differentiation is mediated by a TRAF6-dependent signaling pathway, and its expression is further amplified by the activated NFATc1 protein itself (24). The autoamplification of NFATc1 expression depends on sustained calcium signaling mediated by PLCγ2 activation (24,25). Although decreased compared to the levels detected in wild-type samples, RANKL treatment significantly induced the expression of NFATc1 mRNA (~50% increase) (Figure 4F). This low level of induction was not affected by CsA treatment, suggesting that the NFATc1 mRNA expression observed in Btk mutant cells was not a result of positive feedback by activated NFATc1. When wild-type cells were treated with CsA, the level of NFATc1 mRNA was significantly reduced to approximately the level observed in the RANKL-treated Btk-deficient cells, rather than that measured in untreated wild-type or Btk mutant cells. These results suggest that Btk deficiency inhibits the PLCγ2-dependent positive feedback loop governing NFATc1 expression, thus leading to decreased levels of NFATc1 mRNA and protein (Figure 4A and B).

Taken together, these results suggest that the defect in osteoclast fusion induced by the Btk mutation was a result of reduced NFATc1 activity. Indeed, we recently showed that NFATc1 contributes to osteoclast fusion by directly upregulating the expression of various genes involved in cell-cell fusion (10). In agreement with these results, the expression levels of several genes that encode cell fusion- and adhesion-related proteins, including Atp6v0d2, DC-STAMP, integrin αv, and integrin β3, were significantly lower in Btk-deficient cells than in wild-type cells, whereas the expression levels of various representative markers of osteoclast differentiation, including TRAP, OSCAR, and cathepsin K, were comparable in these two samples (Figure 5).

Ectopic expression of NFATc1 restores osteoclast formation from BMMs derived from Xid mice.

Because the cell-cell fusion defect in Btk mutants resulted from a downregulation of NFATc1 expression, overexpression of NFATc1 should restore cell fusion in Btk mutants. Hence, we overexpressed NFATc1 in BMM osteoclast precursors prepared from Xid mice and assessed RANKL-induced osteoclastogenesis. We introduced a recombinant retroviral construct encoding a constitutively active form of NFATc1 (Ca-NFATc1) into osteoclast precursors. The expression of Ca-NFATc1 was confirmed by immunoblot analysis (Figure 6A). Osteoclast formation was found to be significantly restored by the introduction of Ca-NFATc1 to Btk mutant cells (Figure 6B and Supplementary Figure 1).
Taken together, these results demonstrate that Btk-mediated NFATc1 activation is critical for RANKL-induced osteoclast maturation.

**DISCUSSION**

The roles of Btk in B-cell development have been extensively studied (19,20,26-28), whereas comparatively little is known about its role in osteoclastogenesis. Here, we have elucidated a role of Btk in osteoclastogenesis. Bone marrow cells derived from Xid mice, which carried a mutation in the Btk gene, showed a dramatically decreased ability to form TRAP(+) MNCs (≥ 3 nuclei). For the formation of large TRAP(+) MNCs (≥ 3 nuclei and 100 μm in diameter), the defect was more severe due to decreased fusion efficiency of TRAP(+) mononuclear preosteoclasts. The Btk deficiency produced impaired preosteoclast fusion as a result of reduced NFATc1 activity. Therefore, our findings have identified osteoclasts as yet another cell type that requires Btk, and indicate that Btk regulates NFATc1 expression and activation during osteoclast differentiation.

It is now well-established that NFATc1 is a master regulator of osteoclast differentiation (8,9,24,25). In addition to its role during the early commitment of monocytic precursors to osteoclast differentiation, accumulating data support the importance of NFATc1 during later stages of osteoclast maturation, such as cell-cell fusion; this transcription factor regulates the expression of various genes that encode proteins involved in osteoclast fusion, including Atp6v0d2 and DC-STAMP (4), and osteoclast-mediated bone resorption, such as TRAP, cathepsin K, c-Src, and β3 integrin (8,9,24,25). Our data suggest that Btk regulates the former aspects of NFATc1-mediated functions during osteoclast maturation.

It has been reported that costimulatory signals mediated by ITAM-containing adaptors, such as FcRγ and DAP12, play critical roles in RANKL-induced osteoclast formation (25,29,30). Mice lacking both FcRγ and DAP12 develop severe osteopetrosis owing to impaired osteoclast differentiation (25,29). These ITAM-containing adaptors link to downstream pathways through the Syk kinases, a non-Src family of protein tyrosine kinases, in a manner similar to BCR signaling during B-cell development (31-33). The osteoclasts from FcRγ- and DAP12-deficient mice showed impaired activation of calcium signaling through PLCγ, followed by decreased NFATc1 expression (25). In addition, mice harboring a targeted deletion of PLCγ2 develop an osteopetrotic phenotype and exhibit decreased RANKL-mediated NFATc1 expression (23). Our results have identified another player in this pathway, the Tec family kinase Btk, which is consistent with signaling cascades examined in other cell types, such as B cells (Fig. 7). Detailed investigations of the signaling cascades underlying all of the functions of NFATc1 will provide insights into the regulation of osteoclastogenesis and its relationship with the pathologies of bone diseases, such as osteoporosis and rheumatoid arthritis.

**REFERENCES**

FOOTNOTES

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The abbreviations used are: BMMs, Bone marrow derived macrophages; Btk, Bruton’s tyrosine kinase; Xid, X-linked immunodeficiency; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of NF-κB ligand; NFATc1, nuclear factor of activated T cells 1; TRAP(+) MNCs, tartrate-resistant acid phosphatase-positive multinucleated cells, ITAM, immunoreceptor tyrosine-based activation motif.

FIGURE LEGENDS

Figure. 1. Decreased osteoclast formation from the bone marrow cells of Xid mice in a coculture system. A, Induction of Btk expression during osteoclastogenesis. BMMs from wild-type (WT) mice were cultured with M-CSF and RANKL for 4 days. Total lysates were subjected to SDS-PAGE and Western blot analysis to detect Btk. B and C, Osteoclast formation in a coculture system. Bone marrow cells derived from WT and Xid mice were cultured with osteoblasts in the presence of 1α,25(OH)2D3 and PGE2 for 9 days. B, Cells were fixed and stained for TRAP. C, TRAP(+) MNCs with more than three nuclei (left panel) or more than three nuclei and larger than 100 μm in diameter (right panel) were counted as osteoclasts. D, A TRAP solution assay. TRAP activity was assessed at an absorbance of 405 nm. Data are expressed as the mean ± S.D. and are representative of at least three experiments. NS, not significant; *P < 0.05 vs. WT; and †P < 0.005 vs. WT. Scale bar, 200 μm.

Figure. 2. Decreased TRAP(+) MNCs and pit formation by bone marrow cells derived from Xid mice in stromal cell-free BMM cultures. A and B, Osteoclast formation in BMM cultures. BMMs were cultured with 30 ng/ml M-CSF and RANKL at the indicated concentrations for 4 days. A, Cells were fixed and stained with rhodamine-phalloidin to label the F-actin ring, followed by TRAP staining. B, TRAP(+) MNCs with more than three nuclei (left panel) or more than three nuclei and larger than 100 μm in diameter (right panel) were counted as osteoclasts. C, The TRAP solution assay was performed as described in the material and methods. D, Pit formation. BMMs were cultured on dentine slices with M-CSF and RANKL for 4 days. Resorption pits were stained with 0.5% toluidine blue and counted. Data are expressed as the mean ± S.D. and are representative of at least three experiments. *P < 0.05 vs. WT and †P < 0.01 vs. WT. Scale bar, 200 μm.

Figure. 3. Impaired fusion of preosteoclasts from Xid mice. A, The formation of TRAP(+) mononuclear preosteoclasts. The formation of preosteoclasts (pOCs) was induced by coculturing bone marrow cells derived from wild-type (WT) or Xid mice with osteoblasts in the presence of 1α,25(OH)2D3 and PGE2 for 6 days. Cells were stained for TRAP (left panel), and TRAP(+) mononuclear cells were counted as preosteoclasts (right panel). B, Mature osteoclast formation from preosteoclasts. Preosteoclasts obtained as described in (A) were differentiated into osteoclasts in the presence of M-CSF and RANKL for 24 hours. Cells were fixed and stained for TRAP (left panel). TRAP(+) MNCs with more than three nuclei (upper right panel) or more than three nuclei and larger than 100 μm in diameter (lower right panel) were counted as fused osteoclasts. C, A fusion-efficiency assay. The efficiency of preosteoclast fusion was calculated by dividing the total number of nuclei stained with DAPI within TRAP(+) MNCs.
by the number of TRAP(+) MNCs. D, The formation of FBGCs. BMMs from wild-type and Xid mice were treated with IL-3 and IL-4 to induce giant cell formation. Cells were stained with May-Grünwald-Giemsa (left). Giant cells (> 100 μm in diameter) were counted (right). Data are represented as the mean ± S.D. of experiments performed in triplicate. NS, not significant; *P < 0.01 vs. WT; and †P < 0.001 vs. WT. Scale bar, 200 μm.

Figure. 4. Decreased expression and nuclear translocation of NFATc1 in preosteoclasts from Xid. A, NFATc1 mRNA levels during osteoclast differentiation in BMM cultures. BMMs from wild-type (WT) and Xid mice were treated with M-CSF and RANKL for 4 days. RNA was isolated at the indicated time points and subjected to real-time quantitative PCRs to analyze the levels of NFATc1 mRNA. B, NFATc1 mRNA levels during preosteoclast fusion. To generate preosteoclasts, bone marrow cells from wild-type (WT) and Xid mice were cultured with osteoblasts in the presence of 1α,25(OH)2D3 and PGE2 for 6 days. Preosteoclasts were treated with M-CSF and RANKL to induce cell-cell fusion. RNA was isolated at the indicated time points and subjected to real-time quantitative PCRs as described in (A). C and D, NFATc1 expression and translocation during preosteoclast fusion. Preosteoclasts obtained as described in (B) were induced to fuse for 12 hours with M-CSF alone or M-CSF plus RANKL in the absence or presence of CsA (1 μg/ml). Total lysates (C) or cytosolic and nuclear fractions (D) were harvested from cultured cells, and subjected to SDS-PAGE and Western blot analysis to detect NFATc1. Antibodies specific for actin and histone H3 were used to normalize the cytosolic and nuclear extracts, respectively. E, PLCγ2 activation by RANKL in WT and Xid mutant cells. BMMs from WT and Xid mice were stimulated with RANKL (100 ng/ml) after 6 hours of starvation in serum-free medium. Total lysates were subjected to SDS-PAGE and Western blot analysis. Data are representative of three independent experiments. F, NFATc1 mRNA levels were measured as described in (A). Cells were treated with RANKL for 24 hours in the absence or presence of CsA (1 μg/ml). *P < 0.05; NS, not significant.

Figure. 5. Expression levels of fusion-related molecules during the differentiation of BMMs derived from Xid mice into osteoclasts. BMMs from wild-type (WT) and Xid mice were cultured with M-CSF and RANKL for 4 days. RNA was isolated on the indicated days and subjected to real-time quantitative PCR analysis. Day 0 indicates that the cells were BMMs. A, Levels of mRNA encoding osteoclast specific markers, including OSCAR, TRAP, and Cathepsin K. B, Levels of mRNA encoding fusion-related (Atp6v0d2 and DC-STAMP) and adhesion molecules (integrin αv and integrin β3).

Figure. 6. Restoration of osteoclast formation by ectopic expression of NFATc1 in BMMs derived from Xid mice. A, Overexpression of NFATc1. BMMs from wild-type (WT) and Xid mice were infected with pMX-puro (control retrovirus) or retrovirus encoding HA-tagged Ca-NFATc1 (constitutively active form), and selected with puromycin (2 μg/ml) for 48 hours. Retroviral-mediated expression of Ca-NFATc1 was analyzed by Western blotting with antibodies specific for HA. B, Effects of NFATc1 overexpression on osteoclast differentiation. BMMs infected with retrovirus were differentiated into osteoclasts in the presence of M-CSF (30 ng/ml) and RANKL (0-200 ng/ml) for 4 days. Cultured cells were fixed and stained for TRAP. TRAP(+) MNCs with more than three nuclei and larger than 100 μm in diameter were counted as osteoclasts. Data are expressed as the mean ± S.D. and are representative of at least three experiments. *P < 0.02 vs. WT and †P < 0.001 vs. WT. Scale bar, 200 μm.

Figure. 7. A schematic model of the induction of NFATc1 activation during RANKL-stimulated osteoclastogenesis. ITAM activation resulting from stimulation via immunoreceptors (OSCAR and TREM-2) or the RANKL-RANK interaction may lead to activation of Btk, and subsequent recruitment of downstream effectors, including PLCγ2. PLCγ2 activation induces calcium signaling, which is critical for NFATc1 activation. Alternatively, Btk may recruit and activate undefined effector molecules, which, in turn, mediate NFATc1 activation. NFATc1 activation is also dependent on c-Fos and TRAF6, both of which are activated by RANKL. Finally, activated NFATc1 may contribute to osteoclast fusion, differentiation, and activation.
Figure 1

A

Days

0 1 2 3 4

Btk

Actin

B

WT  Xid

TRAP(+) MNCs/well

≥ 3 nuclei, ≥ 100 μm in diameter

C

≥ 3 nuclei

≥ 3 nuclei, ≥ 100 μm in diameter

D

TRAP activity (A405nm)

NS

Btk

Actin

WT

Xid

TRAP(+) MNCs/well

WT

Xid

TRAP activity (A405nm)

WT

XID

Figure 1

0 1 2 3 4

Days

Btk

Actin

WT  Xid

TRAP(+) MNCs/well

≥ 3 nuclei, ≥ 100 μm in diameter

D

TRAP activity (A405nm)

NS

Btk

Actin

WT

Xid

TRAP(+) MNCs/well

WT

XID

TRAP activity (A405nm)

WT

XID

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Figure 2

A  TRAP  F-actin

WT

Xid

B

≥ 3 nuclei

≥ 3 nuclei, ≥ 100 μm in diameter

TRAP(+) MNCs/well

0 20 50 100 200

RANKL (ng/ml)

WT  Xid

C

D

No. of Pit

WT  Xid

WT  Xid

TRAP activity (A405nm)

0 0.2 0.4 0.6 0.8 1 1.2

RANKL (ng/ml)

WT  Xid

WT  Xid

No. of Pits

0 100 200 300 400 500 600 700 800 900 1000

WT  Xid

†
Figure 3

A

WT

Xid

WT

Xid

B

TRAP(+) pOCS/field

WT

Xid

3 nuclei

NS

TRAP(+) MNCs/well

WT

Xid

≥ 3 nuclei, ≥ 100 μm in diameter

C

Relative fusion efficiency (%)

WT

Xid

*  

D

WT

Xid

FBGCs/well

WT

Xid

†
Figure 5

A

- **TRAP**
  - WT
  - Xid

- **OSCAR**
  - WT
  - Xid

- **Cathepsin K**
  - WT
  - Xid

B

- **Atp6v0d2**
  - WT
  - Xid

- **DC-STAMP**
  - WT
  - Xid

- **Integrin αv**
  - WT
  - Xid

- **Integrin β3**
  - WT
  - Xid
Figure 6

A

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NFATc1-HA

Actin

B

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WT

Xid

TRAP(+) MNCs/well

* †
Figure 7
Supplementary Figure 1. Restoration of osteoclast formation by introducing NFATc1 to bone marrow cells from Xid mice. BMMs from WT and Xid mice were infected with retrovirus bearing pMX-puro (control) or retrovirus encoding Ca-NFATc1 (a constitutively active form of the protein), and selected with puromycin (2 μg/ml) for 48 hours as described in the material and methods. BMMs infected with retroviral vectors were stimulated to differentiate into osteoclasts with 30 ng/ml M-CSF and the indicated concentrations of RANKL for 4 days. Cultured cells were fixed and stained for TRAP.
Supplementary Figure 1

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The Tec family tyrosine kinase Btk regulates RANKL-induced osteoclast maturation
Seoung Hoon Lee, Taesoo Kim, Daewon Jeong, Nacksung Kim and Yongwon Choi
J. Biol. Chem. published online February 14, 2008

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