Osteoclast Differentiation Is Impaired in the Absence of Inhibitor of κB Kinase α

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Signaling through the receptor activator of nuclear factor κB (RANK) is required for both osteoclast differentiation and mammary gland development, yet the extent to which RANK utilizes similar signaling pathways in these tissues remains unclear. Mice expressing a kinase-inactive form of the inhibitor of κB kinase α (IKKα) have mammary gland defects similar to those of RANK-null mice yet have apparently normal osteoclast function. Because mice that completely lack IKKα have severe skin and skeletal defects that are not associated with IKKα-kinase activity, we wished to directly examine osteoclastogenesis in IKKα−/− mice. We found that unlike RANK-null mice, which completely lack osteoclasts, IKKα−/− mice did possess normal numbers of TRAP+ osteoclasts. However, only 32% of these cells were multinucleated compared with 57% in wild-type littermates. A more profound defect in osteoclastogenesis was observed in vitro using IKKα−/− hematopoietic cells treated with colony-stimulating factor 1 and RANK ligand (RANKL), as the cells failed to form large, multinucleated osteoclasts. Additionally, overall RANK-induced global gene expression was significantly blunted in IKKα−/− cells, including osteoclast-specific genes such as TRAP, MMP-9, and c-Src. IKKα was not required for RANKL-mediated IκBα degradation or phosphorylation of mitogen-activated protein kinases but was required for RANKL-induced p100 processing. Treatment of IKKα−/− cells with tumor necrosis factor α (TNFα) in combination with RANKL led to partial rescue of osteoclastogenesis despite a lack of p100 processing. However, the ability of TNFα alone or in combination with transforming growth factor β to induce osteoclast differentiation was dependent on IKKα, suggesting that synergy between RANKL and TNFα can overcome p100 processing defects in IKKα−/− cells.

Skeletal mass homeostasis is maintained by the coupled activities of bone-building osteoblasts and bone-resorbing osteoclasts. Dysregulation of osteoclast differentiation or function underlies many diseases affecting the skeletal system, including osteoporosis, joint inflammation, tumor metastasis to bone, and Paget disease (1). Osteoclasts are highly specialized multinucleated cells that differentiate from monocyte/macrophage lineage hematopoietic precursors. The key factors regulating osteoclastogenesis are the TNF receptor family member RANK, its ligand (RANKL), and the RANKL inhibitor, osteoprotegerin. Ablation of RANK or RANKL in mice results in a complete absence of osteoclasts and severe osteopetrosis, whereas lack of osteoprotegerin leads to excessive osteoclast activity and osteoporosis (2–5).

Genetic studies in mice, as well as in vitro culture of osteoclasts using CSF-1 and RANKL, have elucidated many key components of RANK signaling during osteoclastogenesis. Binding of RANKL to RANK stimulates recruitment of TRAF2, -5, and -6 (6) followed by activation of mitogen-activated protein kinases and IκB kinases, which ultimately lead to activation of the transcription factors AP-1 and NF-κB. Osteopetrosis has been observed in c-fos−/− mice (7), Nfkb1/2 double knock-out mice (8), and TRAF6−/− mice (9). The contribution of other signaling components to RANK-mediated osteoclastogenesis remains unclear because ablation of many of these genes, such as the NF-κB family member p65, TRAF2, and IKKβ, results in embryonic or neonatal lethality (10–12). Additionally, although cytokines that contribute to osteoclast differentiation during inflammation, such as TNFα and interleukin 1, can activate NF-κB and AP-1 (13, 14) in osteoclast precursor cells, RANK and RANKL remain essential for osteoclastogenesis in vivo. For instance, administering osteoprotegerin to rats with adjuvant-induced arthritis abrogates osteoclastogenesis and subsequent bone loss without inhibiting inflammation (15). Furthermore, bone erosion induced by overexpression of TNFα in transgenic mice can be alleviated by administering RANK-Fc or by crossing TNFα transgenic mice into a RANK-null background (16). These studies indicate that RANK may activate unique signaling pathways in osteoclast progenitor cells.

In addition to osteoclast differentiation, RANK signaling is required for mammary gland development during pregnancy (17). Female RANK- or RANKL-null mice are unable support live birth because of an inability to lactate. This defect is because of insufficient mammary epithelial cell proliferation during pregnancy as well as an increase in mammary epithelial cell apoptosis, indicating that RANK signaling is required for both the proliferation and survival of mammary epithelial cells that are necessary for the full development of the mammary

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† The abbreviations used are: TNF, tumor necrosis factor; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; TGFβ, transforming growth factor β; IKK, inhibitor of κB kinase; CSF, colony-stimulating factor; E18.5 dpc, embryonic day 18.5 postconitus; FLC, fetal liver cells; WT, wild type; GM-CFU, granulocyte-macrophage colony-forming unit; TRAP, tartrate-resistant acid phosphatase; TRAF, tumor necrosis factor receptor-associated factor; NIK, NF-κB induced kinase.
lobuloalveolar structures during pregnancy. As with osteoclasts, genetic studies in mice have been useful for determining the specific signaling pathways utilized by RANK in the mammary gland. Specifically, mice expressing a kinase-inactive allele of the inhibitor of x kinase α (IKKaAA) had defects in mammary gland differentiation similar to those in RANK or RANKL-null mice (18). These defects were linked to a lack of NF-κB activation and subsequent up-regulation of cyclin D1 in mammary epithelial cells. Interestingly, IKKaαα mice had no apparent defects in osteoclast differentiation, indicating that RANK may utilize tissue-specific signaling pathways to activate NF-κB.

The transcription factor NF-κB is composed of homo- or heterodimers of the NF-κB family members RelA/p65, RelB, cRel, p50, and p52 (reviewed in Ref. 12). The latter two proteins are synthesized as precursors p105 and p100, respectively. Processing of p105 into p50 is constitutively regulated, and p50 is most commonly associated with p65 and the inhibitor protein IkBα in an inactive complex in the cytosol. Activation of the IKK complex containing the catalytic IKKα and β subunits and the regulatory IKKγ subunit leads to phosphorylation, ubiquitination, and subsequent proteasome-mediated degradation of IkBα, allowing nuclear translocation of p65/p50. In many cell types, such as fibroblasts or lymphocytes, IKKα kinase activity is dispensable for phosphorylation of IkBα and activation of p65/p50 in response to TNFα or interleukin 1, but it is required for the processing of p100 into p52 in response to stimuli including lymphoxygen β, CD40 ligand, and B cell activating factor. In contrast, IKKγ is required for the activation of p65/p50 by RANKL in mammary epithelial cells (18). The contribution of IKKα to RANKL-mediated osteoclastogenesis remains unclear, although both p100 processing and p65/p50 nuclear translocation have been implicated as important components of this process (19, 20). To determine whether IKKα plays a role in osteoclast differentiation, we used mice completely lacking IKKα protein (IKKα−/−) (21). Because these mice die at birth, we examined osteoclast differentiation in vivo in embryonic day 18.5 postconception (E18.5 dpc) IKKα−/− embryos and in vitro by culturing IKKα−/− hematopoietic cells with CSF-1 and RANKL. Using this system, we determined that the ability of RANKL to induce the formation of large multinucleated osteoclasts in vitro is impaired in the absence of IKKα. This observation was correlated with a lack of p100 processing into p52. However, adding TNFα to RANKL-treated IKKα−/− cells partially restored osteoclastogenesis despite the presence of accumulated p100. Interestingly, we also observed a partial absence of multinucleated osteoclasts in vivo in IKKα−/− embryos, indicating that a lack of IKKα in osteoclast precursor cells in vivo may be overcome by cooperation between RANKL and other cytokines, such as TNFα.

MATERIALS AND METHODS

Precursor Cell Analysis and Osteoclast Differentiation Assays— Timed matings were set up for IKKα hemizygous male and female mice as described previously (21) to obtain IKKα wild-type and knock-out embryos. Fetal livers were harvested from E18.5 dpc embryos and disrupted to obtain single-cell suspensions in α minimum Eagle’s medium (Invitrogen). Red blood cells were lysed in solution (Sigma) followed by resuspension in α minimum Eagle’s medium containing 10% fetal bovine serum and antibiotics. To determine progenitor cell frequency, cells were plated in Methocult™ medium 3534 containing stem cell factor, interleukin 6, and interleukin 3 (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) according to the manufacturer’s directions, and morphologically distinct GM-CFU colonies were counted for duplicate wells of three separate wild-type and IKKα−/− embryos. For analysis of macrophage lineage markers, fetal liver cells were grown for 6 days in medium containing a minimum Eagle’s-medium and 40 ng/ml CSF-1 (R&D Systems, Minneapolis, MN). Cells were trypsinized and incubated with fluorescein labeled anti–bodies to CD11b (BD Biosciences) and MOMA-2 (Serotec, Inc., Raleigh, NC) followed by flow cytometric analysis. For osteoclast differentiation assays, cells were plated at 5 × 10⁴ cells/well in 96-well plates in the presence of 40 ng/ml CSF-1 with or without 300 ng/ml recombinant murine RANKL (22). Where indicated, cells were also treated with recombinant murine TNFα at 20 ng/ml and/or TGFR (R&D Systems). Every 48 h, one-half of the cell medium was replaced with medium containing fresh cytokines. Cells were stained after 6 days in culture for TRAP in situ using a commercially available kit (Sigma). For direct analysis of osteoclasts in vivo, E18.5 dpc embryos were fixed in 4% neutral buffered formalin for 24 h and decalcified in 10% EDTA for 5 days. Embryos were step-sectioned into 40 sections and stained for TRAP activity.

RNA Analysis—RNA was extracted from wild-type and IKKα−/− fetal liver cell cultures after 6 days treatment with CSF-1 and murine RANKL using the RNaseasyTM kit (Qiagen). For quantitative reverse transcription-PCR, cDNA was prepared from 1 μg of total RNA and used for PCR reactions using specific primers and SYBR Green dye (Applied Biosystems, Foster City, CA) as described previously (23). For microarray expression profiling, 5 μg of total RNA from individual samples was labeled according to standard protocols (Affymetrix, Santa Clara, CA) and 10 μg of biotinylated cRNA was hybridized to Mouse Affymetrix U430A (MOE430A) chips consisting of ∼22,500 probe sets (Affymetrix, Santa Clara, CA). Hybridized chips were washed and annealed on an Affymetrix FS400 fluidics station using the antibody amplification protocol and scanned using an Affymetrix GeneArray 2500 scanner. Scanned images were loaded into the ResolverTM system (Rosetta Bio- software, Kirkland, WA) for analysis.

Western Blots—Cells were grown for 6 days in CSF-1 and treated with murine RANKL for indicated times prior to lysis in 1% cell lysis buffer (Cell Signaling Technologies, Beverly, MA) supplemented with 1 mm phenylmethylsulfonyl fluoride and 1 mm NaF. Insoluble material was removed by centrifugation at 13,000 rpm in a microcentrifuge at 4 °C for 10 min. Supernatants were stored in aliquota at −70 °C. Protein concentration was determined using a BCA kit (Fierce), and 10 μg of each sample were separated on 8–16% gradient gels (Invitrogen) and transferred to nitrocellulose membranes. Equivalent loading was confirmed by staining membranes with Ponceau S reagent followed by blocking in 5% milk in Tris-buffered saline, 0.1% Tween 20. Primary antibodies were applied in a solution containing 3% bovine serum albumin and 0.1% Tween 20 overnight at 4 °C. Horse radish peroxidase-labeled secondary antibodies were applied after washing, which was followed by more washing and then detection using ECL reagent (Amer sham Biosciences). IκBα antibody (C-21) was obtained from Santa Cruz Biotechnology. Antibodies for phosphorylated proteins and corresponding total proteins were obtained from Cell Signaling Technologies. Antibody to mouse p190 was created by injecting rabbits with the NH-terminus peptide DNCYDPGLGDIPEYD, as described previously (24).

RESULTS

Defective Osteoclast Differentiation in the Absence of IKKα—IKKα-deficient mice die immediately after birth because of keratinocyte differentiation defects (21). Therefore, to determine whether IKKα was required for osteoclast differentiation, we analyzed osteoclasts in embryos of IKKα wild-type (+/+) and IKKα-null (−/−) littermates at E18.5 dpc. Although similar numbers of cells in both wild-type and knock-out embryos stained positive for the osteoclast marker TRAP (data not shown), the morphology and size of the TRAP− cells observed in IKKα−/− embryos was dramatically different from those in wild-type littermates (Fig. 1A). In IKKα wild-type embryos, osteoclasts were predominantly multinucleated (57% multinucleated in +/+ and 61% in +/−, Fig. 1B) and contained a highly vacuolated cytoplasm. In contrast, significantly fewer multinucleated TRAP+ cells were identified in IKKα−/− embryos (32%, p < 0.001 compared with +/+; Fig. 1B), and these cells had an altered morphology compared with wild type, often containing a reduced dense granulated cytoplasm.

To further characterize the role of IKKα in osteoclastogenesis, we next determined whether IKKα-null fetal liver-derived hematopoietic cells could differentiate into osteoclasts in vitro. Fetal liver cells (FLC) from wild-type or IKKα−/− E18.5 dpc embryos were treated with CSF-1 and RANKL for 6 days and stained for TRAP activity. Treatment of wild-type FLC with
CSF-1 and RANKL led to the formation of large multinucleated TRAP\(^+\) osteoclasts (Fig. 2A). However, in IKK\(\alpha^{-/-}\) FLC treated with CSF-1 and RANKL, only small TRAP\(^+\) cells were observed. The number of multinucleated osteoclasts generated from IKK\(\alpha^{-/-}\) FLC was decreased by >15-fold compared with wild type (Fig. 2). To determine whether the lack of osteoclast differentiation observed in IKK\(\alpha^{-/-}\) FLC was because of a difference in osteoclast precursor cell numbers, cells were grown in methylcellulose medium containing monocyte/macrophage growth factors, and the frequency of GM-CFU, the earliest defined myeloid progenitor (25), was determined. No differences in GM-CFU frequency were observed between wild-type and IKK\(\alpha^{-/-}\) cells (Fig. 3A), indicating that the lack of osteoclast differentiation observed in IKK\(\alpha^{-/-}\) FLC is not because of a defect in the myeloid precursor cell compartment. The defect observed in IKK\(\alpha^{-/-}\) FLC is specific to osteoclasts but not macrophage, because similar numbers of cells expressing the markers CD11b and MOMA-2 were observed in wild-type and IKK\(\alpha^{-/-}\) FLC after 1 week of growth in CSF-1 (Fig. 3B). Taken together, these results indicate that IKK\(\alpha\) is required for RANKL-mediated osteoclast differentiation from CSF-1-derived precursor cells in vitro.

Lack of Osteoclast-specific Gene Up-regulation in RANKL-treated IKK\(\alpha^{-/-}\) Cells—To further analyze the extent to which RANK-mediated osteoclast differentiation was inhibited in the absence of IKK\(\alpha\), we assessed the expression of osteoclast-specific genes using quantitative reverse transcription-PCR (Fig. 4). In wild-type FLC grown in CSF-1 and RANKL for 6 days, expression of Mmp-9, c-Src, TRAP, and \(\beta\)_3 integrin was greatly increased compared with cells grown in CSF-1 alone. In contrast, RANKL-induced expression of these genes in IKK\(\alpha\) FLC was blunted, with a >16-fold decrease in expression compared with wild type. Importantly, however, expression of RANK was not different between wild-type or IKK\(\alpha^{-/-}\) FLC, indicating that loss of RANK expression was not responsible for the lack of response to RANKL in IKK\(\alpha^{-/-}\) FLC.

To determine whether IKK\(\alpha\) was required for a specific subset of RANKL-induced genes or for overall RANKL-induced gene expression, we compared global gene expression in both knock-out and wild-type FLC grown for 6 days in CSF-1 with or without RANKL using an oligonucleotide array representing ~22,500 mouse genes (Affymetrix). A striking difference was observed in overall RANKL-induced gene expression in IKK\(\alpha^{-/-}\) cells compared with wild type. Although 209 genes were up-regulated >5-fold \((p < 0.001)\) consistently in duplicate cultures of wild-type FLC treated with CSF-1 and RANKL compared with cells treated with CSF-1 alone (Fig. 5A and supplemental material), only 29 genes were up-regulated >5-fold consistently in duplicate cultures of IKK\(\alpha^{-/-}\) FLC in response to RANKL. Conversely, 226 genes were down-regulated >5-fold in RANKL-treated wild-type FLC, but only three were down-regulated >5-fold in RANKL-treated IKK\(\alpha^{-/-}\) FLC. The expression patterns of the genes up-regulated >5-fold in wild-type FLC treated with CSF1 and RANKL fall into two broadly even categories in similarly treated IKK\(\alpha^{-/-}\) FLC. These categories are a group of genes that show no up-regulation and a group of genes that show “diminished” up-regulation. For example, of the 79 most up-regulated genes in wild-type FLC (>20-fold, \(p > 0.001\)), 32 show effectively no up-regulation (with a trend to down-regulation) in IKK\(\alpha^{-/-}\) FLC (Fig. 5B), whereas 47 show up-regulation but with diminished magnitude (Fig. 5C). Taken together, these results indicate that the overall changes in gene expression induced by RANKL during osteoclast differentiation in vitro are dependent on IKK\(\alpha\).

IKK\(\alpha\) Is Required for RANK-mediated p100 Processing but Not for Activation of the Classical NF-kB Pathway in CSF-1-derived Precursor Cells—Activation of the NF-kB transcription factor pathway has been shown previously to occur immediately after binding of RANKL to RANK and has been implicated in osteoclast differentiation, activation, and survival (1).
To determine whether IKK/IKKa was required for activation of NF-κB by RANKL in osteoclast precursor cells, we analyzed the ability of RANKL to stimulate degradation of the NF-κB inhibitor IκBα in CSF-1-derived wild-type and IKKα/IKKa FLK. In both wild-type and knock-out cells, RANKL treatment led to the complete degradation of IκBα by 15 min, which was followed by IκBα resynthesis at 30 min (Fig. 6A, top panel). Because IκBα resynthesis is dependent on NF-κB transcription factor activity, these data indicate that IKKα is not required for initial activation of NF-κB by RANKL in osteoclast precursor cells. In addition to the classical NF-κB pathway, RANKL activates a second, or non-canonical, NF-κB activity involving processing of the inhibitory protein p100 into a smaller active p52 subunit. Previous studies have shown that RANKL-mediated p100 processing is dependent on NIK, an upstream activator of IKKα (19). To directly assess the role of IKKα in RANKL-mediated p100 processing, we analyzed the relative levels of both p100 and p52 in CSF-1-derived wild-type and IKKα/IKKa FLK treated with RANKL (Fig. 6A, bottom panel). In wild-type cells, p52 accumulation was apparent 8 h after RANKL treatment, whereas p100 levels remained unchanged. However, in IKKα/IKKa cells, RANKL treatment led to accumulation of p100 without any increase in p52 levels, indicating that IKKα is required for RANKL-mediated p100 processing in osteoclast progenitor cells.

Next we analyzed additional signaling pathways activated by RANKL, specifically mitogen-activated protein kinases p38, extracellular signal-regulated kinase, and c-Jun NH2-terminal kinase (Fig. 6B). Phosphorylated forms of these proteins were detected 10 min post-RANKL exposure in both wild-type and
Taken together, these data indicate that IKKα is not required for activation of RANKL-activated early signaling pathways involving classical NF-κB and mitogen-activated protein kinases, but is necessary for later RANKL-induced p100 processing.

Rescue of RANKL-mediated Osteoclast Differentiation in IKKα−/− Cells by TNFα—IKKα−/− FLC failed to form large, multinucleated osteoclasts in response to RANKL; however, such cells were observed in skeletal sections of IKKα−/− embryos, although there were a reduced number of cells.
IKKα and Osteoclastogenesis

FIG. 6. IKKα is required for activation of p100 processing by RANKL but not early signaling events. A, wild-type and IKKα−/− FLC were grown in the presence of 40 ng/ml CSF-1, and RANKL was added at 200 ng/ml at indicated times prior to cell harvest on day 6. Whole cell lysates were used for immunoblot analysis with antibodies to IkBα (upper panel) and p100/p52 (lower panel). Arrows indicate specific bands detected for each protein. B, cells were treated as described in A. Lysates were immunoblotted with antibodies for phospho-extracellular signal-regulated kinase (p-ERK), phospho-c-Jun NH2-terminal kinase (p-JNK), and phospho-p38 (p-p38), and then blots were stripped and reprobed with corresponding total protein antibodies.

companied with wild-type littermates. The discrepancy between in vitro and in vivo osteoclastogenesis in IKKα−/− cells may be because of the presence of additional factors such as TNFα or TGFβ that can promote RANKL-mediated differentiation (13, 26). Therefore, we wished to determine whether TNFα or TGFβ could affect RANKL signaling (specifically p100 processing) or ultimately osteoclastogenesis in IKKα−/− cells. Neither TNFα nor TGFβ stimulated processing of p100 into p52, either alone or in combination with RANKL in IKKα−/− cells (Fig. 7A). However, TNFα treatment alone, but not TGFβ, led to accumulation of p100 protein (without p52 formation) in IKKα−/− cells, similar to RANKL treatment. In wild-type cells, TNFα treatment increased both p100 and p52 protein levels, in contrast to RANKL treatment, which only increased p52. Taken together, these data indicated a potential role for IKKα during TNFα signaling in osteoclast precursor cells. Consistent with this, we observed that fewer TRAP+ multinucleated cells formed in TNFα-treated IKKα−/− cells compared with those observed in wild-type cells. Furthermore, treatment with TNFα and TGFβ led to the formation of large, multinucleated TRAP+ osteoclasts in wild-type but not IKKα−/− cells. Interestingly, we found that TNFα in combination with RANKL was able to induce the formation of large, multinucleated TRAP+ osteoclasts in IKKα−/− FLC, despite the accumulation of p100 and lack of p52 formation following this treatment. The overall number of multinucleated TRAP+ cells observed in IKKα−/− cells treated with RANKL and TNFα was similar to that observed in wild-type cells; however the majority of these cells were smaller in IKKα−/− cultures than in wild type (Fig. 7, B and C). The combination of RANKL, TNFα, and TGFβ also resulted in the formation of large, multinucleated TRAP+ osteoclasts in IKKα−/− FLC. This effect was completely inhibited by the RANKL inhibitor osteoprotegerin (data not shown), indicating that signaling via RANK was essential for osteoclast formation.

DISCUSSION

Here we have demonstrated a clear role for IKKα during osteoclastogenesis in vitro. IKKα−/− fetal liver cell cultures treated with CSF-1 and RANKL failed to form large, multinucleated TRAP+ osteoclasts, even after extended culture periods or with increased amounts of RANKL (not shown). However, unlike RANK or RANKL knock-out mice, which completely lack TRAP+ osteoclasts in vivo, IKKα−/− E18.5 dpc embryos did contain multinucleated TRAP+ osteoclasts, although the frequency of these cells was less than that observed in wild-type embryos. Because IKKα−/− mice die at birth, it is unclear whether the decreased number of multinucleated osteoclasts observed in these mice would result in a functional deficit in adult animals, such as a defect in tooth eruption or proper bone remodeling. Interestingly, mice lacking the upstream activator of IKKα, NIK, have no defects in basal osteoclastogenesis during development and thus have normal tooth eruption and skeletal morphology but fail to respond to osteoclastogenic stimuli, including RANKL and vitamin D (19). Additionally, bone marrow cells from NIK−/− mice are unable to form osteoclasts when cultured in vitro with CSF-1 and RANKL. Taken together, these studies indicate that the NIK/IKKα pathway is important during stimulated but not basal osteoclastogenesis. However, NIK−/− mice are not phenotypically identical to either IKKα−/− or IKKαAA mice because they have no defects in keratinocyte differentiation, as observed in IKKα−/− mice, or in mammary gland development, as observed in IKKαAA mice (in addition to RANK- and RANKL-null mice) (17, 18). Mice expressing a mutant form of NIK (aly/aly) do have defects in mammary gland development that are similar to, albeit less severe than, defects in IKKαAA mice (27); yet it is unknown whether either aly/aly mice or IKKαAA mice have defects in stimulated osteoclastogenesis. Given the differences between NIK−/−, aly/aly, IKKα−/−, and IKKαAA mice, it remains to be determined which functions of IKKα overlap with NIK during osteoclastogenesis (such as p100 processing) and which functions, if any, are independent of NIK.

RANKL and TNFα share many common downstream signaling activities. In particular, the ability of both cytokines to activate the transcription factors AP-1 and NF-kB, which are required for osteoclastogenesis (7, 28, 29), indicates the potential for redundant functions of RANKL and TNFα in osteoclast precursor cells. One distinction between TNFα- and RANKL-signaling pathways is the utilization of IKKα. We and others (19) have demonstrated that IKKα activates p100 processing, which is dependent on IKKα and its upstream activator NIK (30). In addition to p100 processing in osteoclast precursor cells, RANKL requires IKKε for activation of the p65/p50 NF-κB transcription factor in mammary epithelial cells via phosphorylation and degradation of the inhibitor IκBα (18). In contrast, TNFα has no such requirement for IKKα during activation of p65/p50 but instead relies solely upon IKKβ and IKKγ (31, 32). Furthermore, TNFα has not been reported previously to induce p100 processing but instead causes accumulation of p100 mRNA and protein via p65/p50-mediated up-regulation of the nfkβ1 gene (33). Here we have shown that treatment of wild-type but not IKKα−/− osteoclast precursor cells with TNFα does result in a small amount of p52 processing, although the overall net effect is to increase p100 levels compared with p52. RANKL treatment, on the other hand, results in complete processing of p100 into p52. In IKKα−/− cells, both RANKL and TNFα increased p100 levels without
formation of p52, effects that have also been observed in NIK−/− cells. However, although Novack et al. (19) reported that the result of p100 accumulation was to inhibit osteoclast differentiation in NIK−/− cells (19), we found that RANKL and TNFα in combination could stimulate osteoclast formation despite the presence of high levels of p100 protein. As previous studies have shown that TNFα can synergize with RANKL to activate NF-κB and c-Jun N-terminal kinase (13), it remains possible that these types of signals are sufficient to overcome inhibition of osteoclast differentiation by p100.

TGFβ has been reported to have both stimulatory and inhibitory effects on osteoclastogenesis (26). In contrast to a recent report indicating that TGFβ could directly stimulate osteoclast differentiation in the presence of CSF-1 (34), we did not observe any effect of TGFβ and CSF-1 on TRAP+ cell formation in wild-type fetal liver cells after 6 days in culture. However, TGFβ did augment TNFα- and RANKL-mediated osteoclast differentiation in wild-type fetal liver cells, which increased the size of the TRAP+ cells but not the overall number. Although TGFβ has been linked to IKKα and NF-κB p65/p50 activation via the upstream kinase TAK1 (35), we did not observe p100 processing in response to TGFβ treatment alone in either wild-type or IKKα−/− cells. Despite a lack of p100 processing, the ability of TNFα and TGFβ to stimulate osteoclast differentiation was IKKα-dependent. It remains to be determined whether these effects are mediated via TNFα or TGFβ signaling to IKKα via TAK1 or some other mediator.

In addition to its multiple roles in diverse tissues, including mammary gland, skin, and lymphocytes, we have now shown a role for IKKα during osteoclast differentiation. Ohazama et al. (36) have recently reported that IKKα−/− mice have defects in tooth development, specifically abnormal cusp formation, a defect that has been linked previously to the TNF receptor family member ectodysplasin A receptor (37). Although osteoclast activity is required for eruption of teeth, it is unlikely that these defects are linked to deficient osteoclast activity because cusp formation is regulated through epithelial cell signaling interactions. IKKα−/− mice have also been reported to have multiple skeletal abnormalities, including syndactyly and shortened limbs (21, 38, 39). Taken together, these findings indicate that IKKα plays multiple roles in the regulation of skeletal development and may contribute to pathological conditions associated with increased osteoclast activity.

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