

# Defective Osteoclastogenesis by IKK $\beta$ -null Precursors Is a Result of Receptor Activator of NF- $\kappa$ B Ligand (RANKL)-induced JNK-dependent Apoptosis and Impaired Differentiation\*

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It has been reported previously that inhibitory  $\kappa$ B kinase (IKK) supports osteoclastogenesis through NF- $\kappa$ B-mediated prevention of apoptosis. This finding suggests that the ligand for receptor activator of NF- $\kappa$ B (RANKL), the master osteoclastogenic cytokine, induces apoptosis of osteoclast precursors (OCPs) in the absence of IKK $\beta$ /NF- $\kappa$ B competency. To validate this hypothesis, we sought to determine the pro-apoptotic signaling factors induced by RANKL in IKK $\beta$ -null osteoclast OCPs and to rescue osteoclast differentiation in the absence of IKK $\beta$  through their inhibition. To accomplish this, we generated mice that lack IKK $\beta$  in multiple hematopoietic lineages, including OCPs. We found that these mice possess both *in vitro* and *in vivo* defects in osteoclast generation, in concurrence with previous reports, and that this defect is a result of susceptibility to RANKL-mediated apoptosis as a result of gain-of-function of JNK activation. We demonstrate that differentiation of OCPs depends on IKK $\beta$  because reduced IKK $\beta$  mRNA expression correlates with impaired induction of osteoclast differentiation markers in response to RANKL stimulation. We further show that fine-tuned inhibition of JNK activation in these cells inhibits RANKL-induced apoptosis and restores the ability of IKK $\beta$ -null OCPs to become mature osteoclasts. Our data highlight the pro-osteoclastogenic and anti-apoptotic roles of IKK $\beta$  in OCPs and identify a pro-apoptotic mechanism activated within the RANK signalingome.

Osteoclasts develop from bone marrow macrophage precursors under the control of two cytokines, receptor activator of NF- $\kappa$ B ligand (RANKL)<sup>2</sup> (1) and m-CSF (2). RANKL induces

osteoclast commitment and development by signaling downstream to several transcription factors, the most important of which is NF- $\kappa$ B (3). NF- $\kappa$ B is a family of transcription factors whose activity coordinates a major component of the cellular inflammatory program, and its function is essential for osteoclastogenesis (4, 5). NF- $\kappa$ B signaling involves two distinct but cooperating pathways, one canonical and one alternative pathway (6), which has recently been shown to be critical in osteoclast biology (7).

NF- $\kappa$ B is activated by the inhibitory  $\kappa$ B kinase (IKK) complex, which is crucial for osteoclastogenesis. The IKK complex is composed of two catalytically active members, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit IKK $\gamma$ /NEMO. IKK $\alpha$  mediates activation of the alternative pathway by phosphorylation of NF- $\kappa$ B2/p100 (6), whereas IKK $\beta$  is important for activation of the canonical pathway through phosphorylation of I $\kappa$ B (8). The importance of the signaling activity of the IKK complex in osteoclasts is demonstrated by the defect in osteoclastogenesis noted in mice lacking IKK $\alpha$  (9) or IKK $\beta$  (10). Despite the sequence homology of these two kinases, their relative importance in osteoclastogenesis is strikingly different. For example, osteoclasts devoid of active IKK $\alpha$  only demonstrate an *in vitro* defect in osteoclastogenesis, whereas the bone phenotype of the mouse is remarkably normal. On the other hand, mice with an inducible osteoclast precursor-specific deletion of IKK $\beta$  demonstrate both *in vitro* and *in vivo* defects in osteoclastogenesis and are resistant to inflammatory osteolysis (10). Given these findings, it is evident that investigating the mechanism by which IKK2 supports osteoclastogenesis will improve our understanding of osteoclast biology and diseases attributable to overactive osteoclasts.

We and others have shown that diverse methods of IKK blockade arrest osteoclastogenesis by induction of apoptosis (10–12). We were interested in the pro-apoptotic signals

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<sup>2</sup> The abbreviations used are: RANKL, receptor activator of NF- $\kappa$ B ligand; m-CSF, macrophage colony-stimulating factor; OCP, osteoclast precursor; WT, wild type; TRAP, tartrate-resistant acid phosphatase; NFAT, nuclear

factor and activator of T-cells; MMP, matrix metalloproteinase; cIAP, cellular inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis; MKP, MAPK phosphatase; PARP, poly(ADP-ribose) polymerase; TUNEL, TdT-mediated dUTP nick end labeling; JIP, JNK-interacting protein; OCP, osteoclast precursor; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; LPS, lipopolysaccharide; IKK, inhibitory  $\kappa$ B kinase.

downstream of RANKL in the absence of IKK $\beta$ , and we hypothesized that inhibition of these signals would be sufficient to rescue the osteoclast defect of cells lacking IKK $\beta$ . Our findings reveal that loss of IKK $\beta$  in osteoclast precursors (OCPs) results in a gain-of-function of JNK activation in response to RANKL that results in apoptosis. Furthermore, fine-tuned inhibition of this gain-of-function in JNK activation is sufficient to rescue osteoclastogenesis in OCPs lacking IKK $\beta$ . This finding demonstrates that the necessity of IKK $\beta$  for osteoclastogenesis may be evaded by inhibiting the pro-apoptotic effects of RANKL and designates JNK activation in the osteoclast as a potential means to induce cell death in OCPs.

## MATERIALS AND METHODS

**Reagents**—Antibodies against IKK $\beta$ , IKK $\alpha$ , NEMO, actin, JNK, p38, Akt, MKP1, phospho-c-Jun, and c-Jun as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-JNK, phospho-p38, phospho-Akt, and PARP were purchased from Cell Signaling Technologies, Inc. (Danvers, MA). Antibody against MKP5 was purchased from Abcam (Cambridge, MA). Cytokines were purchased from R & D Systems (Minneapolis, MN). TAT-TI-JIP was purchased from EMD Biosciences, Inc. (La Jolla, CA). Enhanced chemiluminescence kit was purchased from Pierce. All other chemicals were purchased from Sigma unless otherwise indicated.

**Animals**—CD11b Cre Y-chromosomal transgenic and floxed IKK $\beta$  mice on a C57BL/6 background were reported previously (12, 13). Male Cre+ Floxed IKK $\beta$  homozygotes were generated by crossing the above mice.

**Cell Culture**—Osteoclast precursors were enriched from bone marrow of 2–3-week-old mice. Briefly, whole marrow was flushed from long bones into  $\alpha$ -minimum Eagle's medium and was centrifuged at 453 relative centrifugal force. Marrow pellets were resuspended in whole media ( $\alpha$ -minimum Eagle's medium with penicillin/streptomycin, 10% heat-inactivated fetal bovine serum) supplemented with 10 ng/ml m-CSF. Cell suspensions were plated onto Petri dishes at 37 °C in 5% CO<sub>2</sub> for 5 days and then were plated according to experimental conditions.

**Osteoclast Formation Assay**—Osteoclast precursors were plated in triplicate at a density of  $3.0 \times 10^4$  cells in 200  $\mu$ l of whole media supplemented with 10 ng/ml m-CSF and RANKL in 96-well tissue culture plates. TAT-TI-JIP was added at the time of cell plating (day 1). TNF- $\alpha$  and LPS were added at day 4 of the assay. Mature osteoclasts form between day 5 and day 6 of culture, at which point the cells are fixed and stained for tartrate-resistant acid phosphatase (TRAP) to visualize osteoclasts (leukocyte acid phosphatase kit, Sigma). TRAP-positive multinucleated cells with three or more nuclei were scored as osteoclasts.

**Protein Phosphorylation Assay**—Osteoclast precursors were plated onto tissue culture dishes overnight in whole media supplemented with m-CSF. Cells were then serum-starved for 4–6 h and stimulated with the indicated cytokine for a planned time course. At the allotted time, cells were lysed in cell lysis buffer

containing (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and distilled deionized H<sub>2</sub>O). Protein concentration was measured by standard BCA assay (Pierce). 10–20  $\mu$ g of total cell protein was used for Western blot.

**Apoptosis Assay**—Osteoclast precursors were plated onto tissue culture dishes overnight in whole media supplemented with m-CSF. Cells were serum-starved for 6 h and stimulated with 10 ng/ml RANKL for the indicated time. At the allotted time, cells were lysed as described above; protein was normalized, and samples were analyzed by Western blot.

**Rescue of Apoptosis**—Osteoclast precursors were plated onto tissue culture dishes for 24 h in whole media supplemented with m-CSF. Four groups of two plates of cells were plated in this assay. Each group was treated with either sterile PBS, 0.4, 1.0, or 2.0  $\mu$ M TAT-TI-JIP. Also at the time of plating, one plate from each group was stimulated with either sterile PBS or 20 ng/ml RANKL. After 24 h, cells were lysed as described above; protein was normalized, and samples were analyzed by Western blot.

**Osteoclast Differentiation Assay**—Osteoclast precursors were plated in whole media supplemented with m-CSF. Cells were either not stimulated or were stimulated with 10 ng/ml RANKL for 5 days. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's standard protocol.

**Reverse Transcription**—1.0  $\mu$ g of total RNA was subjected to reverse transcription under the following conditions. 1.0  $\mu$ g of RNA and 1.0  $\mu$ g of random hexamer primer in 10  $\mu$ l of nuclease-free deionized H<sub>2</sub>O in PCR tubes were heated to 70 °C for 5 min, cooled to 42 °C, and set on ice. The following components were then added at the indicated amounts or concentrations for a total reaction volume of 20  $\mu$ l: 1 $\times$  RT avian myeloblastosis virus buffer (Roche Applied Science), 40 units of RNasin (Promega, San Luis Obispo, CA), 1.25 mM dNTPs, 5 mM sodium pyruvate, 5 units of reverse transcriptase enzyme (Roche Applied Science). To produce cDNA, tubes were placed in a thermocycler programmed as follows: 42 °C for 60 min, 50 °C for 10 min, 95 °C for 5 min, and 4 °C to hold.

**Bone Resorption Assay**—Bone marrow osteoclast precursors were plated onto BD Biocoat osteologic tissue culture slides (BD Biosciences) in the presence of 10 ng/ml m-CSF and RANKL with or without 0.4  $\mu$ M TAT-TI-JIP for the indicated times. Resorption pits were determined as clear areas in the osteologic matrix. Representative photographs were taken at  $\times 10$  Magnification.

**Real Time PCR**—Triplicate samples of 4  $\mu$ l of cDNA product (5 $\times$  diluted), 10  $\mu$ l of SybrGreen PCR Master Mix (Applied Biosystems, Inc. Foster City, CA), 0.1  $\mu$ l each of 10  $\mu$ M forward and reverse primer stocks, and 6  $\mu$ l of nuclease-free deionized H<sub>2</sub>O were subjected to real time PCR according to the following program in a 7300 AB real time PCR system: 50 °C for 2 min, 95 °C for 10 min (95 °C for 15 s, 60 °C for 1 min) for 40 cycles. Results were analyzed using AB RQ Study software. Real time PCR primers were designed using Primer Express software (Applied Biosystems, Inc.) as follows: mouse actin forward 5'-

CTTCTACAATGAGCTGCGTG-3' and mouse actin reverse 5'-TCATGAGGTAGTCTGTCAGG-3'; mouse TRAP forward 5'-CGACCATTGTTAGCCACATACG-3' and mouse TRAP reverse 5'-CACATAGCCCACACCGTTCTC-3'; mouse calcitonin receptor forward 5'-CAAGAACCTTAGCTGCCA-GAG-3' and mouse calcitonin receptor reverse 5'-CAAGCAGC-CGGACAATGTTG-3'; mouse MMP9 forward 5'-CAGGGA-GATGCCCATTTTCG-3' and mouse MMP9 reverse 5'-GGGC-ACCATTTGGAGTTTCCA-3'; mouse A20 forward 5'-CAG-AAAAAAGTGGTGAAGGTGTGA-3' and mouse A20 reverse 5'-CCAGGCTCTGACCTCTGTTACA-3'; mouse cIAP1 (birc2) forward 5'-GTGATGGTGGCTTGAGATGTTG-3' and mouse cIAP1 (birc2) reverse 5'-CAAGAACTCACACCT-TGGAAACC-3'; mouse cIAP2 (birc3) forward 5'-GAAGTG-GGCTGCGGTATCA-3' and mouse cIAP2 (birc3) reverse 5'-GCGCTGTCTTGAACCATGTTTC-3'; mouse Bcl-x<sub>L</sub> forward 5'-GCGGCTGGGACACTTTTG-3' and mouse Bcl-x<sub>L</sub> reverse 5'-CAGAACCACACCAGCCACAGT-3'; mouse XIAP forward 5'-CGGATCGTTACTTTTGGAAACATG-3' and mouse XIAP reverse 5'-CGCCTTCACCTAAAGCATAAAATC-3'; mouse cathepsin K forward 5'-GGAAGAAGACTCACCAG-AAGC-3' and mouse cathepsin K reverse 5'-GTCATATAGC-CGCCTCCACAG-3'; mouse  $\beta_3$  integrin forward 5'-TTACC-CCGTGGACATCTACTA-3' and mouse  $\beta_3$  integrin reverse 5'-AGTCTTCCATCCAGGGCAATA-3'; and mouse GAPDH forward 5'-CTTACCACCATGGAGAAGGC-3' and mouse GAPDH reverse 5'-GACGGACACATTGGGGGTAG-3'.

**Western Blot Assay**—Total cell lysates were boiled in the presence of an equal volume of 2 $\times$  SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromophenol blue, 3%  $\beta$ -mercaptoethanol, and distilled water) for 5 min and subjected to electrophoresis on 8–10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad) and incubated in blocking solution (10% skim milk prepared in phosphate-buffered saline containing 0.05% Tween 20) to reduce nonspecific binding. The membranes were washed with phosphate-buffered saline/Tween buffer and exposed to primary antibodies (16 h at 4 °C), washed again four times, and incubated with the respective secondary horseradish peroxidase-conjugated antibodies (1 h at room temperature). The membranes were washed extensively (four times for 15 min), and an ECL detection assay was performed following the manufacturer's directions.

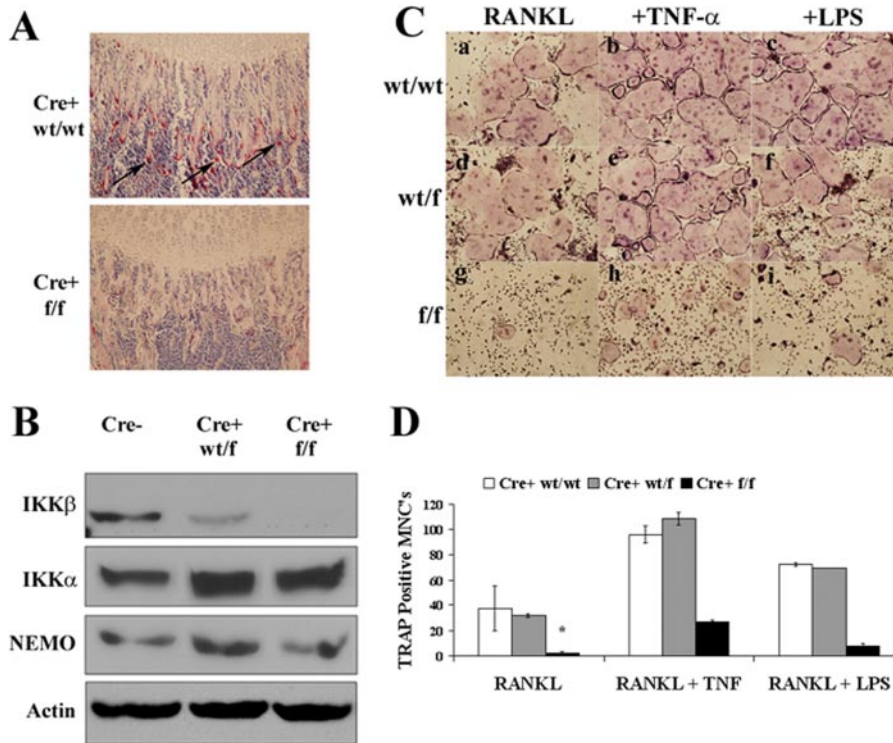
**Western Blot Quantification**—Where indicated, protein expression was quantified using Quantity One 1-D Analysis Software (Bio-Rad).

**Histology**—Long bones were collected from mice and fixed in 10% buffered formalin for 24 h. Bones were then decalcified for 7 days in decalcification buffer (14% (w/v) EDTA, H<sub>4</sub>NOH, pH 7.2), dehydrated in graded ethanol (30–70%), cleared through xylene, and embedded in paraffin. Paraffin sections were stained histochemically for TRAP to visualize osteoclasts or immunohistochemically for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) with the ApopTag peroxidase *in situ* apoptosis detection kit (Millipore/Chemicon International, Temecula, CA) to detect apoptotic cells.

## RESULTS

**Mice with an Osteoclast Precursor IKK $\beta$  Deficiency Demonstrate *In Vitro* and *In Vivo* Defects in Osteoclastogenesis**—IKK $\beta$  has been shown to be necessary for osteoclast formation (10). To define the mechanism through which IKK $\beta$  supports osteoclastogenesis, we generated mice with a deficiency of IKK $\beta$  in multiple hematopoietic lineages, including OCPs by crossing CD11b Cre recombinase transgenic mice (13) with mice possessing floxed IKK $\beta$  (14). In this study, we focus on the osteoclast phenotype, so we will refer to the resultant knock-out (Cre-positive floxed/floxed (*f/f*) IKK $\beta$ ) mice as OCP <sup>$\Delta$ IKK $\beta$</sup>  or Cre+ *f/f*. OCP <sup>$\Delta$ IKK $\beta$</sup>  mice possess a hampered ability to generate osteoclasts *in vivo* as evidenced by a significantly reduced number of TRAP-positive osteoclasts compared with controls observed by histochemical staining for TRAP in long bones (Fig. 1A). This is further supported by lack of IKK $\beta$  protein in osteoclast precursors of OCP <sup>$\Delta$ IKK $\beta$</sup>  mice (Fig. 1B). We demonstrate that this defect is cell-autonomous by culturing bone marrow-derived OCPs in the presence of m-CSF and RANKL. OCP <sup>$\Delta$ IKK $\beta$</sup>  cells form significantly fewer multinucleated osteoclasts (Fig. 1C, *panel g*) compared with Cre-positive IKK $\beta$  wild-type/wild-type (WT/WT) (Fig. 1C, *panel a*) and Cre-positive IKK $\beta$  wild-type/floxed (WT/*f*) heterozygous littermate controls (Fig. 1C, *panel d*). Furthermore, stimulation with TNF- $\alpha$  or LPS (Fig. 1C, *panels h* and *i*) is insufficient to rescue the osteoclast defect of OCP <sup>$\Delta$ IKK $\beta$</sup>  cells. Compared with WT cells, Cre-positive IKK $\beta$  (WT/*f*) OCPs also show decreased IKK $\beta$  protein expression (Fig. 1B), but this difference does not result in impaired osteoclastogenesis (Fig. 1C, *panels d–f*).

**OCP <sup>$\Delta$ IKK $\beta$</sup>  Are Prone to RANKL-induced Apoptosis and Display Defective Osteoclast Differentiation**—IKK $\beta$  has previously been demonstrated to protect OCPs from TNF- $\alpha$ -mediated apoptosis (10). We sought to determine whether IKK $\beta$ -deficient OCPs are similarly susceptible to apoptosis in response to RANKL and to test whether the absence of IKK $\beta$  results in defective osteoclast differentiation. To accomplish this, we cultured OCP <sup>$\Delta$ IKK $\beta$</sup>  and control OCPs in the presence of m-CSF and RANKL for 0 or 5 days to induce osteoclast differentiation. We measured by real time PCR the expression of several markers for osteoclast differentiation. We observe a significant decrease in the expression of mRNA for the osteoclast markers  $\beta_3$  integrin (Fig. 2A), cathepsin K (Fig. 2B), calcitonin receptor (Fig. 2C), matrix metalloproteinase 9 (Fig. 2D), and TRAP (Fig. 2E) in RANKL-stimulated OCP <sup>$\Delta$ IKK $\beta$</sup>  cells compared with controls. This failure to express osteoclast markers in osteoclastogenic conditions correlates with up to an 81% reduction in expression of IKK $\beta$  mRNA in OCP <sup>$\Delta$ IKK $\beta$</sup>  cells compared with controls (Fig. 2F). Interestingly, in OCP <sup>$\Delta$ IKK $\beta$</sup>  cells that express higher levels of IKK $\beta$  mRNA—63% reduction compared with control OCPs, osteoclast marker expression after RANKL stimulation is not affected, yet they still fail to form multinucleated osteoclasts in *in vitro* culture. This finding indicates that IKK $\beta$  also serves a differentiation-independent function to support osteoclastogenesis. These observations led us to surmise that IKK $\beta$  is essential at various stages for differentiation and survival of RANKL-stimulated OCPs.



**FIGURE 1. Mice with IKK $\beta$ -deleted osteoclast precursors possess a defect in *in vivo* and *in vitro* osteoclastogenesis.** *A*, histochemical TRAP stain at growth plate of femur of CD11b Cre-positive WT/WT IKK $\beta$  and CD11b Cre-positive floxed/floxed IKK $\beta$  (OCP $\Delta$ IKK $\beta$ ) mice to visualize osteoclasts. *Arrows* indicate osteoclasts. *B*, Western blot for indicated proteins in total cell lysates of osteoclast precursors from CD11b Cre-negative, CD11b Cre-positive WT/floxed IKK $\beta$ , and CD11b Cre-positive floxed/floxed (*f/f*) IKK $\beta$  mice. *C* and *D*, osteoclast precursors from CD11b Cre-positive wt/wtIKK $\beta$ , CD11b Cre-positive WT/floxed IKK $\beta$ , and CD11b Cre-positive floxed/floxed IKK $\beta$  mice were cultured in osteoclastogenic conditions. *C*, cells were either not stimulated (*panels a, d, and g*) or were further stimulated with 10 ng/ml TNF- $\alpha$  (*panels b, e, and h*) or 100 ng/ml LPS (*panels c, f, and i*) on day 4 of culture. Cells were fixed and histochemically stained for TRAP to visualize osteoclasts on day 6 of culture. *D*, quantification of *C*. TRAP-positive multinucleated cells (MNCs) with three or more nuclei were scored as osteoclasts. *Asterisk* indicates  $p < 0.005$  for difference between number of TRAP-positive MNCs in wells represented by *panels d* and *g*.

Therefore, we tested whether OCP $\Delta$ IKK $\beta$  cells were more susceptible to apoptosis than control cells. First, we cultured OCP $\Delta$ IKK $\beta$  and control OCPs in the presence of whole media supplemented with fetal bovine serum and m-CSF to promote survival and measured by real time PCR the expression of mRNA for several NF- $\kappa$ B-controlled anti-apoptotic proteins (15). We note significant reduction in expression of mRNA for A20, cellular inhibitor of apoptosis 2 (c-IAP2), c-IAP1, Bcl-x $_L$ , and X-linked inhibitor of apoptosis (XIAP) in OCP $\Delta$ IKK $\beta$  compared with control cells (Fig. 2G).

To determine whether OCP $\Delta$ IKK $\beta$  undergo apoptosis in response to RANKL, we exposed serum-starved OCP $\Delta$ IKK $\beta$  and control OCPs to RANKL for a time course of 4 h. We detected the kinetics of PARP cleavage by Western blot as a molecular signature of apoptosis. We note the disappearance of full-length PARP in OCP $\Delta$ IKK $\beta$  cells after 1 h, whereas in control cells, the integrity of full-length PARP is preserved over the time course of RANKL exposure (Fig. 2H), which indicates that RANKL has a pro-apoptotic effect on osteoclast precursors deficient in IKK $\beta$ . We conclude that IKK $\beta$  is necessary for osteoclast differentiation and the survival of osteoclast precursors exposed to RANKL.

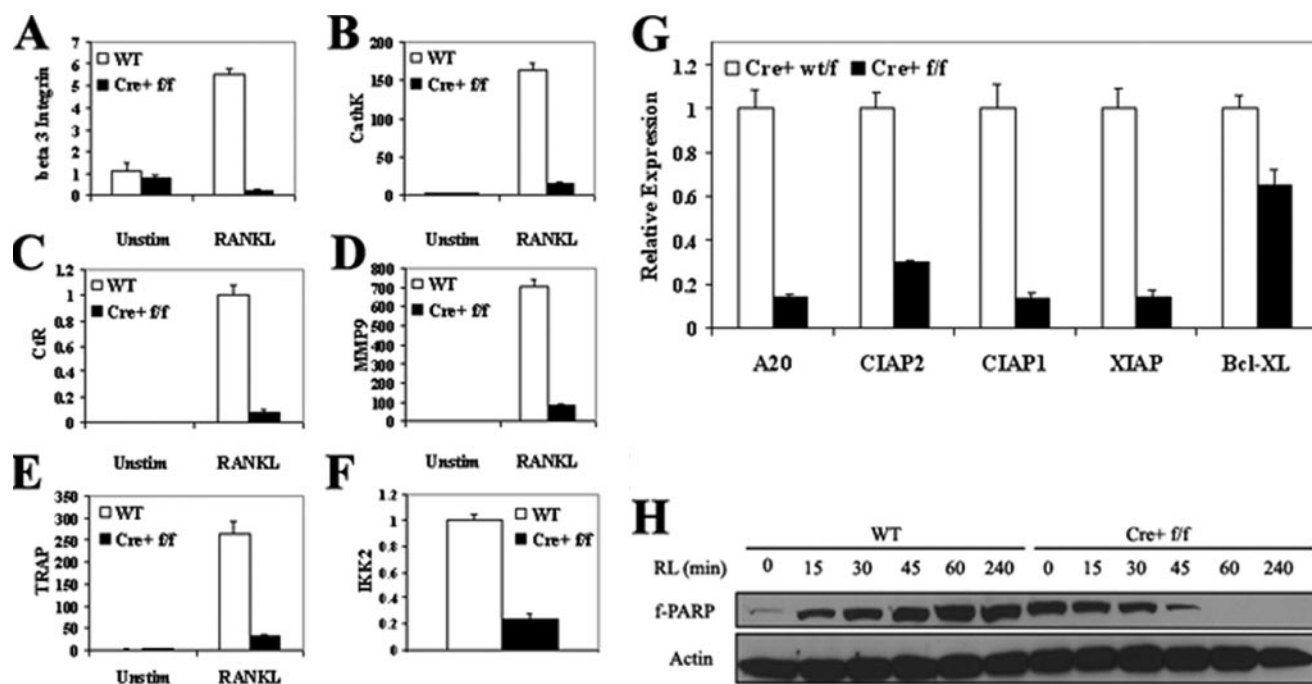
To determine whether apoptosis of osteoclasts or OCPs from OCP $\Delta$ IKK $\beta$  mice contributes to the paucity of osteoclasts observed *in vivo*, we stained sections of long bones of OCP $\Delta$ IKK $\beta$

and control mice immunohistochemically with the TUNEL method to detect apoptosis. We note a significantly greater number of TUNEL-positive peritrabecular nuclei resembling apoptotic osteoclasts and OCPs in OCP $\Delta$ IKK $\beta$  compared with control long bones (Fig. 3, *A* and *B*). Based on our data, we conclude that apoptosis contributes to the osteoclast defect in OCP $\Delta$ IKK $\beta$  mice.

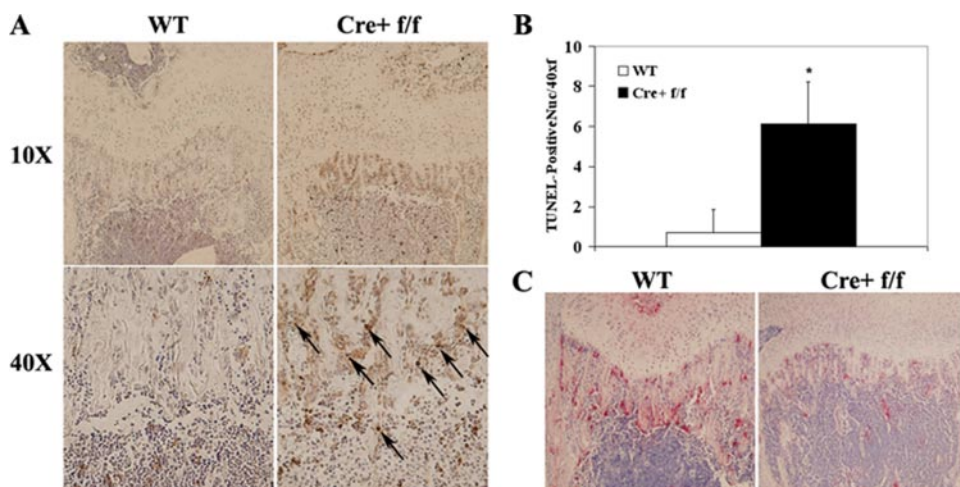
**OCP $\Delta$ IKK $\beta$  Possesses a Gain-of-Function in JNK Activation**—We were interested in potential pro-apoptotic signals induced by RANKL in differentiating IKK $\beta$ -deficient OCPs. To address this, we performed a phosphoprotein screen by Western blot analysis in OCP $\Delta$ IKK $\beta$  and control OCPs after stimulation with a time course of RANKL or TNF- $\alpha$ . We postulated that OCP $\Delta$ IKK $\beta$  kinase signaling would possess a signature that would favor apoptosis. We noted several aberrations in the pattern of protein phosphorylation in OCP $\Delta$ IKK $\beta$  compared with control cells. Of particular interest was an increase and prolongation of JNK phosphorylation in response to RANKL (Fig. 4, *A* and *B*) and TNF- $\alpha$  (Fig. 4A) in OCP $\Delta$ IKK $\beta$  compared with control

OCPs. Interestingly, p38 (Fig. 4A) and Akt (Fig. 4B) phosphorylation remain unaffected in the absence of IKK $\beta$ , suggesting that JNK down-regulation is a specific function of IKK $\beta$ . We observe that MAP kinase phosphatase 1 (MKP1) protein resynthesis after degradation and MKP5 protein synthesis are dampened after RANKL stimulation of OCP $\Delta$ IKK $\beta$  cells compared with controls. In particular, MKP1 and MKP5 protein levels maximize after 30 min of RANKL stimulation of control OCPs (Fig. 4C). This time point correlates with down-regulation of JNK phosphorylation after RANKL stimulation (Fig. 4, *A* and *B*). In OCP $\Delta$ IKK $\beta$  cells, JNK phosphorylation is sustained at 30 min of RANKL stimulation (Fig. 4, *A* and *B*), which correlates with the absence of detectable MKP1 and MKP5 protein at this time point in OCP $\Delta$ IKK $\beta$  cells (Fig. 4C). Because MKP1 (16) and MKP5 (17) serve as JNK phosphatases, MKP induction may serve as an IKK $\beta$ -dependent mechanism for JNK down-regulation after RANKL stimulation. We postulated that the gain-of-function of JNK activation may result in apoptosis of OCP $\Delta$ IKK $\beta$  cells.

**Inhibition of JNK Blocks RANKL-induced Apoptosis of Osteoclast Precursors and Rescues Osteoclastogenesis in IKK $\beta$ -deficient Osteoclast Precursors**—JNK activation has been linked to RANKL-induced apoptosis of differentiating osteoclasts (18). We hypothesized that because OCP $\Delta$ IKK $\beta$  cells are susceptible



**FIGURE 2. OCP $\Delta$ IKK $\beta$  are defective in osteoclast differentiation and demonstrate increased susceptibility to apoptosis.** OCP $\Delta$ IKK $\beta$  and control OCPs were plated in whole media supplemented with 10 ng/ml m-CSF. Cells were either not stimulated (*Unstim*) or were stimulated with 10 ng/ml RANKL for 5 days to induce osteoclast differentiation. mRNA was collected and analyzed by real time PCR for markers of osteoclast differentiation: *A*,  $\beta_3$  integrin; *B*, cathepsin K (*Cath K*); *C*, calcitonin receptor (*Ctr*); *D*, matrix metalloproteinase 9 (*MMP9*); *E*, tartrate-resistant acid phosphatase (*TRAP*); *F*, IKK $\beta$  (*IKK2*). GAPDH served as the internal standard for cDNA normalization. Data are presented as relative quantification with WT nonstimulated levels serving as the reference point (relative expression value of 1). Values represent mean quantification plus the S.E. *G*, OCP $\Delta$ IKK $\beta$  and control OCPs were plated in whole media supplemented with 10 ng/ml m-CSF. mRNA was collected and analyzed by real time PCR for the indicated NF- $\kappa$ B-regulated anti-apoptotic proteins as follows: *A20*, *ciAP2* (cellular inhibitor of apoptosis 2); *ciAP1* (cellular inhibitor of apoptosis 1); *XIAP* (X-linked inhibitor of apoptosis); and *Bcl-x<sub>L</sub>*.  $\beta$ -Actin served as the internal standard for cDNA normalization. Data are presented as relative quantification with control levels serving as the reference point (relative expression value of 1). Values represent mean quantification plus the S.E. Data are representative of three independent experiments. *H*, OCP $\Delta$ IKK $\beta$  and control OCPs were serum-starved and were either not stimulated or were stimulated with 10 ng/ml RANKL for 15, 30, 45, 60, or 240 min. Total cell lysates were analyzed by Western blot for integrity of full-length PARP (*f-PARP*).  $\beta$ -Actin served as the loading control.

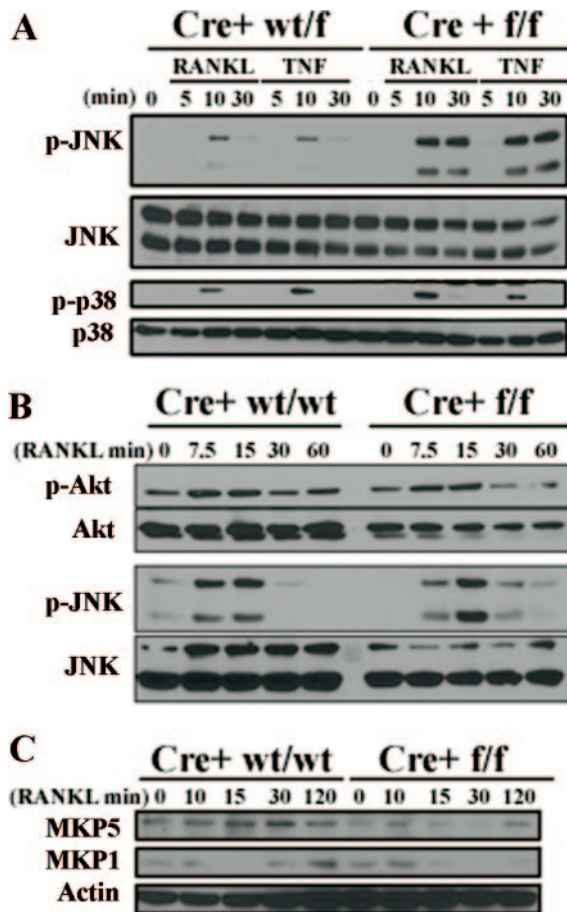


**FIGURE 3. Apoptosis contributes to the *in vivo* deficiency of osteoclasts in OCP $\Delta$ IKK $\beta$  mice.** *A*, immunoperoxidase TUNEL stain and hematoxylin counterstain of histological sections of growth plate of humerus from OCP $\Delta$ IKK $\beta$  and control mice to visualize apoptosis of peritrabecular osteoclasts and OCPs. *Upper images* were taken at  $\times 10$  magnification, and *lower images* are panels from upper images taken at  $\times 40$  magnification. *B*, graph depicting quantification of TUNEL-positive peritrabecular nuclei per  $\times 40$  field visualized by light microscopy. *Arrows* indicate apoptotic nuclei. *Asterisk* denotes  $p < 0.001$ . *C*, TRAP stain of sections taken from same paraffin-embedded bones used for TUNEL stain in *A* to demonstrate correlation between apoptosis and defective *in vivo* osteoclastogenesis.

to RANKL-induced apoptosis, inhibition of RANKL-mediated JNK activation in these cells would rescue osteoclastogenesis. We took advantage of a cell-permeable peptide (TAT-TI-JIP)

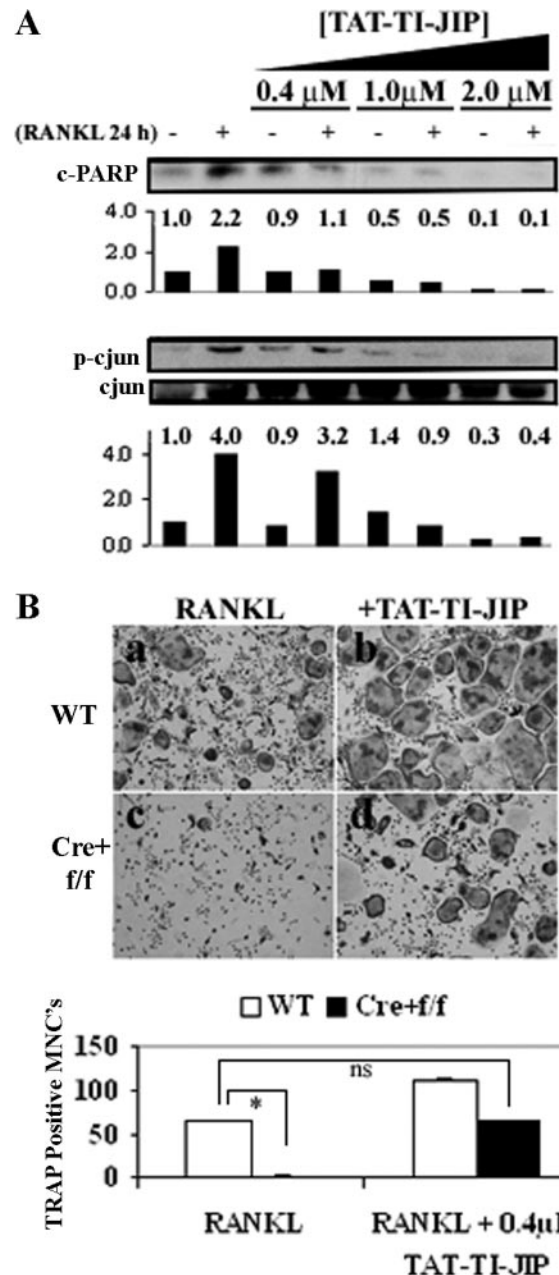
to specifically inhibit JNK activation (19) after RANKL stimulation. In WT OCPs, TAT-TI-JIP peptides inhibit osteoclastogenesis at concentrations above 1  $\mu$ M (not shown). This finding is expected given the established importance of c-Jun in osteoclast differentiation (20, 21). Surprisingly, TAT-TI-JIP peptides enhance osteoclastogenesis in WT cells at a concentration of 0.4  $\mu$ M (Fig. 5*B*, panel *b*). We hypothesized that this concentration of TAT-TI-JIP blocks RANKL-induced PARP cleavage without dramatically altering RANKL-induced phosphorylation of c-Jun (Fig. 5*A*). At a concentration of 1  $\mu$ M, TAT-TI-JIP inhibits RANKL-induced PARP cleavage in

OCPs, but it also inhibits c-Jun phosphorylation (Fig. 5*A*), which explains the inhibitory effect of this concentration on osteoclastogenesis. These results suggest that JNK serves two



**FIGURE 4. Loss of IKK $\beta$  in OCPs results in a gain-of-function in JNK phosphorylation.** OCP $\Delta$ IKK $\beta$  and control OCPs were serum-starved for 4–6 h. *A*, cells were either not stimulated or were stimulated with 10 ng/ml RANKL or TNF- $\alpha$  for 5, 10, or 30 min. *B*, cells were either not stimulated or were stimulated with 10 ng/ml RANKL for 7.5, 15, 30, or 60 min. Total cell lysates were then analyzed by Western blot for the indicated phosphorylated proteins and whole proteins. Equal loading for phosphorylated proteins was determined by stripping the membrane and re-probing for the respective whole protein (*A* and *B*). *C*, cells were stimulated with 10 ng/ml RANKL for 10, 15, 30, or 120 min. Total cell lysates were then analyzed by Western blot for MKP5 and MKP1.  $\beta$ -Actin served as the loading control.

distinct functions in osteoclast differentiation and survival. Because low dose TAT-TI-JIP inhibits apoptosis of OCPs induced by RANKL stimulation without affecting c-Jun activation, we tested this concentration for its potential to rescue osteoclastogenesis of OCPs in the absence of IKK $\beta$ . Indeed, in the *in vitro* osteoclastogenesis assay, 0.4  $\mu$ M TAT-TI-JIP peptide rescues osteoclastogenesis of OCP $\Delta$ IKK $\beta$  cells (Fig. 5*B*). For example, OCP $\Delta$ IKK $\beta$  cells (Fig. 5*B*, panel *c*) treated with RANKL produce less than 5% of the number of TRAP-positive osteoclasts produced by control OCPs (Fig. 5*B*, panel *a*). However, TAT-TI-JIP treatment of OCP $\Delta$ IKK $\beta$  cells (Fig. 5*B*, panel *d*) results in slightly, but statistically not significant, higher number of TRAP-positive osteoclasts compared with non-TAT-TI-JIP-treated controls (Fig. 5*B*, panel *a*). Importantly, JIP peptide-treated control OCPs (Fig. 5*B*, panel *b*) produced more osteoclasts in *in vitro* culture than JIP peptide-treated IKK $\beta$ -deficient OCPs (Fig. 5*B*, panel *d*), indicating that OCP $\Delta$ IKK $\beta$  possess defects in osteoclast differentiation that are independent of JNK-mediated apoptosis.



**FIGURE 5. Inhibition of RANKL-mediated JNK-induced apoptosis rescues osteoclastogenesis defect in OCPs deficient in IKK $\beta$ .** *A*, OCPs from Cre+ wild-type/floxed IKK $\beta$  mice were plated in whole media supplemented with 10 ng/ml m-CSF. Four groups of cells were treated at the time of plating with either no TAT-TI-JIP or with 0.4, 1.0, or 2.0  $\mu$ M TAT-TI-JIP. Also at the time of plating, one sample in each group was stimulated with 20 ng/ml RANKL. Cells were lysed after 24 h of stimulation, and total cell lysates were analyzed by Western blot for cleaved PARP (*c-PARP*), phosphorylated c-Jun (*p-c jun*), and total c-Jun. Cleaved PARP and phospho-c-Jun quantification in the different conditions is shown in numerical and graph form under the corresponding blot image. *B*, OCP $\Delta$ IKK $\beta$  and control OCPs were plated in osteoclastogenic conditions. At the time of plating, one group of cells from each population was either left untreated (panels *a* and *c*) or treated with 0.4  $\mu$ M TAT-TI-JIP (panels *b* and *d*). Cells were fixed and histochemically stained for TRAP to visualize osteoclasts on day 6 of culture. Quantification is shown in graph below. TRAP-positive multinucleated cells (MNCs) with three or more nuclei were scored as osteoclasts. Data are representative of three independent experiments, and error bars represent S.E. Asterisk indicates  $p < 0.0001$  for difference between number of TRAP-positive MNCs in wells represented by panels *a* and *c*. No significant difference exists between panels *a* and *d*.

## JNK-dependent Apoptosis in OCPs Lacking IKK $\beta$

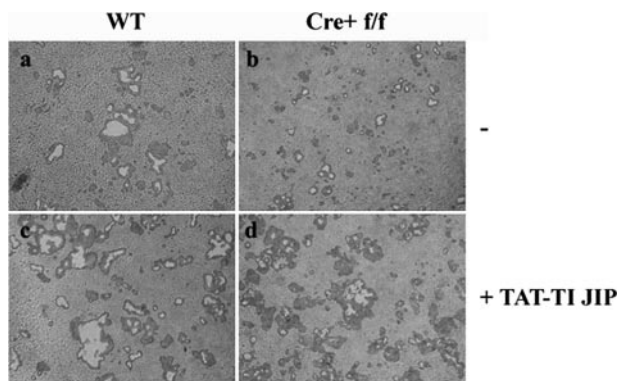


FIGURE 6. Inhibition of JNK in OCP $\Delta$ IKK $\beta$  cells rescues bone resorption. Control (a and c) and OCP $\Delta$ IKK $\beta$  (b and d) osteoclast precursors were plated onto BD Biocoat Osteologic tissue culture slides in osteoclastogenic conditions in the absence (a and b) or presence (c and d) of 0.4  $\mu$ M TAT-TI-JIP. Cells were removed from the slides with deionized water, and resorption pits were noted as clear areas. Images were taken at  $\times 10$  magnification.

Finally, we sought to determine whether rescue of osteoclastogenesis in OCP $\Delta$ IKK $\beta$  through JNK inhibition results in a concomitant rescue of bone resorption. To accomplish this, we plated WT and OCP $\Delta$ IKK $\beta$  osteoclast precursors on an artificial bone substrate in osteoclastogenic conditions in the presence and absence of 0.4  $\mu$ M TAT-TI-JIP. Resorption pits created by RANKL-treated OCP $\Delta$ IKK $\beta$  cells (Fig. 6b) were significantly smaller ( $22 \pm 3\%$  resorption area compared with controls) than those created by control osteoclasts (Fig. 6a), indicating that OCP $\Delta$ IKK $\beta$  are defective in resorbing bone. However, when we treated OCP $\Delta$ IKK $\beta$  cells with TAT-TI-JIP (Fig. 6d), we restored resorption pit size over that noted in WT non-TAT-TI-JIP treated cells ( $165 \pm 7\%$  of control) (Fig. 6a). Consistent with our *in vitro* osteoclastogenesis assay data shown in Fig. 5, resorption pit size of TAT-TI-JIP-treated WT osteoclasts (Fig. 6c) is 2-fold larger than that of TAT-TI-JIP-treated OCP $\Delta$ IKK $\beta$  ( $210 \pm 14\%$ ) indicating that IKK $\beta$  also acts through mechanisms independent of JNK inhibition to support osteoclastogenesis.

## DISCUSSION

In previous studies, we and others have shown that IKK-NF- $\kappa$ B function is necessary for osteoclastogenesis (4, 10, 12). The diverse activities of the individual IKK and NF- $\kappa$ B members suggest that each molecule plays a unique role in the overall program of osteoclast development. It has been demonstrated that IKK $\beta$  protects OCPs from apoptosis in response to TNF- $\alpha$  (10). Although this finding is consistent with previous reports regarding the function of IKK $\beta$  in other settings (22), its precise role downstream of RANK during osteoclastogenesis has remained unclear. The most likely possibility is that IKK $\beta$  serves some capacity in osteoclastogenesis that is dependent upon NF- $\kappa$ B-p65 action. However, the exact function of p65 in the osteoclast remains to be elucidated. It has been suggested that IKK $\beta$  may be important for the survival and differentiation of OCPs (10). We show that OCPs deficient in IKK $\beta$  display a defect in osteoclast differentiation, which is consistent with impaired induction of mRNA for the following osteoclast markers:  $\beta_3$  integrin, cathepsin K, calcitonin receptor, MMP9, and TRAP after RANKL stimulation.

We also show that IKK $\beta$ -deficient OCPs are susceptible to apoptosis in response to RANKL stimulation. Based on our real time PCR data, this phenomenon is likely to partially result from impaired NF- $\kappa$ B-mediated transcription of anti-apoptotic genes. However, the pro-apoptotic function of RANKL in osteoclast precursors has not been fully described. Importantly, in OCP $\Delta$ IKK $\beta$  cells, which expressed higher levels of IKK $\beta$  mRNA, induction of osteoclast differentiation markers was not affected, although impaired *in vitro* osteoclastogenesis was still observed. We examined the expression of mRNA for dendritic cell-specific transmembrane protein (DC-STAMP) (23) and the d2 isoform of the vacuolar ATPase V<sub>0</sub> domain (ATP6v0d2) (24), two gene products known to be important in osteoclast fusion, in these cells to determine whether a fusion deficiency was responsible for the defect. Induction of these markers was equivalent to controls in this population of OCP $\Delta$ IKK $\beta$  cells (data not shown). Although induction of other unknown osteoclast fusogenic genes may be impaired in RANKL-treated OCP $\Delta$ IKK $\beta$  cells, our data suggest that RANKL-induced apoptosis is a major contributor to the osteoclast defect in these cells.

We hypothesized that the kinase signaling environment downstream of RANK in OCP $\Delta$ IKK $\beta$  cells would reveal pro-apoptotic signaling changes. Among several observed signaling aberrations, we noted in particular that JNK displayed a more robust and prolonged phosphorylation profile after RANKL stimulation in OCPs that lack IKK $\beta$ . Because JNK activation has been correlated with RANKL-induced apoptosis previously (18), we postulated that OCP $\Delta$ IKK $\beta$  cells undergo apoptosis as a result of RANKL-mediated JNK activation. Indeed, by inhibiting JNK-dependent apoptosis, we rescued osteoclastogenesis in OCP $\Delta$ IKK $\beta$  cells.

We believe that OCP $\Delta$ IKK $\beta$  cells undergo RANKL-induced JNK-dependent apoptosis early after RANKL stimulation, at a stage prior to the mature osteoclast, for two reasons. First, the number of multinucleated osteoclasts of RANKL-stimulated OCP $\Delta$ IKK $\beta$  cells never approaches that of controls cells.<sup>3</sup> Second, TAT-TI-JIP only rescues the osteoclast defect of OCP $\Delta$ IKK $\beta$  if added to the culture at the same time as RANKL stimulation. When treated with TAT-TI-JIP 48 h after RANKL stimulation, JNK inhibition does not fully rescue osteoclastogenesis of OCP $\Delta$ IKK $\beta$  cells.

Whether the absence of IKK $\beta$  protein is essential for the gain-of-function of JNK in response to RANKL is unknown. For example, it is probable that a downstream effector of IKK $\beta$  signaling and not IKK $\beta$  itself is responsible for inhibition of JNK after RANKL stimulation. In any case, we demonstrate that fine-tuned inhibition of JNK in OCP $\Delta$ IKK $\beta$  cells rescues osteoclastogenesis. Our data suggest a model whereby RANKL stimulation of OCPs leads to activation and eventual down-regulation of JNK through MAP kinase phosphatase synthesis. In the absence of IKK $\beta$ , RANKL-mediated MKP1 and MKP5 synthesis are defective leading to enhancement and prolongation of JNK phosphorylation and activation resulting in apoptosis. One may postulate that blockade of JNK down-regulation through inhibition of the JNK phosphatases will result in

<sup>3</sup> Y. Abu-Amer and J. Otero, unpublished observations.

enhanced and prolonged RANKL-induced JNK activity that is anti-osteoclastogenic.

The mechanism of JNK-mediated apoptosis in response to RANKL is not well defined. It has been demonstrated previously that in response to TNF- $\alpha$  in NF- $\kappa$ B-deficient cells, JNK activation leads to caspase-8-independent cleavage of the pro-apoptotic protein, BID, resulting in mitochondrial release of Smac and apoptosis (25). Because RANKL stimulation of OCPs does not result in caspase-8 activation, it is likely that enhanced JNK activity in the absence of IKK $\beta$  directly leads to apoptosis of OCPs after RANKL stimulation. Because lower expression of IKK $\beta$  in OCP <sup>$\Delta$ IKK $\beta$</sup>  cells prevents osteoclast differentiation, it is unlikely that inhibition of JNK-mediated apoptosis will rescue osteoclastogenesis in the complete absence of IKK $\beta$ . Observing the rescuing effect of a low dose JNK inhibitor on osteoclastogenesis in OCP <sup>$\Delta$ IKK $\beta$</sup>  therefore requires a permissive level of IKK $\beta$  expression that allows differentiation to occur but does not inhibit apoptosis. We believe our CD11b Cre-mediated deletion of IKK $\beta$  was a successful tool in this regard.

In addition to MAP kinase phosphatases, several potential connections between the absence of IKK $\beta$  and enhanced JNK activation exist. For example, known target genes of NF- $\kappa$ B serve to down-regulate JNK activation such as Gadd45 $\beta$ , which specifically inhibits TNF- $\alpha$ -mediated MKK7 activation of JNK (26). Additionally, XIAP is a target of NF- $\kappa$ B that down-regulates JNK activation in response to TNF- $\alpha$  (27). Furthermore, A20 has been postulated to play a role in NF- $\kappa$ B-mediated inhibition of JNK activation by down-regulating TRAF2 (28), although this hypothesis has never been validated. Because OCP <sup>$\Delta$ IKK $\beta$</sup>  display reduced XIAP and A20 expression, it will be interesting to test whether these mechanisms of cross-talk between IKK $\beta$  and JNK hold true in RANKL signaling during osteoclastogenesis.

It is important to note that JNK-mediated c-Jun activation is required for efficient osteoclastogenesis (20). c-Jun activation leads to a partnership between AP-1 and NFAT1, which induces expression of NFAT2 and differentiation of osteoclasts (21). Therefore, inhibition of the RANKL-RANK-JNK pathway is a candidate for treatment of osteoporosis (29). In light of the opposing effects of the two arms of JNK activation in the osteoclast, it will be critical to sort out the pro- and anti-osteoclastogenic means of RANKL-mediated JNK activation.

Our results highlight the necessity of IKK $\beta$  in osteoclastogenesis. We demonstrate that IKK $\beta$  is important for both differentiation and survival of osteoclasts. Given that we are able to rescue osteoclastogenesis in OCP <sup>$\Delta$ IKK $\beta$</sup>  cells through inhibition of JNK-induced apoptosis, we conclude that IKK $\beta$  acts, at least partially, through down-modulation of JNK activity to support cell survival during osteoclastogenesis. Our results therefore suggest that hyperactivation of JNK and inhibition of IKK $\beta$  in OCPs are potential means to treat osteoclast-mediated disease.

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