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Division of Biology and Biomedical Sciences

Molecular Cell Biology

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THE ROLE OF IKKβ IN OSTEOCLASTOGENESIS AND INFLAMMATORY OSTEOLYSIS

by

Jesse E. Otero

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

The Role of IKKβ in Osteoclastogenesis and Inflammatory Osteolysis

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Jesse E. Otero

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2011

Professor Yousef Abu-Amer, Chairperson

Bone homeostasis is essential for health and is altered in many pathological conditions. A balance in the activity of osteoblasts (bone-building cells) and osteoclasts (bone-resorbing cells) determines the state of bone metabolism, and a tip in this balance toward either cell type is detrimental to health. In clinical settings, the most common bone diseases favor increased osteoclast activity and include osteoporosis and rheumatoid arthritis. Heightened osteoclast differentiation and activation in these conditions causes bone loss which results in increased fracture risk, bone pain, and deformity. Understanding the mechanisms by which osteoclasts develop will elucidate important targets for therapy in these conditions. Osteoclasts differentiate from monocyte precursors when stimulated by the Ligand for Receptor Activator of NF-κB (RANKL). Recent research has identified many transcription factors that are activated by RANKL

and are important for osteoclast differentiation. One such family of transcription factors is NF-κB. We hypothesized that activators of NF-κB are necessary for RANKL-induced osteoclast differentiation. The Inhibitory kappaB Kinase (IKK) Complex, which consists of two catalytically active subunits, IKKα and IKKβ, and one regulatory subunit, NEMO, is the main stimulator of NF-κB downstream of RANK in osteoclast progenitors. Our lab and others show that this activation is critical for stimulation of NF-κB and osteoclastogenesis. We sought to further characterize the role of the IKK complex in osteoclastogenesis by conditionally deleting IKKβ from osteoclast precursors. Using this mouse model, we demonstrated that IKKβ is critical for differentiation of osteoclasts *in vivo* and *in vitro*, and that IKKβ supports osteoclastogenesis at the levels of differentiation and survival. Our model provided a useful tool to study the structural components of IKKβ which are important for its function in osteoclast differentiation. Retroviral rescue experiments in which IKKβ KO monocytes were reconstituted with mutant forms of IKKβ revealed that activation loop serines are critical for IKKβ to support osteoclast differentiation. These experiments led to the discovery that constitutive activation of IKKβ results in spontaneous, RANK-independent osteoclast differentiation *in vitro* and osteolysis *in vivo*. Our work demonstrates that IKKβ is central to osteoclast differentiation and is therefore an important target in therapy for osteoclast-mediated disease.

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CHAPTER 1

INTRODUCTION

RATIONALE AND OBJECTIVE OF THESIS

Arthritis is a medical condition defined as inflammation of the joints and surrounding connective tissue. Currently, it is estimated that over 46 million Americans suffer from arthritis, and in 2003, total direct and indirect costs attributable to the condition were approximately $128 billion [1]. Arthritis and its medical complications represent the leading cause of disability in the United States, and it has been projected that by the year 2020, more than 60 million Americans will suffer from the condition [2]. For this reason, it has been a priority of basic science research to understand the mechanisms underlying the onset and progression of arthritis and its complications in order to direct and guide implementation of preventative and therapeutic strategies.

The most devastating complication of arthritis is the destruction of bone associated with inflammation of joints (osteolysis) which results in pain, deformity, and immobility. Osteolysis in arthritis is triggered by inflammatory mediators and accomplished by osteoclasts, cells that resorb bone [3]. The discovery that osteoclasts are required for bone destruction associated with inflammatory conditions [4,5] has led to an intense effort to characterize the cell and the mechanisms involved in its differentiation and activity. Indeed, drugs targeting the osteoclast have been demonstrated to improve arthritis-associated osteolysis and are now being considered in the therapy for osteolytic conditions [6].

The objective of this thesis is to investigate the molecular mechanisms utilized by the osteoclast focusing on the NF-κB pathway (see Background) as a means to better understand the process of osteoclast differentiation and to offer insight that will guide therapy of pathological conditions in which the osteoclast is the culprit.

BACKGROUND

Arthritis and the Osteoclast

Arthritis is the leading cause of disability in the United States. Bone-loss associated with certain types of arthritis is a well-recognized relationship [7,8] and contributes greatly to the joint pain, deformation, and immobility which often accompanies arthritis. The discovery of bone-resorbing osteoclasts in arthritic joint tissue led to the hypothesis that osteoclasts are the culprits in arthritic osteolysis [9]. Since this discovery, genetic studies in mice have confirmed that inflammatory cytokines present in inflamed joint tissue contribute to the hyperactivation of osteoclasts in this setting [5,10,11], and therapy targeting the osteoclast in patients with rheumatoid arthritis reduces the osteolysis associated with their condition [6]. It is therefore critical to gain a deeper understanding of the osteoclast and the molecular mechanisms it utilizes to differentiate and become active in order to more effectively treat arthritis-associated osteolysis.

Osteoclast Biology

The osteoclast is a multinucleated bone-resorbing cell which differentiates from monocyte precursors under the control of stimulation by two cytokines, Macrophage Colony Stimulating Factor (M-CSF) [12] and Ligand for the Receptor Activator of NF-κB (RANKL) [13]. Mice devoid of either cytokine suffer from severe osteopetrosis owing to failure of osteoclast differentiation, which has become a well-established phenotype in animals that possess genetic defects in osteoclast differentiation and activity [14]. The identification of genes whose dysfunction results in osteopetrosis has contributed tremendously to the understanding of the osteoclast.

The mature osteoclast forms through stimulation of monocytes with M-CSF and RANKL which triggers a differentiation program leading to fusion of precursors, attachment to bone matrix, release of protons and proteases, and degradation of bone material [14]. Inhibition of each of these parameters involved in formation and function of osteoclasts results in impairment of osteoclast activity. For example, mice devoid of the d2 subunit of the v_0 v-ATPase [15] or DC-STAMP [16], which are critical in osteoclast precursor fusion display impaired osteoclast activity and increased bone mass. Deficiency in β_3-Integrin results in an osteoclast which is unable to attach effectively to bone, and therefore is ineffective in resorbing bone substrate [17]. Further, mice with a deficiency in Cathepsin K (Cath K), a protease released by active osteoclasts demonstrate high bone mass [18]. Moreover, failure to acidify secretory vesicles in osteoclasts results in inefficient bone resorption [19] (Figure 1). Identification of additional proteins which contribute to the

functions of osteoclasts will likely yield a more complete understanding of osteoclast biology.

Differentiation of the mature osteoclast from monocyte precursors involves integration of complex signaling cascades activated by RANKL, through TRAF6 [20,21], including ERK and p38 MAP Kinases [22], PI3K [23], Akt [24], c-Src [25], PKC [26], and JNK [27] (Figure 2). Cooperation of these signals leads to a hierarchy of transcriptional regulation that results in osteoclast differentiation from precursors. Research has identified several transcription factors, such as NF-κB [28], AP-1 [27,29], and NFATc1 [30] whose deficiency results in impaired differentiation of osteoclasts from monocyte progenitors. It is now believed that the interplay between these transcription factors is critical for osteoclast differentiation [31].

The most important transcription factor for the differentiation of the osteoclast is NF-κB. NF-κB is activated by RANKL, and several murine genetic models have demonstrated that NF-κB signaling defects result in impaired differentiation [28,32-39]. Furthermore, NF-κB appears to be the most upstream transcriptional regulator involved in osteoclast differentiation [31]. Therefore, understanding NF-κB signaling in the context of osteoclast differentiation will likely contribute to new therapeutic approaches in the treatment of osteoclast mediated disease.

NF-κB Signaling

NF-κB is a transcription factor activated primarily through receptor stimulation by extracellular ligands. NF-κB signaling is divided for convenience into two separate pathways, classical (or canonical) and non-canonical (alternative) [40] which have both recently been shown to play a role in osteoclast differentiation [32,33]. In the classical pathway, signals such as RANKL, TNF-α, IL-1, and LPS result in activation of the Inhibitory κB Kinase (IKK) complex, the catalytic complex which is responsible for activation of NF-κB. The classical IKK complex consists of two kinases, IKKα and IKKβ, and a regulatory subunit, NEMO (or IKKγ). Once activated, IKK phosphorylates IκBα [41], which normally sequesters the NF-κB heterodimers p50/p65 in the cytosol in an inactive state. Phosphorylation of IκBα results in its ubiquitination and proteasomal degradation [42], which releases p50/p65(RelA) NF-κB heterodimers in the nucleus to activate gene transcription (Figure 3). In the alternative NF-κB pathway [43], extracellular signals such as RANKL, CD40L, and Lymphotoxin β stimulate activation of IKKα homodimers which phosphorylate the C-terminus of *NF-κB2*(p100) which serves an IκB function for RelB, holding it inactive in the cytosol. This phosphorylation results in proteasomal processing of p100 to p52, which partners with RelB to enter the nucleus and activate gene transcription as the alternative NF-κB pathway heterodimer (Figure 4).

Efforts to define the relative contribution of each of these pathways to osteoclastogenesis have led to disparate conclusions. For example, mice devoid of active IKKα and alternative NF-κB activation by RANKL demonstrate no apparent bone phenotype and

are susceptible to inflammatory osteolysis [37]. However, mice lacking RelB, the transactivating NF-κB member of the alternative pathway, show impaired osteoclast differentiation *in vitro*, and are resistant to pathological bone destruction induced by tumor metastasis and TNF injection [32]. The reason for this discrepancy most likely rests in the individual systems used to challenge the osteoclast. Nevertheless, it is well accepted that the classical NF-κB pathway is indispensable for osteoclast differentiation, since deficiency of IKKβ or RelA results in impaired osteoclastogenesis and inflammatory osteolysis [33,37,38]. Therefore investigation of classical NF-κB signaling downstream of RANKL will yield valuable insight into the basic mechanisms utilized by differentiating osteoclasts.

The Kinase IKKβ

IKKβ is the kinase responsible for activation of the classical NF-κB signaling pathway [43,44]. It is a 756-amino acid cytosolic serine kinase with three general domains, a kinase domain important for catalytic activity, a leucine zipper domain which is important for protein-protein interactions, and a helix-loop-helix domain which regulates the molecule's kinase activity [45]. Activation of IKKβ requires its association with NEMO through two Tryptophan residues (739 and 741) located in a caryoxyl terminal domain termed the NEMO-binding Domain (NBD) [46,47]. Phosphorylation of two activation loop Serines (177 and 181) on IKKβ by an unknown kinase results in full activation of IKKβ [48] which phosphorylates IκBα leading to NF-κB activation. Pharmacologic inhibition of IKKβ association with NEMO through the use of decoy peptides blocks activation of NF-

κB, inhibits osteoclastogenesis, and prevents arthritis and associated bone loss in mice [35,36]. Phosphomimmetic mutation of IKKβ by mutating Serines 177 and 181 to Glutamic Acid results in constitutive activation, and mutation of Lysine 44 of IKKβ abrogates kinase activity [49] (Figure 5).

IKKβ in the Osteoclast

In order to determine the precise role of IKKβ in osteoclastogenesis, we created a mouse with a conditional deletion of IKKβ in osteoclast precursors by breeding mice possessing a loxP-flanked IKKβ gene [50] and mice transgenically expressing Cre recombinase under the control of the CD11b promoter [51]. Conditional IKKβ knockout mice generated in this manner displayed many phenotypic anomalies including aberrant bone metabolism as a result of defective osteoclastogenesis (Figure 6). The primary goal of this thesis was to characterize the osteoclast phenotype of these mice, to use the model to study the structure function relationship of IKKβ in the differentiating osteoclast, and to draw conclusions regarding the importance of IKKβ in arthritic osteolysis.

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FIGURE LEGENDS

Figure 1: Schematic of Osteoclast Differentiation

Osteoclasts differentiate from monocyte precursors when stimulated by M-CSF and RANKL, two cytokines secreted by osteoblast cells in normal bone metabolism. Stimulation of the receptors, c-fms and RANK, by M-CSF and RANKL, respectively, triggers the activation of the transcription factors, NF-κB, AP-1, and NFATc1 in the differentiating pre-osteoclast. These transcription factors coordinate the osteoclast differentiation program resulting in expression of proteins necessary for fusion of precursors (DC-STAMP), for bone attachment (β3-Interin), for cytoskeletal organization (c-Src), and for degradation of bone matrix (Cathepsin K and MMP9). Calcitonin Receptor and Tartrate-Resistant Acid Phosphatase (TRAP) are useful biomarkers for the mature osteoclast.

Figure 2: Osteoclastogenic Signaling

Stimulation of the receptor, RANK, by its ligand, RANKL, leads to activation of several kinases including IKK, JNK, ERK, p38, and Akt. These signals require association of the E3 Ubiquitin Ligase, TRAF6, with the RANK receptor. Interference with each of these kinases, as well as loss of TRAF6, results in impaired osteoclast differentiation. IKK activates NF-κB which induces the transcription factor, c-fos. NFATc1, considered the master regulator of osteoclastogenesis, is activated downstream of NF-κB and AP-1.

Figure 3: Classical NF-κB Signaling Pathway

The Classical NF-κB heterodimer, p50/p65(RelA), is bound in an inactive state in the cytosol by IκBα. Extracellular Signals lead to activation of the classical IKK complex consisting of IKKα, IKKβ, and IKKγ (NEMO). IKKβ phosphorylates IκBα leading to its ubiquitylation and rapid degradation by the proteasome. As a result, P50/p65 (RelA) heterodimers translocate into the nucleus to activate gene transcription.

Figure 4: Alternative NF-κB Signaling Pathway

RelB, the transactivating factor of the alternative pathway, is sequestered in the cytosol by NF-κB p100. Extracellular signals result in activation of IKKα homodimers. IKKα phosphorylates p100 resulting in its proteasomal processing to p52. p52/RelB heterodimers then translocate into the nucleus to regulate gene transcription.

Figure 5: Schematic Structure of IKKβ

IKKβ consists of an N-terminal kinase domain (Kinase) which transfers phosphate from ATP to target serine and Threonine residues of substrate proteins, a Leucine Zipper domain (LZ) important for protein-protein interactions, a Helix-Loop-Helix domain (HLH) which modulates IKKβ kinase activity, and a C-terminal domain (NBD) that is critical for binding to NEMO. Binding of IKKβ to NEMO, through Tryptophan residues 739 and 741, results in phosphorylation by an upstream kinase of Serines 177 and 181 in the activation loop in the kinase domain of IKKβ, leading to activation of IKKβ. Lysine

44 is the ATP-binding residue in IKKβ, and its mutation results in a kinase-inactive, dominant-negative molecule.

Figure 6: Generation of Mice with a Conditional Deletion of IKKβ in Osteoclast Precursors

We generated mice with a conditional loss of IKKβ in osteoclast precursors (OCPΔIKKβ) by crossing mice possessing loxP-flanked (floxed) IKKβ with mice harboring a Cre recombinase transgene under the control of the CD11b promoter. Resultant knockout mice (OCPΔIKKβ) displayed dense femurs (arrows) on X-ray compared with control littermates suggesting that IKKβ is critical for osteoclast differentiation from precursors.

FIGURES

Figure 1

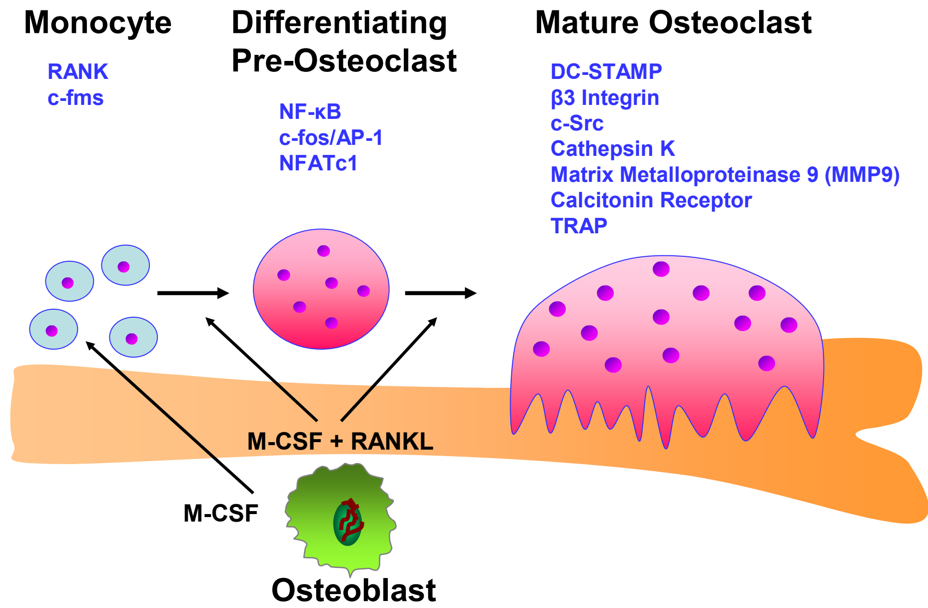


Figure 2

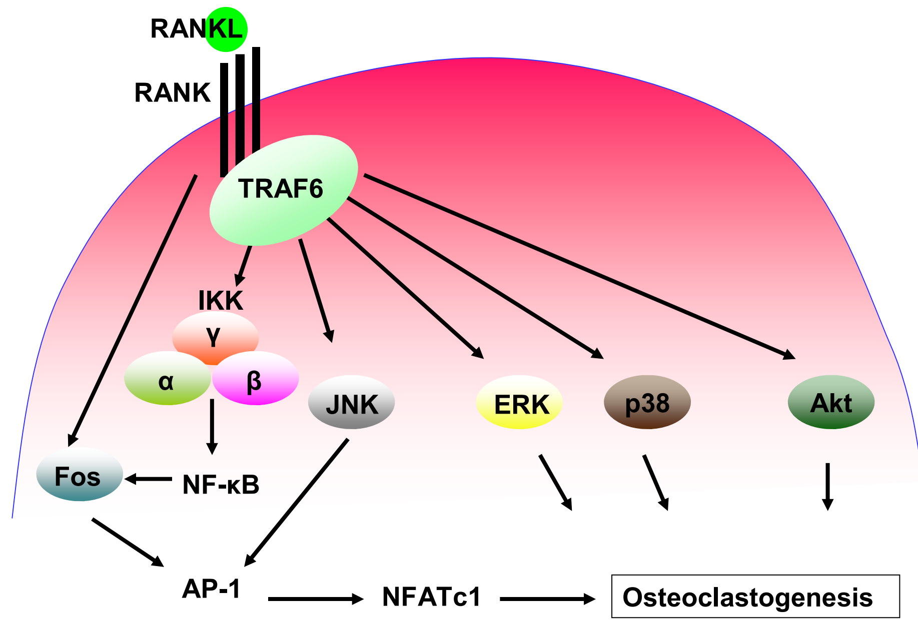


Figure 3

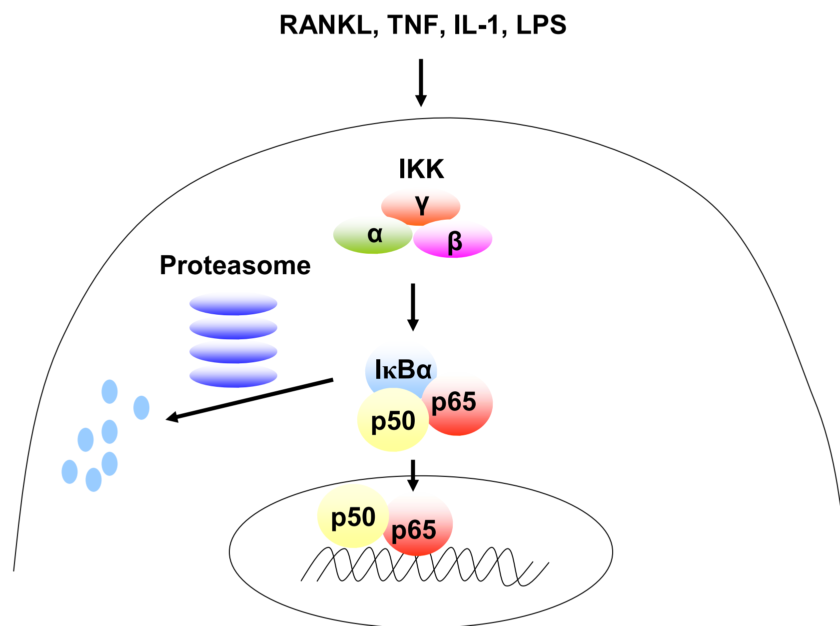


Figure 4

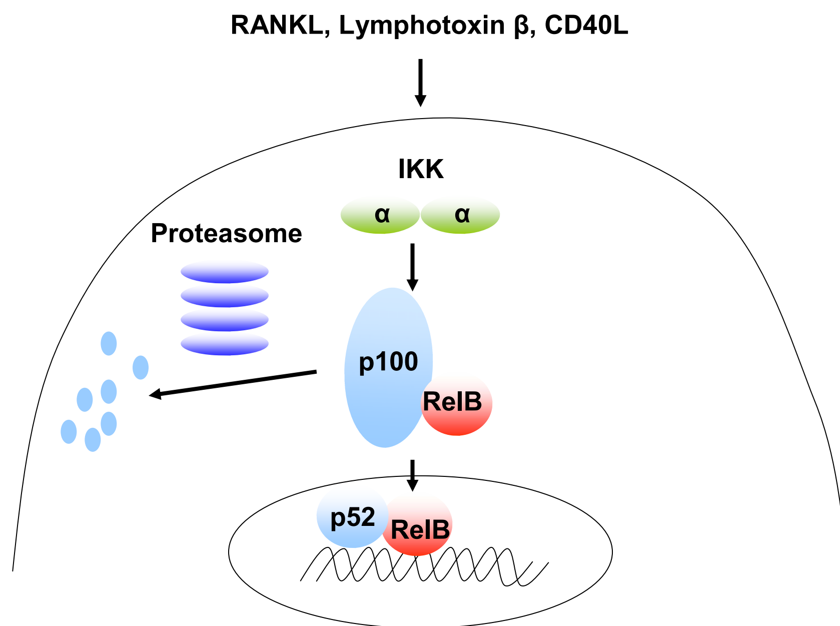


Figure 5

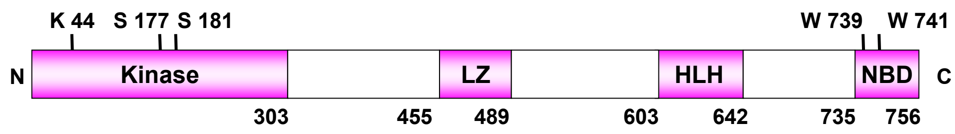
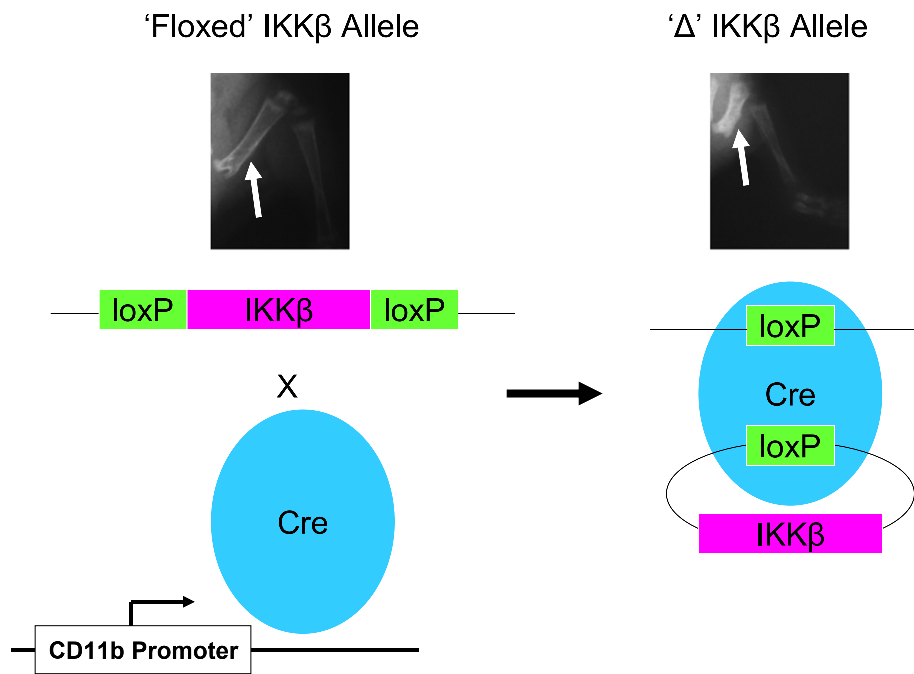


Figure 6



CHAPTER 2

DEFECTIVE OSTEOCLASTOGENESIS BY IKKβ-NULL PRECURSORS IS A RESULT OF RANKL-INDUCED JNK-DEPENDENT APOPTOSIS AND IMPAIRED DIFFERENTIATION

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DEFECTIVE OSTEOCLASTOGENESIS BY IKKβ-NULL PRECURSORS IS A RESULT OF RANKL-INDUCED JNK-DEPENDENT APOPTOSIS AND IMPAIRED DIFFERENTIATION*

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Running Head: JNK-Dependent Apoptosis in OCP's lacking IKKβ

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It has previously been reported that IKKβ supports osteoclastogenesis through NF-κB-mediated prevention of apoptosis. This finding suggests that the ligand for Receptor Activator of NF-κB (RANKL), the master osteoclastogenic cytokine, induces apoptosis of osteoclast precursors (OCP's) in the absence of IKKβ/NF-κB competency. To validate this hypothesis, we sought to determine the pro-apoptotic signaling factors induced by RANKL in IKKβ-null osteoclast OCP's and to rescue osteoclast differentiation in the absence of IKKβ through their inhibition. To accomplish this, we generated mice which lack IKKβ in multiple

hematopoietic lineages including OCP's. 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 OCP's depends on IKKβ since reduced IKKβ 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 theses cells inhibits RANKL-induced apoptosis and restores the ability of IKKβ-null OCP's to become mature osteoclasts. Our data highlight the pro-osteoclastogenic and anti-apoptotic roles of IKKβ in OCP's and identify a pro-apoptotic mechanism activated within the RANK signalosome.

INTRODUCTION

Osteoclasts develop from bone marrow macrophage precursors under the control of two cytokines, Receptor Activator of NF-κB Ligand[1] (RANKL) (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-κB (3). NF-κB 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-κB 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-κB is activated by the inhibitory κB kinase (IKK) complex, which is crucial for osteoclastogenesis. The IKK complex is composed of two catalytically active members, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO. IKKα mediates activation of the alternative pathway by phosphorylation of NF-κB2/p100 (6), while IKKβ is important for activation of the canonical pathway through phosphorylation of IκB (8). The importance of the signaling activity of the IKK complex in osteoclasts is demonstrated by the defect in osteoclastogenesis noted in mice lacking IKKα (9) or IKKβ (10). Despite the sequence homology of these two kinases, their relative importance in osteoclastogenesis is strikingly different. For example, osteoclasts devoid of active IKKα only demonstrate an *in vitro* defect in osteoclastogenesis, while the bone phenotype of the mouse is remarkably normal. On the other hand, mice with an inducible osteoclast precursor-specific deletion of IKKβ 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 downstream of RANKL in the absence of IKKβ, and we hypothesized that inhibition of these signals would be sufficient to rescue the osteoclast defect of cells

lacking IKKβ. Our findings reveal that loss of IKKβ in osteoclast precursors (OCP's) 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 OCP's lacking IKKβ. This finding demonstrates that the necessity of IKKβ 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 OCP's.

MATERIALS AND METHODS

Reagents- Antibodies against IKKβ, IKKα, 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 Biotechnology, Inc (Rockford, IL). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Animals- CD11b Cre Y-chromosomal transgenic and floxed IKKβ mice on a C57BL/6 background were reported previously (12, 13). Male Cre+ Floxed IKKβ 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 α-MEM and was centrifuged at 453 rcf. Marrow pellets were resuspended in whole media (α-MEM 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 ^{o}C in 5% CO_2 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 x 10^4 cells in 200 μL 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-α 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, St. Louis, MO). TRAP-positive multinucleated cells with 3 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 hours 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 NaPyrophosphate, 1 mM β-Glycerophosphate, 1% Triton, 1 mM Na_3VO_4, 1 μg/mL Leupeptin, 1 mM NaF, 1 mM PMSF, and distilled deionized H_2O). Protein concentration was measured by standard BCA assay (Pierce). 10-20 μg of total cell protein was used for Western blot. See below.

Apoptosis Assay- Osteoclast precursors were plated onto tissue culture dishes overnight in whole media supplemented with m-CSF. Cells were serum starved for 6 hours 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. See below.

Rescue of Apoptosis- Osteoclast precursors were plated onto tissue culture dishes for 24 hours 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 μM, 1.0 μM, or 2.0 μM 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 hours, cells were lysed as described above, protein was normalized, and samples were analyzed by Western Blot. See below.

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, Carlsbad, CA) according to the manufacturer's standard protocol.

Reverse Transcription- 1.0 μg total RNA was subjected to reverse transcription under the following conditions. 1.0 μg RNA and 1.0 μg random hexamer primer in 10 μL nuclease-free deionized H_2O in PCR tubes were heated to 70 ^{o}C for 5 minutes, cooled to 42 ^{o}C, and set on ice. The following components were then added at the indicated amounts or concentrations for a total reaction volume of 20 μL: 1 x RT AMV buffer (Roche, Palo Alto, CA), 40U RNAseIn (Promega, San Luis Obispo, CA), 1.25 mM dNTP's, 5 mM NaPyruvate, 5 U Reverse Transcriptase Enzyme, AMV (Roche). To produce cDNA, tubes were placed in a thermocycler programmed as follows. 42 ^{o}C for 60 minutes, 50 ^{o}C for 10 minutes, 95 ^{o}C for 5 minutes, and 4 ^{o}C to hold.

Bone Resorption Assay- Bone marrow osteoclast precursors were plated onto BD Biocoat Osteologic tissue culture slides (BD Biosciences, San Jose, CA) in the presence of 10 ng/ml m-CSF and RANKL with or without 0.4 μM TAT-TI-JIP for the indicated times. Resorption pits were determined as clear areas in the osteologic matrix. Representative photographs were taken at 10X magnification.

Real-Time PCR- Triplicate samples of 4 μL cDNA product (5X diluted), 10 μL Sybr Green PCR Master Mix (Applied Biosystems, Inc. Foster City, CA), 0.1 μL each of 10 μM forward and reverse primer stocks, and 6 μL nuclease-free deionized H_2O were subjected to real time PCR according to the following program in a 7300 AB Real Time PCR System: 50 ^oC for 2 minutes, 95 ^oC for 10 minutes, (95 ^oC for 15 seconds, 60 ^oC for 1 minute) x 40 cycles. Results were analyzed using AB RQ Study Software. Real Time PCR primers were designed using Primer Express Software (Applied Biosystems, Inc.) mouse Actin Forward 5'-CTTCTACAATGAGCTGCGTG-3', mouse Actin Reverse 5'-TCATGAGGTAGTCTGTCAGG-3', mouse TRAP Forward 5'-CGACCATTGTTAGCCACATACG-3', mouse TRAP Reverse 5'-CACATAGCCCACACCGTTCTC-3', mouse Calcitonin Receptor Forward 5'-CAAGAACCTTAGCTGCCAGAG-3', mouse Calcitonin Receptor Reverse 5'-CAAGCACGCGGACAATGTTG-3', mouse MMP9 Forward 5'-CAGGGAGATGCCCATTTCG-3', mouse MMP9 Reverse 5'-GGGCACCATTTGGAGTTTCCA-3', mouse A20 Forward 5'-CAGAAAAAAGTGGTGAAGGTGTGA-3', mouse A20 Reverse 5'-CCAGGCTCTGACCTCTGTTACA-3', mouse cIAP1 (birc2) Forward 5'-GTGATGGTGGCTTGAGATGTTG-3', mouse cIAP1 (birc2) Reverse 5'-CAAGAACTCACACCTTGGAAACC-3', mouse cIAP2 (birc3) Forward 5'-GAAGTGGGCTGCGGTATCA-3', mouse cIAP2 (birc3) Reverse 5'-GCGCTGTCTTGAACCATGTTC-3', mouse Bcl-x_L Forward 5'-GCGGCTGGGACACTTTTG-3', mouse Bcl-x_L Reverse 5'-

CAGAACCACACCAGCCACAGT-3', mouse XIAP Forward 5'-CGGATCGTTACTTTTGGAACATG-3', mouse XIAP Reverse 5'-CGCCTTCACCTAAAGCATAAAATC-3', mouse Cathepsin K Forward 5'-GGAAGAAGACTCACCAGAAGC-3', mouse Cathepsin K Reverse 5'-GTCATATAGCCGCCTCCACAG-3', mouse β_3 Integrin Forward 5'-TTACCCCGTGGACATCTACTA-3', mouse β_3 Integrin Reverse 5'-AGTCTTCCATCCAGGGCAATA-3', mouse GAPDH Forward 5'-CTTCACCACCATGGAGAAGGC-3', mouse GAPDH Reverse 5'-GACGGACACATTGGGGGTAG-3'.

Western Blot Assay- Total cell lysates were boiled in the presence of an equal volume of 2X SDS sample buffer consisting of (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, 3% β-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, Hercules, CA) 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 ^{o}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 (4 X 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, BioRad (Hercules, CA).

Histology- Long bones were collected from mice and fixed in 10% buffered formalin for 24 hours. Bones were then decalcified for 7 days in decalcification buffer consisting of (14% (w/v) EDTA, H_4NOH 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 TdT-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- IKKβ–Deficiency Demonstrate in vitro and in vivo Defects in Osteoclastogenesis

IKKβ has been shown to be necessary for osteoclast formation (10). To define the mechanism through which IKKβ supports osteoclastogenesis, we generated mice with a deficiency of IKKβ in multiple hematopoietic lineages including OCP's by crossing CD11b Cre recombinase transgenic mice (13) with mice possessing floxed IKKβ (14) (Supplementary Figure 1). In this study, we focus on the osteoclast phenotype, so we will refer to the resultant knockout (Cre-positive floxed/floxed IKKβ) mice as $OCP^{\Delta IKK\beta}$ or Cre+ f/f. $OCP^{\Delta IKK\beta}$ 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 IKKβ protein in osteoclast precursors of $OCP^{\Delta IKK\beta}$ mice (fig. 1B). We demonstrate that this defect is cell-autonomous by culturing bone marrow-derived OCP's in the presence of m-CSF and RANKL. $OCP^{\Delta IKK\beta}$ cells form significantly fewer multinucleated osteoclasts (fig. 1C, panel g) compared with Cre-positive IKKβ wild-type/wild-type (wt/wt) (fig. 1C, panel a) and Cre-positive IKKβ wild-type/floxed (wt/f) heterozygous littermate controls (fig. 1C, panel d). Furthermore, stimulation with TNF-α or LPS (fig. 1C, panels h and i) is insufficient to rescue the osteoclast defect of $OCP^{\Delta IKK\beta}$ cells. Compared with WT cells, Cre-positive IKKβ (wt/f) OCP's also show decreased IKKβ protein expression (fig. 1B), but this difference does not result in impaired osteoclastogenesis (fig. 1C, panels d-f).

$OCP^{\Delta IKK\beta}$ are Prone to RANKL-Induced Apoptosis and Display Defective Osteoclast Differentiation

IKKβ has previously been demonstrated to protect OCP's from TNF-α-mediated apoptosis (10). We sought to determine whether IKKβ-deficient OCP's are similarly susceptible to apoptosis in response to RANKL and to test whether the absence of IKKβ results in defective osteoclast differentiation. To accomplish this, we cultured $OCP^{\Delta IKK\beta}$ and control OCP's in the presence of m-CSF and RANKL for zero or five 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 m-RNA for the osteoclast markers β_3 Integrin (Fig. 2A), Cathepsin K (Fig. 2 B), Calcitonin Receptor (Fig. 2C), Matrix Metalloproteinase 9 (Fig. 2D), and TRAP (Fig. 2E) in RANKL-stimulated $OCP^{\Delta IKK\beta}$ cells compared with controls. This failure to express osteoclast markers in osteoclastogenic conditions correlates with up to an 81% reduction in expression of IKKβ mRNA in $OCP^{\Delta IKK\beta}$ cells compared with controls (Fig. 2F). Interestingly, in $OCP^{\Delta IKK\beta}$ cells that express higher levels of IKKβ mRNA- 63% reduction compared with control OCP's- osteoclast marker expression after RANKL stimulation is not affected, yet they still fail to form multinucleated osteoclasts in *in vitro* culture (Supplementary Figure 2). This finding indicates that IKKβ also serves a differentiation-independent function to support osteoclastogenesis. These observations lead us to surmise that IKKβ is essential at various stages for differentiation and survival of RANKL-stimulated OCP's.

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 OCP's 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-κB-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 OCP's to RANKL for a time-course of four hours. We detected the kinetics of poly-ADP Ribose Polymerase (PARP) cleavage by

Western blot as a molecular signature of apoptosis. We note disappearance of full-length PARP in OCP$^{\Delta IKK\beta}$ cells after 1 hour while 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 IKKβ. We conclude that IKKβ is necessary for osteoclast differentiation and the survival of osteoclast precursors exposed to RANKL.

In order to determine whether apoptosis of osteoclasts or OCP's 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 OCP's in OCP$^{\Delta IKK\beta}$ compared with control long bones (fig. 3A and B). Based on our data, we conclude that apoptosis contributes to the osteoclast defect in OCP$^{\Delta IKK\beta}$ mice.

OCP$^{\Delta IKK\beta}$ Possess a Gain-of-Function in JNK Activation

We were interested in potential pro-apoptotic signals induced by RANKL in differentiating IKKβ-deficient OCP's. To address this, we performed a phospho-protein screen by Western blot analysis in OCP$^{\Delta IKK\beta}$ and control OCP's after stimulation with a time course of RANKL or TNF-α. We postulated that OCP$^{\Delta IKK\beta}$ kinase signaling would possess a signature which 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. 4A and B) and TNF-α (fig. 4A) in $OCP^{\Delta IKK\beta}$ compared with control OCP's. Interestingly, p38 (fig. 4A) and Akt (fig. 4B) phosphorylation remain unaffected in the absence of IKKβ, suggesting that JNK downregulation is a specific function of IKKβ. 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 minutes of RANKL stimulation of control OCP's (fig. 4C). This time point correlates with downregulation of JNK phosphorylation after RANKL stimulation (fig. 4A and B). In $OCP^{\Delta IKK\beta}$ cells, JNK phosphorylation is sustained at 30 minutes of RANKL stimulation (fig. 4A 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). Since MKP1 (16) and MKP5 (17) serve as JNK phosphatases, MKP induction may serve as an IKKβ-dependent mechanism for JNK downregulation 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 IKKβ-Deficient Osteoclast Precursors

JNK activation has been linked to RANKL-induced apoptosis of differentiating osteoclasts (15). We hypothesized that since $OCP^{\Delta IKK\beta}$ cells are susceptible 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 (18) after RANKL stimulation. In WT OCP's,

TAT-TI-JIP peptides inhibit osteoclastogenesis at concentrations above 1 μM (not shown). This finding is expected given the established importance of c-jun in osteoclast differentiation (19,20). Surprisingly, TAT-TI-JIP peptides enhance osteoclastogenesis in WT cells at a concentration of 0.4 μM (fig. 5B panel b). We hypothesized that this concentration of TAT-TI-JIP is sufficient to block the pro-apoptotic action of JNK without affecting the activity of JNK toward c-jun after RANKL stimulation. Indeed, 0.4 μM of TAT-TI-JIP blocks RANKL-induced PARP cleavage without dramatically altering RANKL-induced phosphorylation of c-jun (fig. 5A). At a concentration of 1 μM, TAT-TI-JIP inhibits RANKL-induced PARP cleavage in OCP's, but it also inhibits c-jun phosphorylation (fig. 5A), which explains the inhibitory affect of this concentration on osteoclastogenesis. These results suggest that JNK serves two distinct functions in osteoclast differentiation and survival. Since low dose TAT-TI-JIP inhibits apoptosis of OCP's induced by RANKL stimulation without affecting c-jun activation, we tested this concentration for its potential to rescue osteoclastogenesis of OCP's in the absence of IKKβ. Indeed, in the *in vitro* osteoclastogenesis assay, 0.4 μM TAT-TI-JIP peptide rescues osteoclastogenesis of $OCP^{\Delta IKK\beta}$ cells (fig. 5B). For example, $OCP^{\Delta IKK\beta}$ cells (fig. 5B, panel c) treated with RANKL produce less than 5% of the number of TRAP-positive osteoclasts produced by control OCP's (fig. 5B, panel a). However, TAT-TI-JIP treatment of $OCP^{\Delta IKK\beta}$ cells (fig. 5B, panel d) results in slightly, but statistically not significant, higher number of TRAP-positive osteoclasts compared with non-TAT-TI-JIP-treated controls (fig. 5B, panel a). Importantly, JIP peptide-treated control OCP's (fig. 5B panel b) produced more osteoclasts in *in vitro* culture than JIP peptide-treated

IKKβ deficient OCP's (fig. 5B panel d), indicating that $OCP^{\Delta IKK\beta}$ possess defects in osteoclast differentiation that are independent of JNK-mediated apoptosis.

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 μM TAT-TI-JIP. Resorption pits created by RANKL-treated $OCP^{\Delta IKK\beta}$ cells (fig.6, panel b) were significantly smaller (22+/-3% resorption area compared with controls) than those created by control osteoclasts (fig. 6, panel a), 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. 6, panel d), we restored resorption pit size over that noted in WT non-TAT-TI-JIP treated cells (165+/-7% of control) (Fig. 6, panel a). Consistent with our *in vitro* osteoclastogenesis assay data shown in figure 5, resorption pit size of TAT-TI-JIP-treated WT osteoclasts (fig. 6, panel c) is two fold larger than that of TAT-TI-JIP-treated $OCP^{\Delta IKK\beta}$ (210+/-14%) indicating that IKKβ also acts through mechanisms independent of JNK inhibition to support osteoclastogenesis.

DISCUSSION

In previous studies, we and others have shown that IKK-NF-κB function is necessary for osteoclastogenesis (4,10,12). The diverse activities of the individual IKK and NF-κB members suggest that each molecule plays a unique role in the overall program of osteoclast development. It has been demonstrated that IKKβ protects OCP's from

apoptosis in response to TNF-α (10). While this finding is consistent with previous reports regarding the function of IKKβ in other settings (21), its precise role downstream of RANK during osteoclastogenesis has remained unclear. The most likely possibility is that IKKβ serves some capacity in osteoclastogenesis that is dependent upon NF-κB-p65 action. However, the exact function of p65 in the osteoclast remains to be elucidated. It has been suggested that IKKβ may be important for the survival and differentiation of OCP's (10). We show that OCP's deficient in IKKβ display a defect in osteoclast differentiation, which is consistent with impaired induction of mRNA for the osteoclast markers: β_3 Integrin, Cathepsin K, Calcitonin Receptor, MMP9, and TRAP after RANKL stimulation.

We also show that IKKβ-deficient OCP's 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-κB-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 IKKβ mRNA, induction of osteoclast differentiation markers was not affected, although impaired *in vitro* osteoclastogenesis was still observed (Supplementary Figure 2). We examined the expression of mRNA for DC-STAMP (22) and ATP6v0d2 (23), two genes 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 suggests 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 OCP's which lack IKKβ. Since JNK activation has been correlated with RANKL-induced apoptosis previously (15), 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 (unpublished observations). 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 (Supplementary Figure 3, panel c). When treated with TAT-TI-JIP 48 hours after RANKL stimulation, JNK-inhibition does not fully rescue osteoclastogenesis of $OCP^{\Delta IKK\beta}$ cells (Supplementary Figure 3, panel d).

Whether the absence of IKKβ 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 IKKβ signaling and not IKKβ 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 OCP's leads to activation and eventual downregulation of JNK through MAP kinase phosphatase synthesis. In the absence of IKKβ, 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-downregulation through inhibition of the JNK phosphatases will result in 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 previously been demonstrated that in response to TNF-α in NF-κB-deficient cells, JNK activation leads to caspase-8 - independent cleavage of the pro-apoptotic protein, bid, resulting in mitochondrial release of smac and apoptosis (24). Since RANKL stimulation of OCP's does not result in caspase-8 activation, it is likely that enhanced JNK activity in the absence of IKKβ directly leads to apoptosis of OCP's after RANKL stimulation. Because lower expression of IKKβ in $OCP^{\Delta IKK\beta}$ cells prevents osteoclast differentiation, it is unlikely that inhibition of JNK-mediated apoptosis will rescue osteoclastogenesis in the complete absence of IKKβ. Observing the rescuing effect of a low-dose JNK inhibitor on osteoclastogenesis in $OCP^{\Delta IKK\beta}$, therefore, requires a permissive level of IKKβ expression that allows differentiation to occur but does not inhibit apoptosis. We believe our CD11b Cre-mediated deletion of IKKβ was a successful tool in this regard.

In addition to MAP kinase phosphatases, several potential connections between the absence of IKKβ and enhanced JNK activation exist. For example, known target

genes of NF-κB serve to downregulate JNK activation such as Gadd45β, which specifically inhibits TNF-α-mediated MKK7 activation of JNK (25). Additionally, XIAP is a target of NF-κB which downregulates JNK activation in response to TNF-α (26). Furthermore, A20 has been postulated to play a role in NF-κB-mediated inhibition of JNK activation by downregulating TRAF2 (27), although this hypothesis has never been validated. Since $OCP^{\Delta IKK\beta}$ display reduced XIAP and A20 expression, it will be interesting to test whether these mechanisms of crosstalk between IKKβ 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 (19). c-jun activation leads to a partnership between AP-1 and NFAT1 which induces expression of NFAT2 and differentiation of osteoclasts (20). Therefore, inhibition of the RANKL-RANK-JNK pathway is a candidate for treatment of osteoporosis (28). 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 IKKβ in osteoclastogenesis. We demonstrate that IKKβ is important for both differentiation and survival of osteoclasts. Given that we are able to rescue osteoclastogenesis in $OCP^{\Delta IKK\beta}$ cells through inhibition of JNK-induced apoptosis, we conclude that IKKβ 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 IKKβ in OCP's are potential means to treat osteoclast-mediated disease.

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FOOTNOTES

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[1] Abbreviations used are: m-CSF, macrophage colony stimulating factor; RANKL, Receptor Activator of NF-κB Ligand; OCP, osteoclast precursor; wt, wild-type; TRAP, tartrate-resistant acid phosphatase; CtR, calcitonin receptor; NFAT, nuclear factor and activator of T-cells; MMP, matrix metalloproteinase; cIAP, cellular inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis; MKP, MAP kinase phosphatase; PARP, poly (ADP-ribose) polymerase; TUNEL, TdT-mediated dUTP Nick End Labeling; JIP, JNK-interacting protein

FIGURE LEGENDS

Figure 1. Mice with IKKβ–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 IKKβ and CD11b Cre-positive floxed/floxed IKKβ ($OCP^{\Delta IKK\beta}$) 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 IKKβ, and CD11b Cre-positive floxed/floxed IKKβ mice. (*C* and *D*) Osteoclast precursors from CD11b Cre-positive wt/wt IKKβ, CD11b Cre-positive wt/floxed IKKβ, and CD11b Cre-positive floxed/floxed IKKβ mice were cultured in osteoclastogenic conditions. (*C*) Cells were either not stimulated (a, d, g), or were further stimulated with 10 ng/ml TNF-α (b, e, h) or 100 ng/ml LPS (c, f, 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 (MNC's) with 3 or more nuclei were scored as osteoclasts. Asterisk indicates $p < 0.005$ for difference between number of TRAP-positive MNC's in wells represented by d and g.

Figure 2. $OCP^{\Delta IKK\beta}$ are defective in osteoclast differentiation and demonstrate increased susceptibility to apoptosis. $OCP^{\Delta IKK\beta}$ and control OCP's were plated in whole media supplemented with 10 ng/ml m-CSF. Cells were either not stimulated 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*) β_3-Integrin, (*B*) Cathepsin K (Cath K), (*C*) Calcitonin Receptor (CtR), (*D*) Matrix Metalloproteinase 9 (MMP9), and (*E*) Tartrate-Resistant Acid Phosphatase (TRAP) as well as (*F*) IKKβ (IKK2). GAPDH served as the internal standard for cDNA normalization. Data are presented as relative quantification with WT non-stimulated levels serving as the reference point (relative expression value of 1). Values represent mean quantification plus the standard error of the mean. (*G*) $OCP^{\Delta IKK\beta}$ and control OCP's 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-κB-regulated anti-apoptotic proteins: A20, cIAP2 (cellular inhibitor of apoptosis 2), cIAP1 (cellular inhibitor of apoptosis 1), XIAP (X-linked inhibitor of apoptosis), and Bcl-x_L. β-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 standard error of the mean. Data are representative of three independent experiments. (*H*) $OCP^{\Delta IKK\beta}$ and control OCP's were serum starved and were either not stimulated or were stimulated with 10 ng/ml RANKL for 15, 30, 45, 60, or 240 minutes. Total cell lysates were analyzed by Western blot for integrity of full length PARP (f-PARP). β-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 OCP's. Upper images taken at 10X magnification, and lower images are panels from upper images taken at 40X magnification. (*B*) Graph depicting quantification of TUNEL positive peritrabecular nuclei per 40X 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.

Figure 4 . Loss of IKKβ in OCP's results in a gain-of-function in JNK phosphorylation. $OCP^{\Delta IKK\beta}$ and control OCP's were serum starved for 4-6 hours. (*A*) Cells were either not stimulated or were stimulated with 10 ng/ml RANKL or TNF-α for 5, 10, or 30 minutes. (*B*) Cells were either not stimulated or were stimulated with 10 ng/ml RANKL for 7.5, 15, 30, or 60 minutes. 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. β-Actin served as the loading control.

Figure 5. Inhibition of RANKL-mediated JNK-induced apoptosis rescues osteoclastogenesis defect in OCP's deficient in IKKβ. (*A*) OCP's from Cre+ wild-type/floxed IKKβ 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 μM, 1.0 μM, or 2.0 μM 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 hours 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 OCP's were plated in osteoclastogenic conditions. At the time of plating, one group of cells from each population was either left untreated (a and c) or treated with 0.4 μM TAT-TI-JIP (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 (MNC's) with 3 or more nuclei were scored as osteoclasts. Data are representative of three independent experiments, and error bars represent standard error of the mean. Asterisk indicates $p < 0.0001$ for difference between number of TRAP-positive MNC's in wells represented by a and c. No significant difference exists between a and d.

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

Supplementary Figure 1. Deletion of IKKβ in multiple hematopoietic tissues. Southern blot for IKKβ gene from DNA of bone, thymus (thym), and spleen of CD11b Cre + floxed/floxed IKKβ mice. Floxed; loxP-flanked, Δ; deleted by Cre.

Supplementary Figure 2. $OCP^{\Delta IKK\beta}$ with insufficient suppression of IKKβ mRNA show defective osteoclast phenotype *in vitro* despite normal induction of osteoclast markers. Control and $OCP^{\Delta IKK\beta}$ osteoclast precursors were either not treated (unstim) or were treated with RANKL for 5 days. At day 5, mRNA was collected from cells for analysis of gene expression for (*A*) β_3-Integrin, (*B*) TRAP, (*C*) Calcitonin Receptor, and (*D*) IKKβ. RANKL-treated cells were also TRAP-stained (*E*) for visualization of osteoclasts.

Supplementary Figure 3. Requirement for early inhibition of JNK for rescue of osteoclastogenesis in $OCP^{\Delta IKK\beta}$. Control (a) and $OCP^{\Delta IKK\beta}$ (b-d) osteoclast precursors were treated with RANKL for 5 days. For JNK inhibition, $OCP^{\Delta IKK\beta}$ cells were either not treated (b) or were treated with 0.4 μM TAT-TI-JIP at the same time as the first RANKL stimulation (c) or 48 hours later (d).

FIGURES

Figure 1

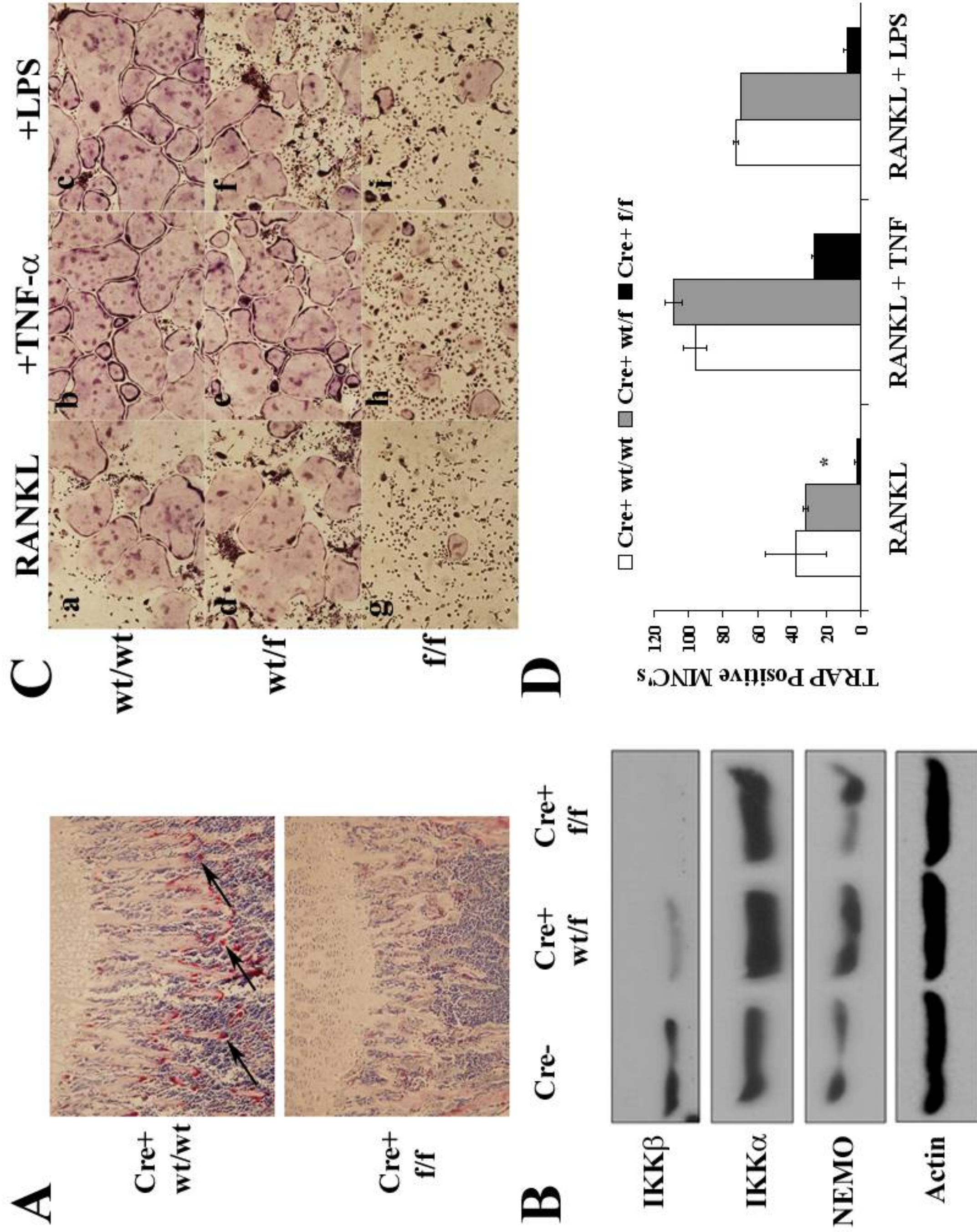


Figure 2

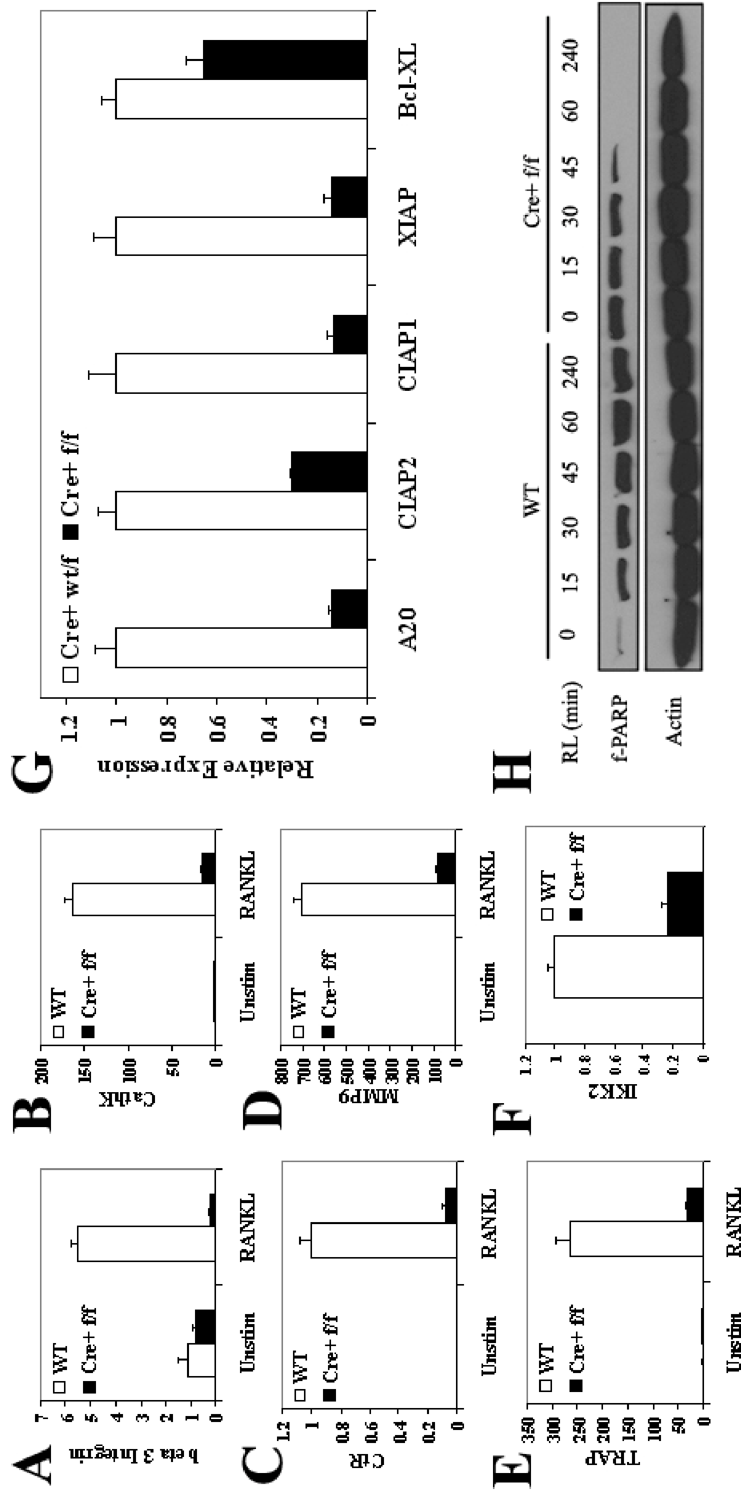


Figure 3

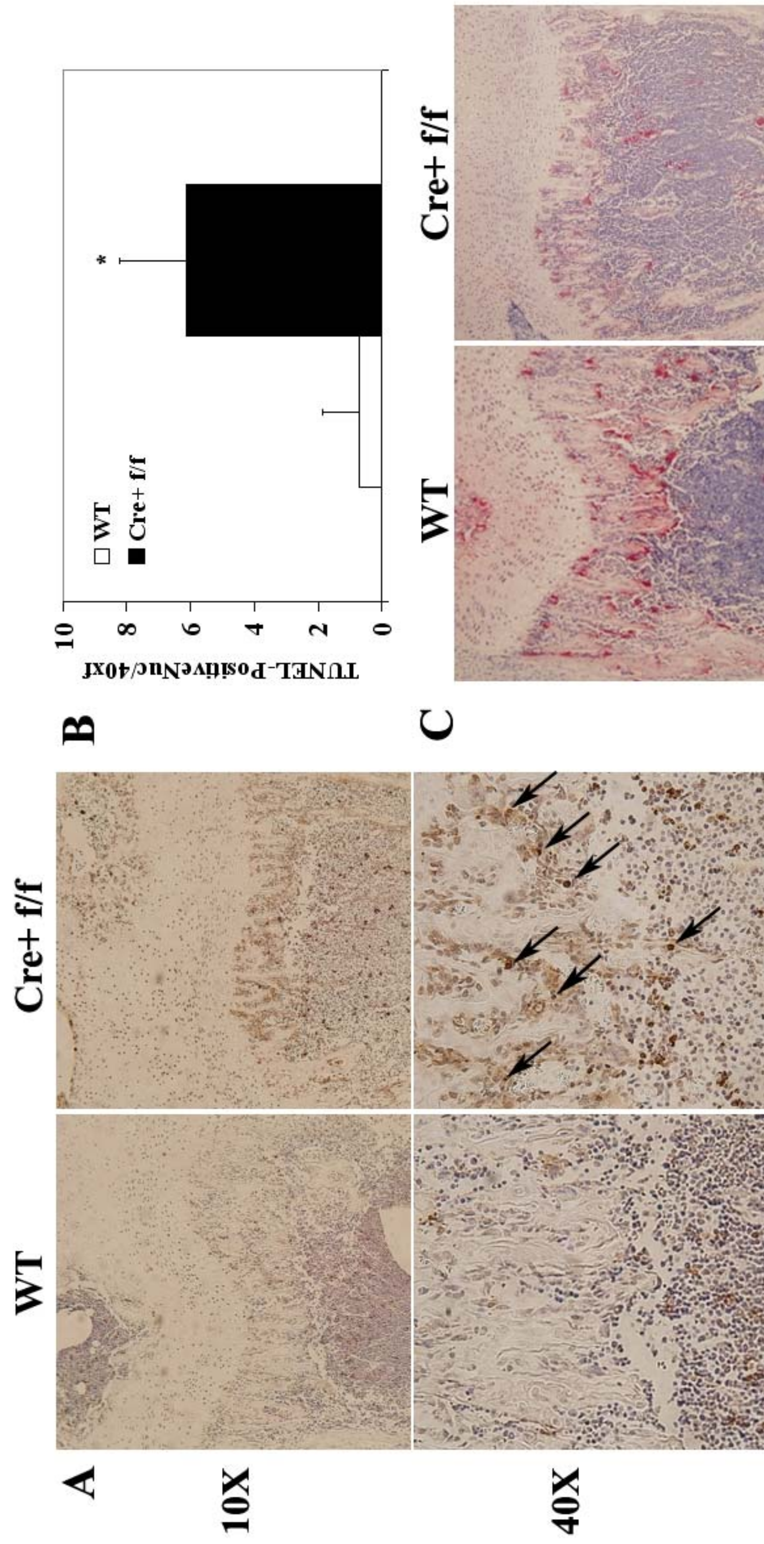


Figure 4

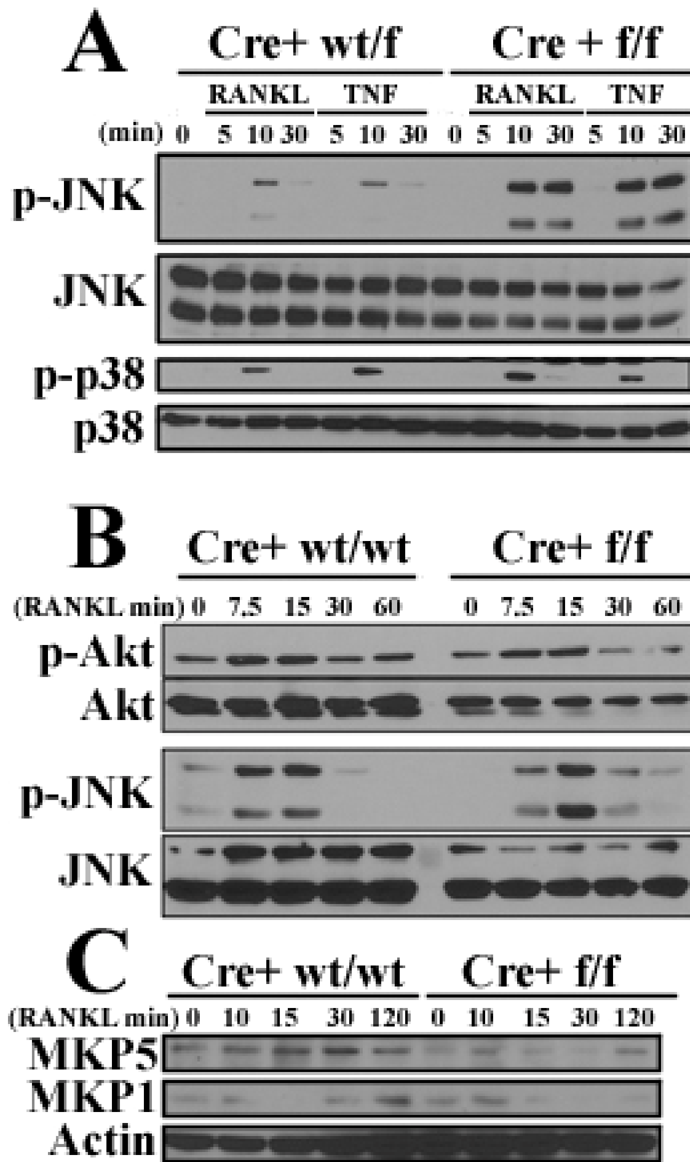


Figure 5

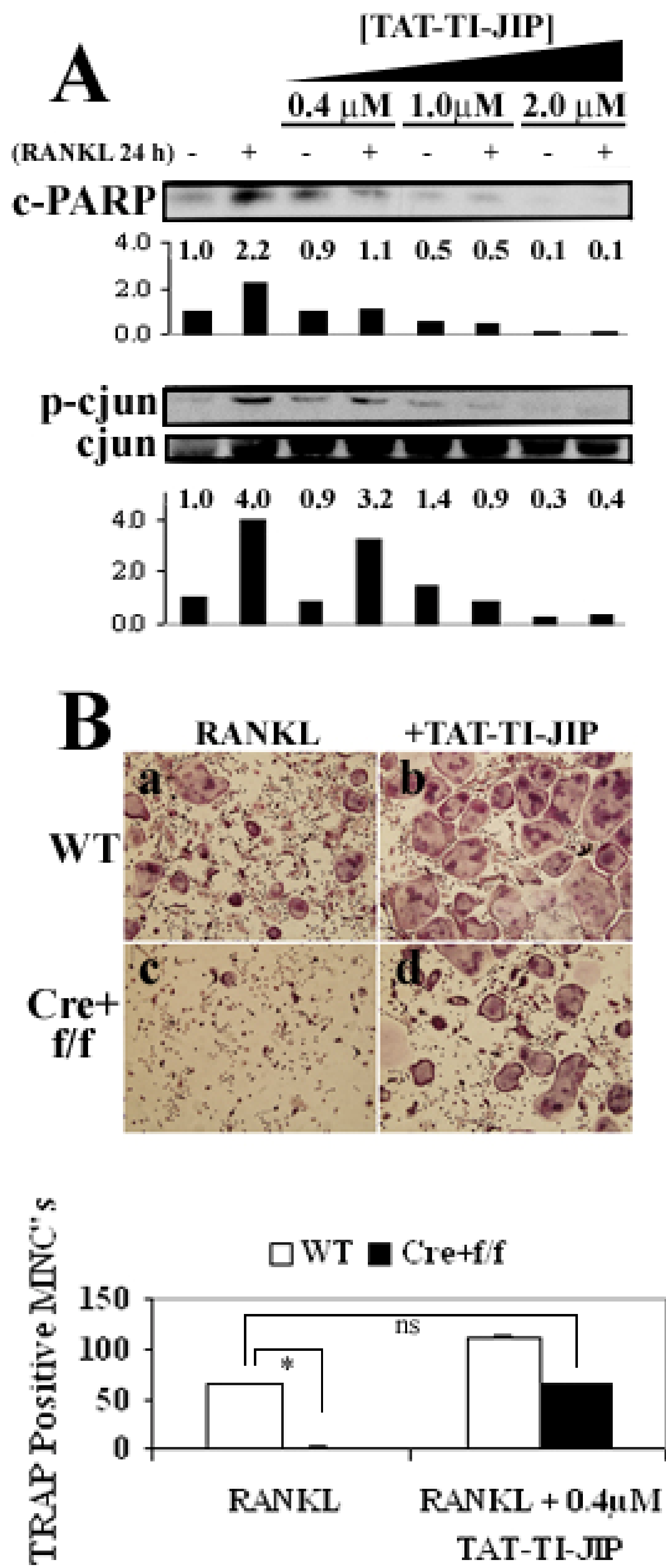
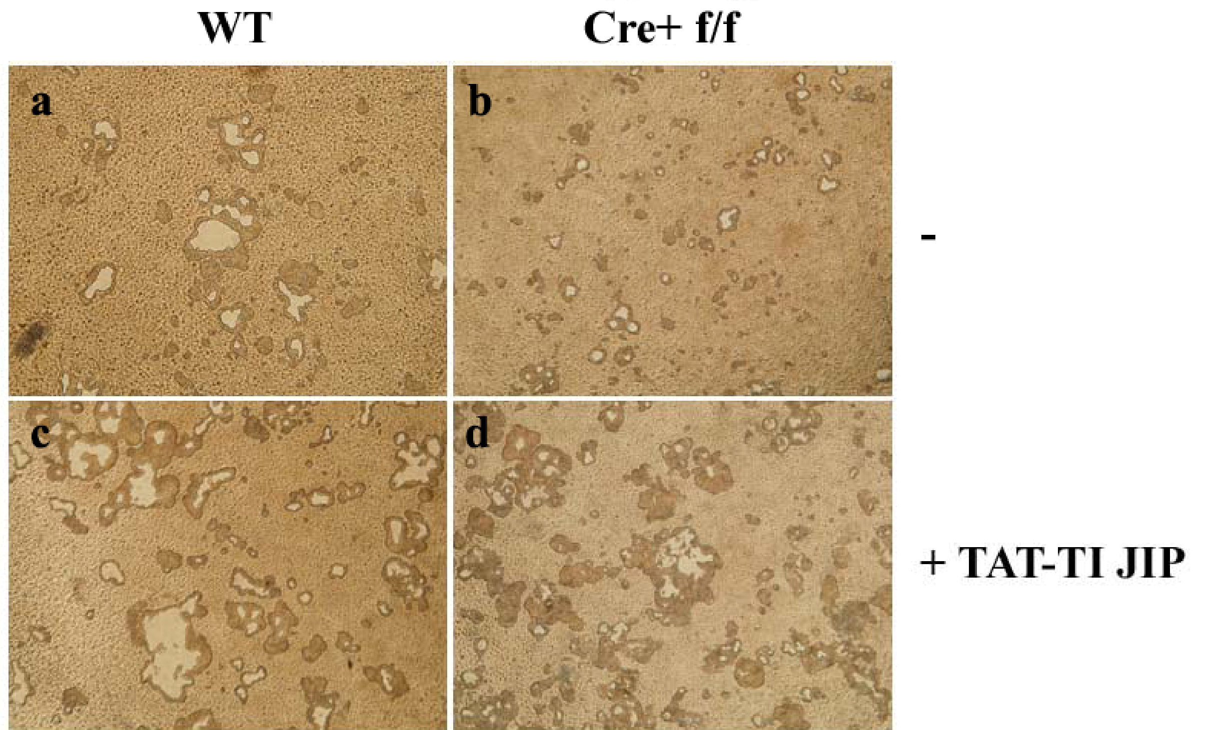
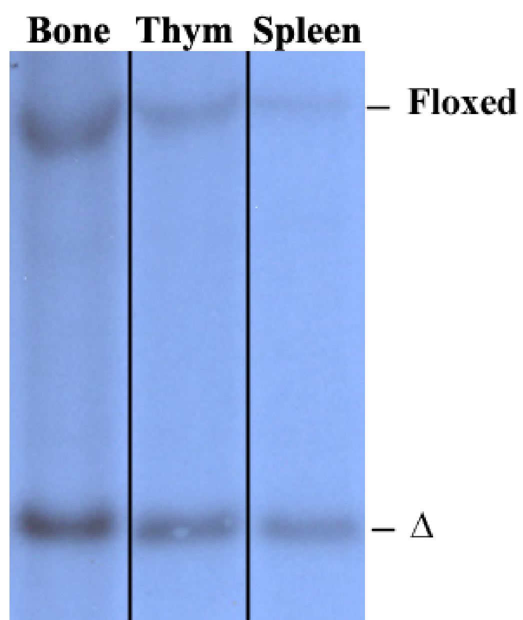


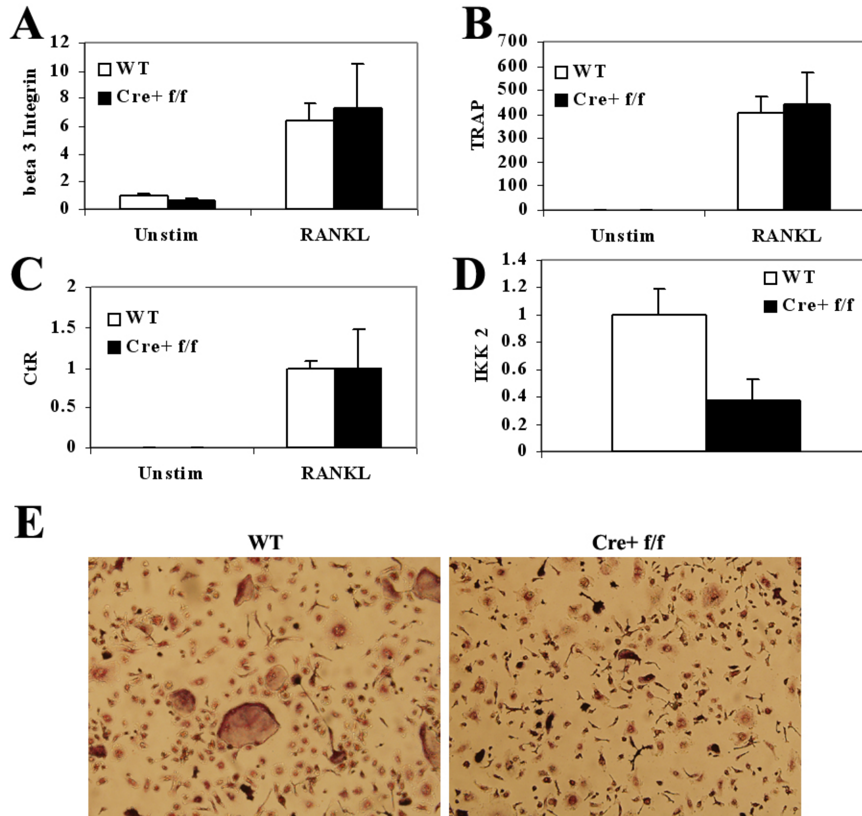
Figure 6



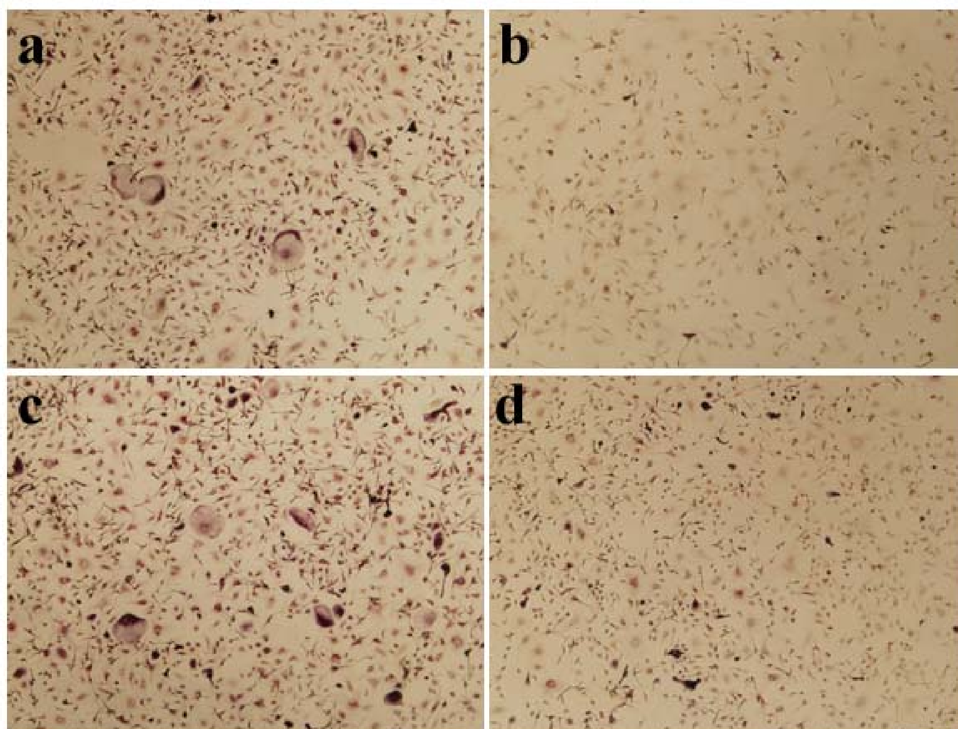
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



CHAPTER 3

IKKβ ACTIVATION IS SUFFICIENT FOR RANK-INDEPENDENT OSTEOCLAST DIFFERENTIATION AND OSTEOLYSIS

IKKβ Activation is Sufficient for RANK-Independent Osteoclast Differentiation and Osteolysis

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Running Title: RANK-independent osteoclastogenesis by IKKβ

SUMMARY:

Osteoclasts differentiate from monocytes through stimulation of Receptor-Activator of NF-κB (RANK). Many downstream effectors of RANK play a positive role in osteoclastogenesis, but their relative importance in osteoclast differentiation is unclear. We report the discovery that introduction of constitutively activated IKKβ ($IKKβ^{SSEE}$) into monocytes stimulates differentiation of *bona-fide* osteoclasts in the absence of RANK Ligand (RANKL). This phenomenon is independent of upstream signals, since $IKKβ^{SSEE}$ induced the development of bone-resorbing osteoclasts from RANK and IKKα-knockout monocytes and in conditions in which NEMO-IKKβ association was inhibited.

NF-κB p100 and p105 -but not RelB- were critical mediators of this effect. More importantly, adenoviral gene transfer of $IKK\beta^{SSEE}$ induced osteoclasts and osteolysis in calvariae and knees of mice. Our data establish the sufficiency of IKKβ activation for osteolysis and suggest that IKKβ gain-of-function may be a causative factor in conditions of pathological bone destruction refractory to RANK/RANKL proximal therapeutic interventions.

INTRODUCTION:

Healthy bone balance is dependent on the concerted activity of osteoblasts, bone forming cells, and osteoclasts, bone resorbing cells. In pathological conditions such as rheumatoid arthritis, osteoporosis, and osteolytic cancer metastasis, bone balance tips in favor of increased osteoclast activity, stemming from heightened osteoclast differentiation and activation[1]. These conditions result in significant bone pain and increased risk of fracture. Therefore, therapies which target the osteoclast are in the armament for treatment of these conditions[2]. On the other hand, gene mutations that disrupt osteoclast differentiation lead to development of osteopetrotic dense bones compromising bone homeostasis[3]. This class of bone diseases is incurable owing to osteoclast deficiency and lack of bone resorption. Undoubtedly, increasing understanding of the factors which regulate the osteoclast in health and disease will offer important insight into new effective therapies for bone loss associated with pathological conditions and for osteopetrosis.

The osteoclast is the sole bone resorbing cell, and it differentiates from a monocyte precursor through the concerted action of two cytokines, Ligand for the Receptor Activator for NF-κB (RANKL) and Macrophage Colony Stimulating Factor (M-CSF)[1]. Upon stimulation of their cognate receptors, RANK and c-Fms respectively, a series of signaling events induces activation of transcription factors such as NF-κB, AP-1, and NFATc1 which results in fusion of precursors and expression of genes required for osteoclast function, including β_3-Integrin, Cathepsin K, Tartrate Resistant Acid Phosphatase (TRAP), and Matrix Metalloproteinase 9 (MMP9)[4]. With expression of all necessary genes for osteoclast differentiation, the ability of the osteoclast to resorb bone requires tight regulation of the actin cytoskeleton. Indeed, genetic murine models have revealed a number of proteins such as c-Src[5], β_3-Integrin[6], ITAM containing adaptors[7,8], and the small GTPase Rac[9] whose activity is required for cytoskeletal regulation and bone resorption. These molecules contribute to formation of the actin ring which is the signature of a polarized osteoclast capable of resorbing bone[10].

The complement of proteins whose expression and activation are important for osteoclast differentiation and function make up a growing list, but the relative importance of these genes remains unclear given the varied phenotypes of osteoclasts or osteoclast precursors devoid of any of them. A well-studied family of transcription factors which is required for osteoclastogenesis is NF-κB. Recent findings have revealed that NF-κB p100 and p105 are both required together for osteoclast differentiation[11]. Additionally, p65/RelA[12] and RelB[13] have been shown to play complementary roles in osteoclast survival and differentiation, respectively. The complex interplay between NF-κB family

members in the context of osteoclast differentiation remains to be worked out, but it is clear that factors which activate NF-κB are logical targets for the treatment of osteoclast-mediated disease.

The IκB Kinase (IKK) Complex is responsible for NF-κB activation downstream of RANK. Upstream signals lead to association of two catalytically active kinases, IKKα and IKKβ with the non-catalytic member, IKKγ/NEMO. This association is required for activation of IKK through phosphorylation of two IKK activation loop serines, via an unidentified upstream kinase. IKK then phosphorylates IκB targeting it for proteasomal degradation allowing NF-κB to enter the nucleus and regulate gene transcription[14-16]. We and others have shown that inhibition of IKK activation through pharmacological inhibition of IKK association with NEMO abrogates osteoclastogenesis and inflammatory osteolysis[17,18]. Furthermore, mice devoid of IKKα[19] or IKKβ[20,21] demonstrate an impaired ability for osteoclast development *in vitro*. In a study comparing the relative contributions of the catalytic IKK members to osteoclast development *in vivo*, mice devoid of IKKβ displayed osteopetrosis and resistance to inflammatory bone erosion, while mice lacking active IKKα showed no obvious skeletal phenotype[20]. This finding implicates IKKβ as an important target for therapy in osteoclast-mediated disease.

We now report that IKKβ is not only necessary for RANKL-mediated osteoclastogenesis, but its activation is sufficient for RANK-independent osteoclast formation. Using retroviral delivery of constitutively active IKKβ ($IKK\beta^{SSEE}$), we reveal a signal for differentiation of functional osteoclasts that occurs downstream of RANK. $IKK\beta^{SSEE}$-but not wild-type IKKβ nor $IKK\alpha^{SSEE}$-induces osteoclast differentiation from

bone marrow, spleen, and fetal liver monocytes. These osteoclasts express all markers for normal osteoclasts, they form actin rings, and they resorb bone demonstrating that they are indeed authentic osteoclasts. Importantly, RANK is not a requirement since IKKβ^{SSEE} induces formation of bone resorbing osteoclasts from RANK-null spleen monocytes. Furthermore, we show that the classical IKK complex is not a requirement for active IKKβ to drive osteoclastogenesis, since IKKβ^{SSEE} induces formation of bone-resorbing osteoclasts from IKKα null fetal liver monocytes and when NEMO binding is inhibited. Finally, adenoviral gene transfer of IKKβ^{SSEE} in knees and calvariae of mice is sufficient for development of massive osteolysis. In summary, our findings demonstrate for the first time that a single activated kinase is sufficient for RANK-independent osteoclast differentiation and that active IKKβ alone leads to the development of osteolytic disease. These data highlight the centrality of IKKβ in osteoclast differentiation and implicate gain-of-function of IKKβ in pathological bone destruction.

RESULTS:

Constitutively Active IKKβ Induces RANKL-Independent Osteoclast Differentiation from Monocytes

Recently, we and others have demonstrated the necessity for IKKβ in osteoclast differentiation [20,21]. In an effort to identify mutations in IKKβ which could prevent or enhance its ability to rescue osteoclast differentiation in IKKβ knockout (KO) osteoclast precursors, we made the surprising observation that introduction of the constitutively activated form of IKKβ (IKKβ^{SSEE}), but not the wild-type (IKKβ^{WT}) form, into wild-type

or IKKβ KO bone marrow-derived macrophages induced the formation of TRAP-positive osteoclasts in the absence of RANKL administration (**Fig. 1A, and Supplementary Figures 1 and 2**). These cells were morphologically indistinguishable from RANKL-induced osteoclasts. Levels of IKKβ^{WT} and IKKβ^{SSEE} protein were comparable (**Fig. 1B**), whereas IKKβ^{SSEE} but not IKKβ^{WT} was recognized by an antibody specific for IKKβ phosphorylated at activation loop serines (**Fig. 1C**). These observations suggest that the kinase domain of IKKβ^{SSEE} exists in an active conformation and further assert that mutation of IKKβ activation loop serines 177 and 181 to glutamic acid, and not overexpression of IKKβ *per se*, is the factor responsible for the formation of osteoclasts in the absence of RANKL.

Further characterization showed that IKKβ^{SSEE}, but not GFP or IKKβ^{WT}, induced expression of RelB and c-fos, two transcription factors known to be critical for normal osteoclast differentiation[13,22]. IKKβ^{SSEE} but not IKKβ^{WT}, also induced the expression of β_3-Integrin and Cathepsin K, two markers for mature osteoclasts and genes whose products are required for osteoclast bone resorption[6, 23], (**Fig. 1C**). Quantitative real-time PCR analysis revealed that IKKβ^{SSEE} induced expression of mRNA for calcitonin receptor, cathepsin K, TRAP, and β_3-integrin, indicating that these cells possess the molecular signature for true osteoclasts (**Fig. 1D**). This notion was further supported by experiments showing that, similar to RANKL-induced cells, osteoclasts derived through introduction of IKKβ^{SSEE} into precursors form actin rings and are capable of resorbing artificial **(Supplementary Figure 3**) and authentic bone matrix (**Fig. 1E**). Expression of IKKβ^{SSEE} by RANKL-independent osteoclasts was demonstrated using IKKβ^{SSEE}-GFP

fusion construct (**Fig. 1F**). These data provide evidence that the TRAP+ multinucleated cells induced through expression of constitutively active IKKβ in macrophages are authentic osteoclasts. Furthermore, the fact that both bone marrow and spleen-derived macrophages were susceptible to osteoclast induction by $IKK\beta^{SSEE}$ argued against the theoretical possibility that $IKK\beta^{SSEE}$ may induce osteoclastogenesis in a paracrine fashion.

Given the implications of this finding in studying osteoclast signaling in various genetic models for osteoclast impairment which result in embryonic lethality, we sought to examine whether stimulation of osteoclast differentiation through introduction of $IKK\beta^{SSEE}$ was a phenomenon restricted to precursors obtained from adult tissue. To this end, $IKK\beta^{SSEE}$– infected but not GFP or $IKK\beta^{WT}$– infected fetal liver cells formed authentic osteoclasts with visible actin rings, a critical step in osteoclast polarization[10], which are capable of resorbing dentin slices (**Fig. 1E**). Actin rings and resorption pits were observed in $IKK\beta^{WT}$-infected cells only after RANKL administration (**Fig. 1E, Supplementary Figure 4**). These observations reveal that $IKK\beta^{SSEE}$ is sufficient to induce an authentic program for functional osteoclasts from adult and fetal precursor cells independent from RANKL. To verify the specificity of the osteoclastogenic effect of the phosphomimmetic mutation, we mutated IKKβ activation loop Serines to Alanine ($IKK\beta^{SSAA}$). This mutation resulted in an activation deficient molecule which failed to rescue basal and IL-1-induced osteoclastogenesis in IKKβ KO monocytes (**Fig. 1G and H**). This result indicates that phosphomimmetic mutation of IKKβ activation loop Serines is a specific inducer of the osteoclast program.

IKKβ^{SSEE} Rescues RANK Knockout Osteoclast Phenotype

Having established that RANKL is dispensable for IKKβ^{SSEE}-mediated osteoclastogenesis, we sought to determine whether intrinsic RANK signaling played a role in this phenomenon. To accomplish this, the RANKL decoy molecule osteoprotegerin (OPG-Fc) [10,24] and RANK-null cells were employed. OPG-Fc completely inhibited RANKL-induced osteoclastogenesis in IKKβ^{WT} -infected macrophages but had absolutely no effect on IKKβ^{SSEE}–induced osteoclast differentiation indicating that IKKβ^{SSEE} induces osteoclastogenesis without RANKL (**fig. 2A**). Furthermore, IKKβ^{SSEE}, but not IKKβ^{WT} nor GFP, induced the formation of osteoclasts from RANK knockout cells (**Fig. 2B**), excluding the possible necessity for an intrinsic effect of RANK. Importantly, RANK KO spleen-derived macrophages expressing GFP or IKKβ^{WT} failed to form osteoclasts in response to RANKL (**Fig. 2B**). IKKβ^{SSEE}- induced osteoclasts were also observed to form actin rings and resorb dentin (**Fig. 2C**). Consistent with this result, Western blot revealed that IKKβ^{SSEE} introduction into, but not RANKL treatment of, RANK KO cells resulted in expression of c-fos and RelB as well as c-src, β_3-Integrin, and Cathepsin K (**Fig. 2D**), indicating that IKKβ^{SSEE}-induced RANK-KO osteoclasts are indeed *bona fide* osteoclasts which appear molecularly identical to those derived from RANKL treatment of wild-type precursors. Furthermore, quantitative real-time PCR revealed that IKKβ^{SSEE} induces expression of mRNA for TRAP, Calcitonin Receptor, Cathepsin K, and β_3-Integrin in RANK KO cells (**Fig. 2E**). These data prove that IKKβ^{SSEE} functions independent of RANK to induce a program for differentiation of functional osteoclasts.

IKKβ^{SSEE} Acts Independently from the Classical IKK Complex to Drive Osteoclastogenesis

Activation of IKKβ by upstream signals requires its association, via two carboxyl-terminal tryptophans (W739 and W741), with the non-catalytic IKK member, NEMO[25, 26]. We and others have shown that inhibition of this association blocks RANKL-induced IKK activity and inhibits osteoclastogenesis and osteolysis[17,18]. Since IKKβ^{SSEE} induces osteoclastogenesis independent from RANK signaling, we tested if IKKβ^{SSEE} could also induce osteoclastogenesis in the absence of NEMO binding. We employed pharmacologic and molecular approaches to test this hypothesis. First, we determined that while administration of cell-permeable NBD peptides, which inhibit the association of IKKβ with NEMO, blocks RANKL-induced osteoclast differentiation, NBD did not inhibit osteoclastogenesis in response to transduction of IKKβ^{SSEE} (**Fig. 3A**). Second, while compound mutations of W739 and W741 to Alanine in the presence of the S177 and S181 to Glutamic Acid (IKKβ$^{SSEE/WA}$) prevent IKKβ^{SSEE} from binding to NEMO (**Fig. 3B,C**), IKKβ$^{SSEE/WA}$ is still capable of inducing RANKL-independent osteoclastogenesis from bone marrow macrophages to the same degree as IKKβ^{SSEE} (**figure 3D**). This quadruple IKK mutant is expressed properly and retains its kinase activity **(Fig 3E, F)**. Collectively, these results solidify the conclusion that IKKβ^{SSEE} induces RANKL-independent osteoclastogenesis without binding to NEMO, uncoupling the mechanism of IKKβ^{SSEE} – induced osteoclastogenesis from virtually all known upstream stimuli important for osteoclast differentiation and activation. Furthermore, this result suggests that in the setting of osteoclast differentiation, IKKβ binding to NEMO is

only important for IKKβ activation loop phosphorylation, after which point the association is not required for IKKβ to induce osteoclastogenesis.

Since NEMO association was not required for this phenomenon, we hypothesized that $IKK\beta^{SSEE}$ could induce osteoclastogenesis without formation of the classical IKK complex which includes IKKα, a kinase that has been shown required for osteoclastogenesis *in vitro*[19]. First, we confirmed that IKKα-null fetal liver-derived macrophages (FLC) transduced with GFP do not differentiate into osteoclasts (**Fig. 4A**) and fail to express mRNA for the osteoclast markers TRAP, Cathepsin K, and calcitonin receptor in response to RANKL stimulation while induction of these markers is normal in littermate control cells treated with RANKL (**Fig. 4C**). However, transduction of IKKα knockout FLCs with $IKK\beta^{SSEE}$ restores osteoclastogenesis even in the absence of RANKL administration, rescues the ability of these cells to form normal actin rings and to resorb bone (**Fig. 4A**), and induces expression of typical signaling proteins (**Fig. 4B**) and expression of mRNA for Cathepsin K, TRAP, and Calcitonin Receptor (**Fig. 4C**). These data indicate that formation of the classical IKK complex and the IKKα-mediated non-canonical NF-κB signaling pathway are not a requirement for $IKK\beta^{SSEE}$ to stimulate RANK-independent osteoclastogenesis.

Requirement for Coordinated NF-κB Activation in $IKK\beta^{SSEE}$–Induced Osteoclastogenesis

In an effort to identify the mechanism underlying $IKK\beta^{SSEE}$-induced osteoclastogenesis, we examined the status of essential NF-κB subunits compared with RANKL-treated

conditions. We observed elevated total levels of RelB in the cytosol of IKKβ^{SSEE} –expressing cells at all time points assessed, including non-stimulated, compared with GFP and IKKβ^{WT} expressing cells. RelB translocation into the nucleus was only slightly stimulated by RANKL in the time course of one hour. We also observed reduced levels of IκBα which coincided with a dramatically increased level of RelA protein in the nucleus in the absence of RANKL stimulation and at all time points tested in IKKβ^{SSEE} compared with GFP and IKKβ^{WT} expressing cells, indicating that the constitutively activated form of IKKβ induces continuous IκBα processing (**Fig. 5A**). These data suggest that IKKβ^{SSEE} may act through an NF-κB-dependent mechanism to induce RANK-independent osteoclast differentiation. To test this hypothesis, we began by challenging RelB knockout cells with IKKβ^{SSEE}, since we repeatedly observed induction of RelB protein expression in response to IKKβ^{SSEE} in macrophages and since a positive role for RelB in osteoclast differentiation was recently described[13]. To our surprise, RelB knockout bone marrow-derived macrophages were capable of differentiating into TRAP+ osteoclasts which express Cathepsin K in the absence of RANKL-administration when expressing IKKβ^{SSEE} but not IKKβ^{WT} (**Fig. 5B,C**). Real-time PCR analysis also revealed that while induction of expression of mRNA for Calcitonin Receptor and TRAP in response to RANKL administration was impaired in RelB knockout cells, IKKβ^{SSEE} rescued the induction to levels equivalent to that seen in WT cells expressing IKKβ^{SSEE} (**Fig. 5D**). These data indicate that IKKβ^{SSEE} does not require RelB to induce osteoclast differentiation.

Given the observation that IKKβ^{SSEE} induces nuclear translocation of RelA and induces increased expression of RelB, we further explored the possibility that these NF-κB subunits may act as mediators of the IKKβ^{SSEE} effect. However, overexpression of RelA, RelB, or a combination of RelA and RelB (**Fig. 5E**) did not induce osteoclast differentiation of bone marrow-derived macrophages. To verify activity of the RelA and RelB in this setting, we observed that RelA induced expression of IκBα and that RelA and RelB as well as the combination of RelA and RelB induced expression of p100. (**Fig. 5F**).Collectively, these results indicate that IKKβ^{SSEE} is a specific activator of NF-κB capable of inducing osteoclast differentiation and simple ectopic overexpression of RelA plus RelB is insufficient to coordinate this effect.

Phosphorylation of T-loop residues is a hallmark of activation for many kinases[27]. Given the specificity of IKKβ T-loop activation as a mediator of osteoclast differentiation, we asked whether constitutive activation of other kinases through phosphomimetic mutations could induce osteoclast differentiation or whether this effect is specific to IKKβ. IKKα and IKKβ share significant primary and secondary structural homology[15], so we reasoned that, in contrast to other less-related kinases, constitutive activation of IKKα through phosphomimetic mutation (S176/180E) would be most likely to induce an osteoclast program like IKKβ^{SSEE}. We found that when expressed at comparable levels (**Fig. 5E**), IKKβ^{SSEE} induces osteoclast differentiation from bone marrow macrophages, whereas IKKα^{SSEE} had no such effect (**Fig. 5F**), demonstrating that IKKβ is the specific kinase activator of the osteoclast program.

It has been established that a combination of both NF-κB1/p50 and NF-κB2/p52 subunits are absolutely required for osteoclast differentiation[11]. We wished to determine whether IKKβ^{SSEE}-induced RANK-independent osteoclastogenesis also requires NF-κB1 and 2. To accomplish this, we transduced control and *NF-κB1-/- / NF-κB2-/-* (NF-κB dKO) spleen macrophages with GFP, IKKβ^{WT}, and IKKβ^{SSEE} (**Fig. 5G**) and performed TRAP staining for osteoclasts in the absence of RANKL administration. While control cells expressing IKKβ^{SSEE} produced a significant number of osteoclasts capable of resorbing bone coinciding with expression of mRNA for Cathepsin K, no osteoclasts were observed in NF-κB dKO cells (**Fig. 5H,I**) despite constitutive IκBα processing (**Supplementary Figure 5**). These results indicate that IKKβ^{SSEE}-mediated induction of osteoclastogenesis requires NF-κB-mediated gene regulation.

Constitutively Active IKKβ is Sufficient for the Establishment of *in vivo* Osteolysis

To determine the relevance of active IKKβ to osteoclastogenesis and osteolysis *in vivo*, we performed gene transfer experiments in mice. We injected mice with adenovirus expressing IKKβ^{SSEE} or lacZ supracalvarially or intra-articularly into the knee joint (**Supplementary Figure 6**). While lacZ did not induce an osteoclast response in either calvariae or knees, IKKβ^{SSEE} stimulated a massive local osteolytic response in both settings characterized by bone destruction and the appearance of osteoclasts at sites of bone erosion (**Fig. 6A,B**). To support the role of kinase activity of IKKβ in mediating this effect, joints injected with adenoviral IKKβ^{SSEE} showed intense immunostaining for phosphorylated IκBα at sites of synovial inflammation and osteoclastic articular bone

erosion, while LacZ-infected knees stained negatively for articular osteoclasts and phosphorylated IκBα (**Fig. 6B**).

DISCUSSION:

We provide novel evidence that osteoclast differentiation from monocyte progenitors can be triggered by an autonomous intracellular signal downstream, yet independent of the RANK receptor. The IKK complex has been implicated in RANKL-induced osteoclast differentiation[17-21], but the sufficiency of this single enzyme to independently induce osteoclastogenesis is surprising. The fact that constitutively activated IKKβ induces osteoclast differentiation raises several questions. First and foremost, phosphorylation of IKKβ is not unique to the RANKL/RANK signaling complex, so why do other receptor signaling complexes which activate IKKβ fail to induce osteoclast differentiation? The answer to this question, most likely, lies in the tuning of IKK-mediated NF-κB activation by RANK. It has recently been demonstrated that at the level of promoter binding and gene activation, the timing of NF-κB oscillations in concert with AP-1 determines transcriptional output[28]. These oscillations are differentially regulated by TNF and RANKL, despite their activation by both cytokines, resulting in transcription at different promoters. Our data predicts that NF-κB activation through IKKβ in monocyte progenitors is more sustained and prolonged by RANKL than other cytokines and that prolonged activation of IKK/NF-κB results in transcription at osteoclast-specific gene promoters. Second, in light of its osteoclastogenic function, it is interesting to consider how closely IKKβ^{SSEE} mimics the molecule when activated

through NEMO association-dependent phosphorylation. It is possible that $IKK\beta^{SSEE}$ takes on functions not performed by IKKβ in normal settings. In support of this speculation, we observe that infection of monocytes with $IKK\beta^{SSEE}$ results in p100 processing to p52, which is usually considered to be a function of IKKα[29]. Perhaps atypical functions like this contribute to its osteoclastogenic activity. Nevertheless, the ability of $IKK\beta^{SSEE}$ to induce the osteoclast is entirely dependent on kinase activity, since mutation of the ATP-binding lysine to methionine in the kinase domain completely abrogated the $IKK\beta^{SSEE}$ osteoclastogenic function (**Supplementary Figure 7**). Another explanation of the high specificity with which active $IKK\beta^{SSEE}$ prompts osteoclastogenesis in monocytes despite the fact that this kinase can be activated by numerous stimuli in other systems devoid of osteoclasts, can be attributed to the fact that upstream ligands prompt complex stimulatory and inhibitory circuits to modulate their responses. These complex circuits emanating from ligand/receptor interactions are absent from the $IKK\beta^{SSEE}$ response, suggesting that the immediate IKK response is induction of osteoclastogenesis whereas upstream receptor-based regulatory signals induced by various stimuli, such as TNF, lipopolysaccharide, IL-1, etc, provide networks that counteract the $IKK\beta^{SSEE}$ osteoclastogenic effect.

In addition to IKKβ, several kinases have been demonstrated to play a role in osteoclast differentiation including ERK and p38 MAP Kinases[30], PI3K[31], Akt[32], c-Src[33], PKC[34], and JNK[35]. Many of these kinases have been demonstrated to be essential to osteoclastogenesis, but it has been difficult to sort out their individual contributions to differentiation for two main reasons. First, survival defects mask the effect of null

mutations on differentiation. Second, no kinase has ever been studied for its sufficiency to activate the osteoclast program. Our discovery that constitutively activated IKKβ stimulates osteoclast differentiation led us to question the uniqueness of IKKβ in accomplishing this function. To address this, we mutated IKKα to create a constitutively active mutant with significant structural and sequence similarity to $IKK\beta^{SSEE}$. This molecule was insufficient for osteoclastogenesis, which led us to conclude that IKKβ is uniquely situated to stimulate the osteoclast program. In this regard, it is interesting that $IKK\beta^{SSEE}$ did not require IKKα or NEMO, its members in the traditional IKK complex [15]. This observation strongly suggests that the function of IKKα and NEMO in the setting of osteoclast differentiation is to facilitate activation of IKKβ.

It is important to consider the extent to which $IKK\beta^{SSEE}$-induced osteoclasts resemble those induced by RANKL. With regard to their ability to resorb bone, they are indeed *bone fide* osteoclasts. This is supported by the fact that the same transcription factors which are important for RANKL-induced osteoclast differentiation are also upregulated by $IKK\beta^{SSEE}$, including RelB, c-Fos, and nuclear p65/RelA. Furthermore, our analysis revealed that $IKK\beta^{SSEE}$ potently induced expression of markers for RANKL-induced osteoclasts, TRAP, Cathepsin K, Calcitonin Receptor, and β_3-Integrin. In addition to these, preliminary mRNA expression array revealed significant upregulation of DC-STAMP, OSCAR, as well as MMP9, further validating the $IKK\beta^{SSEE}$-induced osteoclast phenotype.

Differentiation of the osteoclast requires NF-κB[11]. To determine whether the phenotype we observed similarly requires NF-κB we tested the ability of $IKK\beta^{SSEE}$ to

drive osteoclastogenesis in *NF-κB1/2* double knockout spleen monocytes, in which it failed. In addition to NF-κB, other transcription factors may play a role in the IKKβ^{SSEE} effect. c-Fos has been implicated downstream of NF-κB for osteoclastogenesis, and in the *NF-κB1/2* double knockout, failure of c-Fos upregulation in response to RANKL has been shown to lead to impaired differentiation[36]. We saw no defect in c-Fos upregulation by IKKβ^{SSEE} in the *NF-κB1/2*-null setting meaning that c-Fos induction is not sufficient for osteoclastogenesis (Unpublished data, J.O. and Y.A.). Therefore, NF-κB must serve functions other than c-Fos upregulation which are necessary for osteoclast differentiation. Identification of these targets will lead to a better understanding of NF-κB in the osteoclast.

Another puzzling question prompted by our data regards the mechanism through which IKKβ^{SSEE} induces formation of the Actin ring. It is known that complicated signaling at the membrane of osteoclasts associated with integrin stimulation ultimately results in formation of the actin ring which allows formation of the sealing zone and bone resorption[7,37]. Our data suggests that the initial requirements for actin ring formation are transcriptional and are downstream of IKK, since RANK knockout cells can be induced to form actin rings and resorb bone when expressing IKKβ^{SSEE}. In this setting, integrin signaling at the bone-osteoclast interface most likely leads to actin organization. Our discovery provides a direct approach to identify the essential components for establishment of the actin ring.

NF-κB is a critical regulator of the inflammatory cellular program[38], and inflammatory cytokines have been linked to enhanced osteoclast function[39,40]. Since

TNF-α has been shown to induce osteoclast differentiation in certain settings[41], we sought to determine whether this inflammatory factor was required for IKKβ^{SSEE} to induce osteoclast differentiation. Using IKKβ^{SSEE}-transduced TNF-α knockout bone marrow monocytes, we found that TNF-α is not required for IKKβ^{SSEE} to accomplish its effect in osteoclast differentiation (Unpublished data, J.O. and Y.A.). Therefore, the mechanism we observe for IKKβ^{SSEE}- induced osteoclastogenesis is uncoupled from inflammatory signaling with respect to TNF. However, IKKβ^{SSEE} is capable of inducing secretion of TNF by wild-type cells suggesting that this kinase may modulate osteoclastogenesis as well as inflammatory osteolysis at varying levels.

Consistent with our *in vitro* findings, adenoviral gene transfer experiments revealed that IKKβ^{SSEE} is also sufficient for the establishment of osteolysis *in vivo*. The clinical significance of our findings is highlighted by our observations that IKKβ^{SSEE}-induced osteoclastogenesis is refractory to intervention with OPG and deletion of RANK/RANKL. In this regard, a number of conditions in human patients are associated with heightened bone turnover in the setting of inflammation for which a cause has not been identified[42]. Given the potency with which activated IKKβ induces osteoclast appearance and bone destruction in this model, it is important to consider IKKβ gain-of-function as an independent cause and a target in therapy for these and all conditions of inflammatory bone destruction. Further, given the potency at which IKKβ^{SSEE} induces bone resorption, this molecule positions itself as a strong therapeutic strategy for osteopetrotic diseases.

In summary, our data highlight the critical role of IKKβ in osteoclast differentiation and osteolysis. We have found that constitutively active IKKβ unfolds the osteoclast program in the absence of upstream signals. This result raises the possibility that other kinase effectors of RANK in osteoclast differentiation may be involved in IKKβ activation. We report the first evidence of RANK-independent osteoclast differentiation that is induced through a single kinase, and we propose that gain-of-function in human IKKβ may lead to a subset of genetic diseases resulting in bone destruction which would be refractory to treatments targeting proximal RANK and TNF signaling molecules.

METHODS:

Reagents. Antibodies against IKKβ, IKKα, IκBα, NEMO, Actin, RelA, RelB, c-Fos, c-Src, β_3-Intergin, Histone H1, and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho- IKKβ, p100, and p105 were from Cell Signaling Technologies, Inc. (Danvers, MA). Antibodies against Flag epitope were from Sigma (St. Louis, MO). Antibody against V5 epitope was from Novus (Littleton, CO). Antibody against Cathepsin K was from Millipore/Chemicon (Temecula, CA). HRP-conjugated secondary antibody for immunohistochemistry was from Vector Laboratories (Burlingame, CA) Cytokines were purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Animals and Cells. All mice were housed in a controlled barrier facility at Washington University (St. Louis, MO). TRAP Cre mice [43] were from Dr. Roodman (University of Pittsburgh, PA). Floxed IKKβ [44] mice were from Dr. Pasparakis (University of Cologne, Germany). TRAP Cre floxed/floxed IKKβ mice were generated by crossing TRAP Cre transgenic mice with floxed IKKβ mice. IKKα heterozygous mice [19] were obtained from Dr. Akira (Osaka University, Japan). RelB KO [45] and control bone marrow was from Dr. Novack (Washington University, St. Louis, MO). RANK KO [46] and control spleens as well as NF-κB double KO [36] and control spleens were provided by Dr. Xing (University of Rochester Medical Center, N.Y.) For *in vivo* experiments, wild-type C57BL/6 mice at 5-6 weeks of age were used.

Plasmids. pMxs retroviral expression plasmid was from Dr. T. Kitamura (University of Tokyo, Japan). Mouse cDNA for IKKα was kindly provided by Dr. Kenneth Marcu (Stony Brook, NY). IKKβ and RelB cDNA were purchased from ATCC. RelA cDNA was provided by Dr. C. Sasaki (NIA, Baltimore, MD). All expression constructs were subcloned into pMxs using standard techniques. The following mutations were generated using the QuickChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer pairs in parentheses: IKKβ^{SSEE} (IKKβ_S177_181E_f, GAGCTGGATCAGGGCGAACTGTGCACGGAATTTGTGGGGACTCTGC, and IKKβ_S177_181E_r, GCAGAGTCCCCACAAATTCCGTGCACAGTTCGCCCTGATCCAGCTC). Note that the constitutive activating effect of this mutation of IKKβ has been established

previously[47,48]. IKKβ^{SSAA} (IKKβ_S177_181A_f, GAGCTGGATCAGGGCGCTCTGTGCACGGCATTTGTGGGGACTCTGC, and IKKβ_S177_181A_r, GCAGAGTCCCCACAAATGCCGTGCACAGAGCGCCCTGATCCAGCTC); IKKβ^{WWAA} (IKKβ_W739_741A_f, GACTCTAGACGCGAGCGCGTTACAGATGGAGGATG, and IKKβ_W739_741A_r, CATCCTCCATCTGTAACGCGCTCGCGTCTAGAGTC); IKKβ^{KM} (IKKβ_K44M_f, GTGAACAGATCGCCATCATGCAATGCCGACAGGAGC, and IKKβ_K44M_r, GCTCCTGTCGGCATTGCATGATGGCGATCTGTTCAC); IKKα^{SSEE} (IKKα_S176_180E_f, GATGTTGATCAAGGAGAGCTCTGTACAGAATTTGTGGGAACATTGC, and IKKα-S176_180E_r, GCAATGTTCCCACAAATTCTGTACAGAGCTCTCCTTGATCAACATC).

Generation of Bone Marrow, Spleen, and Fetal Liver-Derived Monocyte/Macrophages. Whole marrow was flushed from long bones into α- Minimum Essential Medium (MEM). Spleens and d18.5 fetal livers were crushed into cell suspensions in α-MEM and were centrifuged at 453 rcf. Cell pellets were resuspended in whole media (α-MEM with 1X penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS)). Monocytes/ macrophages were produced by growing cell suspensions in the presence of 10 ng/ml M-CSF. Monocytes/ macrophages were allowed to proliferate

for 3 days at 37 ^{o}C in 5% CO_2 at which point they were infected with retrovirus. See below.

Generation and Use of Retrovirus. The use of Plat-E retrovirus packaging cells stably expressing retroviral structural proteins gag-pol and env for transient production of high titer retrovirus was described previously[49]. Briefly, 8µg pMx vectors expressing our gene of interest were transfected into 5 million plat-E cells (grown in DMEM supplemented with 10% FBS, 10ng/ml M-CSF, and penicillin/streptomycin) using Fugene 6 (Roche, Palo Alto, CA) according to manufacturer's instructions. Twenty-four hrs post transfection, media was exchanged to remove transfection reagent. Twenty-four and 48 hrs post media exchange, supernatant was collected and pooled. In parallel, monocyte/macrophage cultures from bone marrow, spleen, or fetal liver were developed on petri dishes, washed with PBS, and infected with retrovirus in infection mix (50% virus supernatant, 50% αMEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 4µg/ml hexadimethrine bromide). Twenty-four hrs post infection, cells were selected in αMEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 2µg/ml puromycin for 72 hrs, at which point selection media was removed, cells were washed, and grown for 24 additional hrs without puromycin. At this point, cells were lifted, counted, and plated for downstream experiments. Of note, $IKK\beta^{SSEE}$-expressing cells fused, spread, and assumed osteoclast morphology within 30 minutes of plating on a tissue culture-treated surface, indicating that gene expression which occurred during viral transduction and selection contributed to osteoclast differentiation. A representative image depicting this phenomenon 30 min. post-plating is depicted in supplementary figure 2.

***In vitro* Osteoclastogenesis.** IKKβ^{SSEE} expressing cells were cultured in 10ng/ml M-CSF, while GFP and IKKβ^{WT}-expressing cells were cultured in 10ng/ml M-CSF plus 100ng/ml RANKL for 4 days. IKKβ^{SSEE} induced spontaneous osteoclastogenesis in the absence of RANKL, while RANKL was required for osteoclasts to form from monocyte/macrophages expressing GFP or IKKβ^{WT}. At this point, cells were fixed and TRAP stained using the Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO). TRAP-positive cells with three or more nuclei were scored as osteoclasts.

Inhibitor Studies. For inhibition of osteoclastogenesis, cells were treated with 100ng/ml OPG/Fc Chimera (R&D Systems, Minneapolis, MN), 25µM TAT-NBD (YGRKKRRQRRR-G-TTLDWSWLQME) or 25µM TAT-mutant NBD (YGRKKRRQRRR-G-TTLD<u>A</u>S<u>A</u>LQME) during the entire course of retroviral transduction and *in vitro* osteoclast differentiation. The efficacy of TAT-NBD in inhibition of RANKL-induced osteoclastogenesis has been established previously [17].

RNA Isolation and cDNA Production. RNA was isolated from macrophage or osteoclast cultures using the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. Reverse transcription was performed as follows: 1.0 µg RNA and 1.0 µg random hexamer primer in 10 µl nuclease-free deionized H_2O in PCR tubes were heated to 70 ^{o}C for 5 minutes, cooled to 42 ^{o}C, and set on ice. The following components were then added at the indicated amounts or concentrations for a total reaction volume of 20 µl: 1x AMV RT buffer (Roche, Palo

Alto, CA), 40U RNAseIn (Promega, San Luis Obispo, CA), 1.25 mM dNTPs, 5 mM Sodium Pyruvate, 5 U Reverse Transcriptase Enzyme, AMV (Roche). To produce cDNA, tubes were placed in a thermocycler programmed as follows: 42 ^{o}C for 60 minutes, 50 ^{o}C for 10 minutes, and 95 ^{o}C for 5 minutes.

Quantitative Real-Time PCR. Triplicate samples of 4 µl cDNA product (5X diluted), 10 µl Sybr Green PCR Master Mix (Applied Biosystems, Inc. Foster City, CA), 0.1 µl each of 10 µM forward and reverse primer stocks, and 6 µL nuclease-free deionized H_2O were subjected to real-time PCR according to the following program in an ABI 7300 Real Time PCR System: 50 ^{o}C for 2 minutes, 95 ^{o}C for 10 minutes, (95 ^{o}C for 15 seconds, 60 ^{o}C for 1 minute) x 40 cycles. Results were analyzed using AB RQ Study Software. Real Time PCR primers were designed using Primer Express Software (Applied Biosystems, Inc.) mouse RANK Forward 5’-CTGCCTCCTGGGCTTCTTCT-3’, mouse RANK Reverse 5’-CCCCTGGTGTGCTTCTAGCT-3’, mouse TRAP Forward 5’-CGACCATTGTTAGCCACATACG-3’, mouse TRAP Reverse 5’-CACATAGCCCACACCGTTCTC-3’, mouse Calcitonin Receptor Forward 5’-CAAGAACCTTAGCTGCCAGAG-3’, mouse Calcitonin Receptor Reverse 5’-CAAGCACGCGGACAATGTTG-3’, mouse Cathepsin K Forward 5’-GGAAGAAGACTCACCAGAAGC-3’, mouse Cathepsin K Reverse 5’-GTCATATAGCCGCCTCCACAG-3’, mouse β_3 Integrin Forward 5’-TTACCCCGTGGACATCTACTA-3’, mouse β_3 Integrin Reverse 5’-AGTCTTCCATCCAGGGCAATA-3’, mouse GAPDH Forward 5’-

CTTCACCACCATGGAGAAGGC-3’, mouse GAPDH Reverse 5’-GACGGACACATTGGGGGTAG-3’.

Western Blotting. Equivalent amounts of total cell protein were boiled in the presence of an equal volume of 2X SDS sample buffer consisting of (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, 3% β-Mercaptoethanol (v/v), and distilled water) for 5 min. and subjected to electrophoresis on 8–10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad, Hercules, CA) 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 PBS/Tween buffer and exposed to primary antibodies (16 h at 4 ^{o}C), washed (4 x 15 min.), and incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (1 h at room temperature). The membranes were washed extensively (4 X 15 min.), and an ECL detection assay was performed following the manufacturer’s instructions.

Co-Immunoprecipitation. Cells expressing GFP, flag IKKβ^{WT}, flag IKKβ^{WA}, flag IKKβ^{SSEE}, or flag IKKβ$^{SSEE/WA}$ were lysed in immunoprecipitation (IP) buffer (10mM Tris pH 7.4, 150mM NaCl, 0.5% NP-40 (IGEPAL) 1mM EDTA, 1mM NaF, 1mM PMSF, 1mM Na_3VO_4, and 1X protease inhibitor cocktail) at 4°C. Protein was measured by BCA Assay (Pierce, Rockford, IL) and normalized. Non-specific binding was removed by rocking total cell lysate at 4°C with GammaBind G Sepharose beads (GE

Lifesciences) and 100ng normal mouse IgG for 2 hrs at 4°C. Beads and normal antibody were centrifuged and supernatant was incubated with GammaBind G Sepharose beads and 1 μg/ml mouse anti-Flag M2 antibody (Sigma, St. Louis, MO) at 4°C for 16 hrs. Immune complexes were centrifuged with beads. Supernatant was removed by vacuum suction, and 2X sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, 3% β-Mercaptoethanol, and distilled water) was added to beads which were boiled for 5 minutes to elute the complex components.

***In vitro* Kinase Assay.** Plat E cells expressing indicated flag-tagged IKKβ constructs were lysed in IP buffer. IKKβ was immunoprecipitated with M2 antibody, washed twice with IP buffer, once with kinase assay buffer (Cell Signaling Technologies Danvers, MA), and incubated for 30 minutes at 30°C in 30 μl kinase assay buffer with 1μg GST-IκBα, 2.5mM $MgCl_2$, and 16μM ATP. Reaction was terminated with 30 μl reducing sample buffer. Samples were analyzed by Western blot.

Bone Resorption Assays. Osteoclasts were cultured on 5mm^2 100μm thick dentin slices for 5 days in a 48-well tissue culture plate. To visualize resorption pits and tracks, slices were exposed to 0.5N NaOH, and cells were removed by mechanical agitation. Slices were washed in PBS three times and stained with 0.1% toluidine blue (w/v) in PBS for 10 minutes. Stained slices were rinsed with PBS and blotted dry, and pits were visualized by light microscopy. For assessment of resorption of artificial bone matrix, macrophages or osteoclasts were cultured on BD Biocoat Osteologic tissue culture slides (BD

Biosciences, San Jose, CA) for 4 days, and resorption areas were determined as clear areas in the osteologic matrix as visualized by light microscopy.

Actin Ring Staining. Macrophages expressing IKKβ^{SSEE} cultured on dentin in M-CSF (IKKβ^{SSEE}-induced osteoclasts) or expressing GFP or IKKβ^{WT} cultured on dentin in M-CSF or M-CSF + RANKL were fixed in 4% paraformaldehyde in PBS for 5 minutes at room temperature. Fixed macrophages or osteoclasts on dentin slices were washed with PBS three times and permeabilized in 0.2% Triton X 100 in PBS for 10 minutes at room temperature. Dentin slices were washed three times with PBS and then incubated in a 1:40 dilution of Alexa Fluor-488 phalloidin (Invitrogen Molecular Probes, Eugene, OR) for 10 minutes in a dark humidified chamber at room temperature. Slices were washed with PBS and mounted onto microscope slides for visualization of actin rings with fluorescent microscopy.

Generation and Use of Adenovirus. Adenovirus expressing IKKβ^{SSEE} was generated by subcloning from the pMx parental vector into Ad5 CMV K-NpA Shuttle using EcoR1 and Not1 restriction endonucleases (New England Biolabs, Ipswich, MA). Recombination[50], production and characterization (pfu/particle) of virus was provided by Viraquest, Inc. (North Liberty, IA). For local *in vivo* gene transfer in mice, 1 x 10^7 pfu of virus diluted in 10µl sterile PBS were injected intra-articularly into the knee joint capsule. Contralateral knees on the same mouse served as experimental (Ad IKKβ^{SSEE}) and control (AdntLacZ). For calvarial osteolysis, 1 x 10^7 pfu of virus diluted in 50µl

sterile PBS were injected supracalvarially. Seven days post-injection, knees and calvariae were fixed, decalcified, and analyzed histologically for osteoclasts and osteolysis. In calvarial injection experiment, RANKL and LPS were used as positive controls for osteolysis. 1µg mouse RANKL diluted in 50µl sterile PBS or 20µg LPS (055:B5 Sigma, St. Louis, MO) diluted in 50µl sterile PBS were injected over calvaria. LPS and RANKL were injected every-other-day.

X-Gal Staining. Bones were incubated at 37° C for 15 minutes in staining buffer consisting of 2mM $MgCl_2$, 5mM $K_3FE(CN)_6$, 5mM $K_4FE(CN)_6$, and 1mg/ml X-Gal in PBS, pH 7.4.

Histology. Long bones and calvariae were collected from mice and fixed in 10% buffered formalin for 24 hours. Bones were then decalcified for 7 days in decalcification buffer consisting of (14% (w/v) EDTA, H_4NOH 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 H&E to assess tissue architecture. Immunohistochemistry was performed according to antibody manufacturer's instructions for immunoperoxidase staining.

Microscopy. Cells and histological sections were imaged under white or ultraviolet light on an inverted microscope (Olympus IX-51). For f-actin visualization, ultraviolet light

was passed through a FITC filter cube to localize green phalloidin. Digital images were captured using a CCD camera (Olympus DP70, 12 mega-pixel resolution).

Author contributions

J.E.O., S.D., Y.A.A, planned and designed experiments. M.A.A. maintained animal colony. J.E.O and S.D. designed DNA constructs. J.E.O. performed experiments. I.D. assisted with tissue culture experiments. J.E.O. and Y.A.A. analyzed, interpreted data, and wrote manuscript.

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FIGURE LEGENDS

Figure 1. IKKβ^{SSEE} Induces *bona fide* Osteoclasts from Bone Marrow, Spleen, and Fetal Liver Progenitors

(A) Bone marrow macrophages from IKKβ-KO and littermate mice were infected with retroviruses expressing GFP, IKKβ^{WT} or IKKβ^{SSEE} and cultured with M-CSF alone or M-CSF+RANKL for 4 days. Cells were fixed and TRAP stained to determine osteoclastogenesis. Arrows denote osteoclasts. Lower panel, quantification; flx, loxP flanked.

(B) Expression levels of IKKβ^{WT} and IKKβ^{SSEE} in control and IKKβ KO macrophages were measured by Western blot. Expression of endogenous NEMO is shown as control.

(C) Western blot for expression of NF-κB molecules and osteoclast markers in total cell lysates of spleen cells infected with indicated viruses. Actin expression indicates equal loading. OC+, osteoclast positive control total cell lysate. (B and C) pMx, retroviral expression vector.

(D) Relative expression of mRNA for osteoclast markers assessed by quantitative real-time PCR. GAPDH served as internal standard for cDNA normalization. Values are expressed as relative quantity plus the standard error of the mean.

(E) Fetal liver cells (FLCs) were plated on dentin slices to determine bone resorbing activity. Slices were stained with toluidine (Tol.) blue and phalloidin to visualize resorption tracks (darker staining areas) and actin ring formation, respectively. Cells were cultured in M-CSF alone except where indicated. Scale bars indicate magnification of actin images.

(F) Spleen macrophages infected with retrovirus expressing IKKβ^{SSEE}-EGFP Fusion cultured in M-CSF were visualized by fluorescent microscopy (lower panel) and then TRAP stained to visualize osteoclasts (upper panel). (G) Control and IKKβ KO monocytes were transduced with viruses expressing GFP or the indicated forms of IKKβ. These cells were treated with M-CSF and RANKL and TRAP stained to visualize osteoclasts. (H) Western blot to demonstrate expression of the indicated IKKβ constructs.

Figure 2. IKKβ^{SSEE}-induced Osteoclastogenesis does not Require RANKL/RANK Upstream Signals

(A) Bone marrow macrophages were cultured in the presence of M-CSF alone or in the presence of M-CSF plus RANKL each in the absence or presence of OPG/Fc chimera. IKKβ^{SSEE}- expressing cells were cultured with M-CSF in the absence or presence of OPG/Fc chimera. Cells were TRAP stained to visualize osteoclasts.

(B) WT, RANK +/?, or RANK-/- spleen-derived macrophages were infected with a retrovirus expressing GFP, IKKβ^{WT}, or IKKβ^{SSEE}. These cells were cultured in the presence of M-CSF alone or in combination with RANKL for four days and TRAP stained to visualize osteoclasts.

(C) WT and RANK-/- spleen-derived macrophages were infected with a retrovirus expressing IKKβ^{WT} or IKKβ^{SSEE}. These cells were cultured in the presence of M-CSF alone or in combination with RANKL for four days on dentin and were stained with phalloidin or toluidine (Tol.) blue to visualize actin rings and resorption pits, respectively. Scale bars indicate relative magnification of images.

(D) WT (+/+) or RANK KO (-/-) spleen cells infected with the indicated viruses were cultured in the presence of M-CSF or M-CSF + RANKL (RL), and total cell lysates were analyzed by Wesern blot for expression of indicated proteins. Lower panel, quantitative real-time PCR analysis to verify absence of RANK mRNA expression in RANK KO cells. (E) Relative expression of mRNA for osteoclast markers assessed by quantitative real-time PCR. GAPDH served as internal standard for cDNA normalization. Values are expressed as relative quantity plus the standard error of the mean.

Figure 3. IKKβ^{SSEE} Induces Osteoclastogenesis Independent from NEMO Association

(A) Bone marrow macrophages expressing IKKβ^{SSEE} were cultured in the presence of M-CSF without NBD or with NBD or mutant NBD (mNBD). Cells expressing IKKβ^{WT} were treated with RANKL to induce osteoclast differentiation in the absence of NBD or in the presence of NBD or mNBD. Cells were TRAP stained for osteoclasts. Right panel, quantification.

(B) Schematic diagram of IKKβ constructs utilized in (C and D). Kinase, kinase domain; LZ, leucine zipper; HLH, helix-loop-helix; NBD, NEMO-binding domain. Not shown to scale.

(C) Co-immunoprecipitation depicting ability of Tryptophan 739 and 741 to Alanine mutations to prevent binding of IKKβ^{WT} and IKKβ^{SSEE} to NEMO.

(D) TRAP stain of cells expressing GFP or the IKKβ constructs shown in (B).

(E) Luciferase assay for NF-κB induction by constructs shown in (B).

(F) Kinase assay for constructs shown in B. p-IκBα, phosphorylated IκBα.

Figure 4. IKKβ^{SSEE} Induction of Osteoclasts does not Require IKKα

(A) WT and IKKα-null FLCs expressing GFP were cultured with M-CSF in the absence or presence of RANKL, or IKKβ^{SSEE} with M-CSF alone. TRAP staining for osteoclastogenesis; phalloidin staining for actin ring formation; toluidine (Tol.) blue staining for dentin resorption. Scale bars indicate magnification of actin image. (B) Western blot for expression of selected NF-κB pathway markers. Actin blot shows equal loading. OC+, osteoclast positive control. n.s., non-specific band. (C) Relative expression of mRNA for selected markers of osteoclastogenesis assessed by quantitative real-time PCR. Levels normalized to GAPDH.

Figure 5. IKKβ^{SSEE} Induction of Osteoclastogenesis Requires Coordinated NF-κB Signaling

(A) Western blot for expression levels of key NF-κB signaling molecules in total cell lysates (left panel) and cytosol and nuclear fractions (right panel) of spleen-derived macrophages expressing GFP, IKKβ^{WT}, or IKKβ^{SSEE} not treated or treated with RANKL for the indicated times.

(B) TRAP stain of WT and RelB KO bone marrow macrophages expressing IKKβ^{SSEE} cultured in the presence of M-CSF without RANKL.

(C) Western blot for the indicated proteins in WT and RelB KO cells expressing GFP, IKKβ^{WT}, or IKKβ^{SSEE} cultured with M-CSF. OC+, osteoclast positive control.

(D) Relative mRNA expression of selected osteoclast markers in WT and Rel B KO cells treated with RANKL or expressing IKKβ^{SSEE} not treated with RANKL.

(E and F) Protein expression and osteoclastogenesis, respectively, were measured following expression of GFP, IKKβ^{WT}, IKKβ^{SSEE}, IKKα^{SSEE}, p65(RelA), RelB, or p65+RelB in bone marrow macrophages cultured with M-CSF.

(G-I) Control or NF-κB double knockout (dKO) spleen cells transduced with GFP, IKKβ^{WT}, or IKKβ^{SSEE} were analyzed to determine (G) protein expression levels, (H) osteoclastogenesis and bone resorption, and (I) Cathepsin K mRNA expression. Cells in (I) were cultured in the presence of M-CSF without RANKL.

Figure 6. Active IKKβ is Sufficient for Osteolysis

(A) Images of TRAP stained histological slides of calvarial bones of mice injected supracalvarially with adenovirus expressing lac Z (Ad LacZ) or IKKβ^{SSEE} (Ad IKKβ^{SSEE}), LPS, or RANKL. Arrows indicate areas of osteoclastic bone erosion. Scale bars, 200μm.

(B) Images of H&E, TRAP, and Immunoperoxidase-phospho-IκBα stained histological slides of knees of mice injected intra-articularly with adenovirus expressing lac Z (Ad LacZ) or IKKβ^{SSEE} (Ad IKKβ^{SSEE}). Arrows indicate areas of pathological osteoclastic bone erosion at the articular surface (osteoclasts stain pink). Asterisks denote areas of inflammatory cell infiltrate into the synovial space. Arrowheads show positive stain for phosphorylated IκBα, indicative of active IKKβ. Objective used for capturing image is labeled above panels. Scale bars, 200μm.

Supplementary Figure 1. High Resolution Image Demonstrating RANKL-Independent Osteoclastogenic Effect of IKKβ^{SSEE} in the Absence of Endogenous IKKβ. In an independent experiment from figure 1A of main text, IKKβ KO cells were retrovirally transduced with GFP, IKKβ^{WT}, or IKKβ^{SSEE} and were either cultured in M-CSF or M-CSF + RANKL. Cells were TRAP stained to visualize osteoclasts (indicated with arrows).

Supplementary Figure 2. IKKβ^{SSEE}-Transduced cells 30 min. Post-Plating. Image capturing the phenomenon of the assumption of osteoclast morphology (arrows) by IKKβ^{SSEE} but not GFP - expressing cells 30 minutes after plating on a tissue culture surface. This observation indicates that gene expression which occurs during the retroviral infection and selection process is responsible for osteoclast differentiation.

Supplementary Figure 3. Resorption of Artificial Matrix by Osteoclasts Generated by Expression of IKKβ^{SSEE}. Spleen cells expressing IKKβ^{WT} and IKKβ^{SSEE} were grown in M-CSF alone or in combination with RANKL and were TRAP stained or plated on osteologic (Osteo.) substrate to assess osteoclast activity. Clear areas indicated by arrowheads represent osteoclastic resorption of substrate.

Supplementary Figure 4. High Resolution Image of Resorption Pits Generated by RANKL and IKKβ^{SSEE} – Induced Osteoclasts. Monocytes were retrovirally transduced with either IKKβ^{WT} or IKKβ^{SSEE}. Cells were then plated on dentin slices and grown in the presence of M-CSF (IKKβ^{SSEE}) or M-CSF + RANKL (IKKβ^{WT}) for 5 days. Slices were stained with toluidine blue and visualized by light microscopy. This high resolution image depicts the regular pattern of the dentin slice best visualized when not resorbed (IKKβ^{WT} in M-CSF). Arrows indicate resorption pits and tracks which appear as dark staining irregularities in the dentin slice.

Supplementary Figure 5. Constitutive IκBα Processing Induced by IKKβ^{SSEE} in Control and NF-κB dKO Cells. Monocytes were retrovirally transduced with either GFP, IKKβ^{WT}, or IKKβ^{SSEE}. Total cell lysates were immunoblotted for the indicated proteins. This data indicates that basal IκBα level is normal in the absence of subunits NF-κB1 and 2. IKKβ^{SSEE} induces constitutive processing of IκBα independently from these two subunits, but this phenomenon is insufficient to induce osteoclasts without NF-κB1 and 2. OC+, osteoclast positive control.

Supplementary Figure 6. Adenoviral Gene Transfer *in vivo*. Knees of mice were intra-articularly injected with adenovirus expressing LacZ or IKKβ^{SSEE}. One week post-injection, legs were analyzed by X-gal staining for LacZ expression. Retro-patellar location of blue stain (arrowhead) indicates the intra-joint localization of gene expression. Scale bar, 1 cm.

Supplementary Figure 7. Kinase Activity is responsible for IKKβ^{SSEE}-induced osteoclastogenesis. (A) Spleen macrophages were infected with retrovirus expressing GFP, IKKβ, IKKβ^{SSEE}, or IKKβ$^{SSEE\ KM}$. Cells were TRAP-stained to visualize osteoclasts. (B) NF-κB luciferase and (C) *in vitro* kinase assays for indicated constructs.

FIGURES:

Figure 1

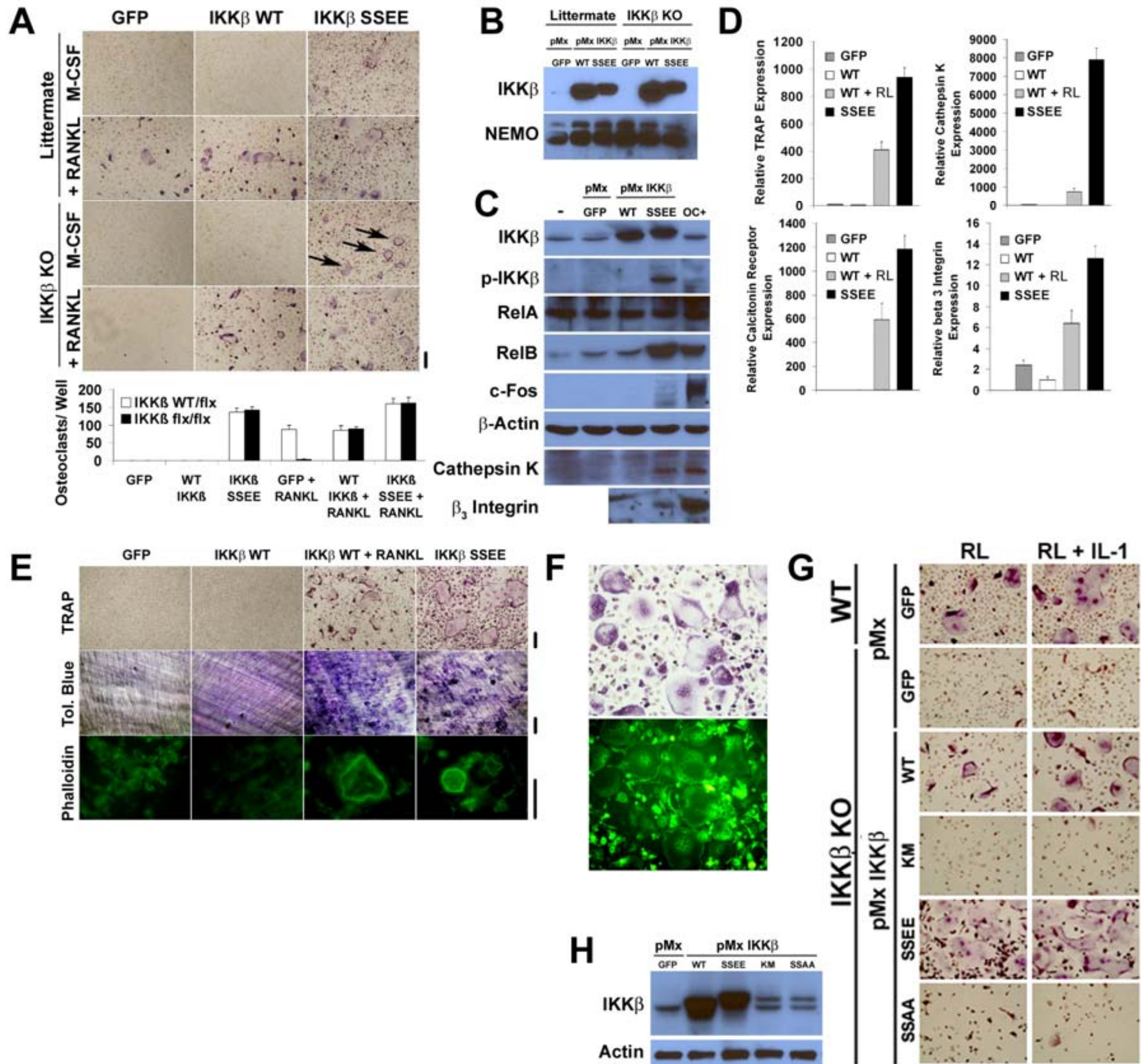
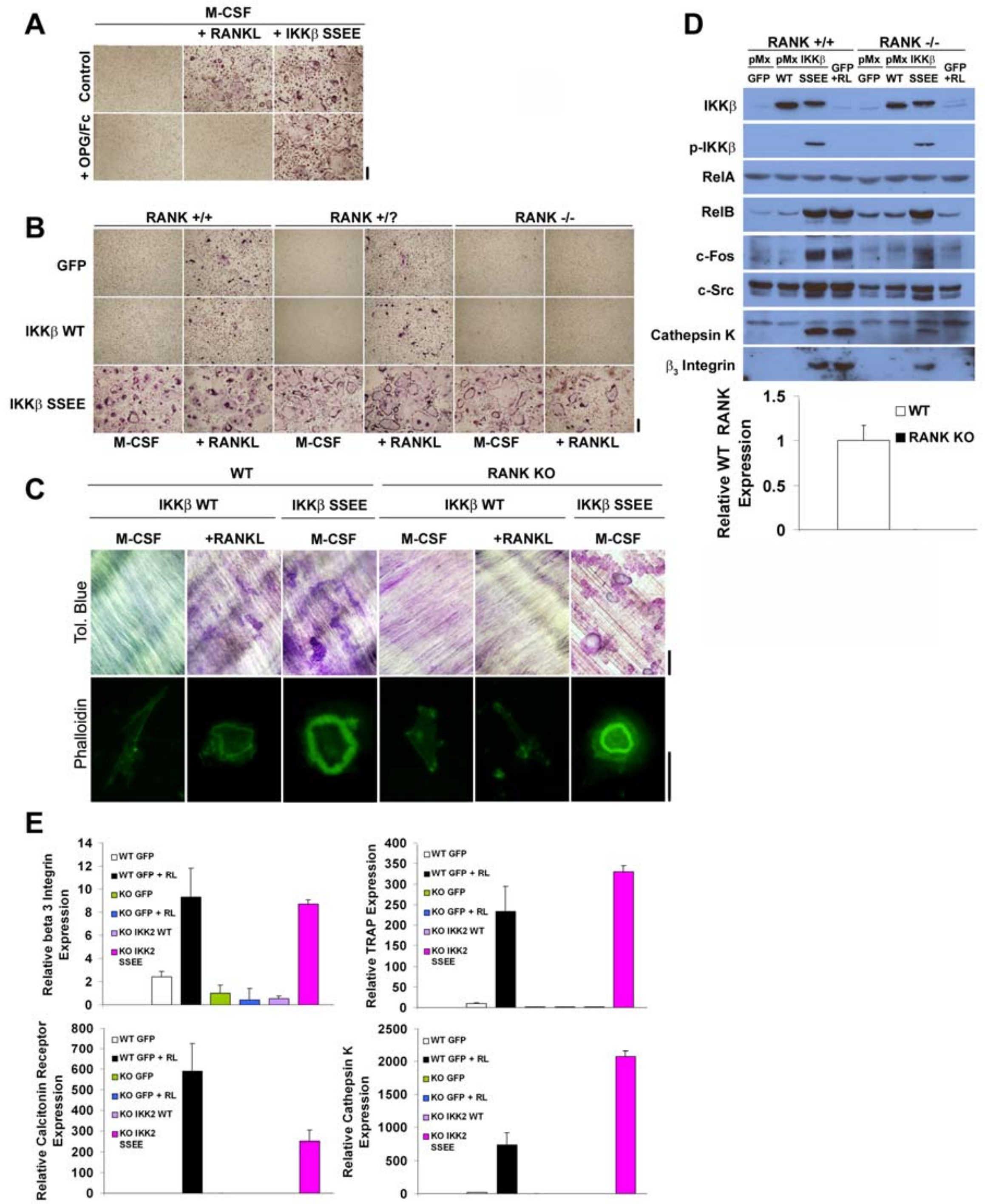


Figure 2



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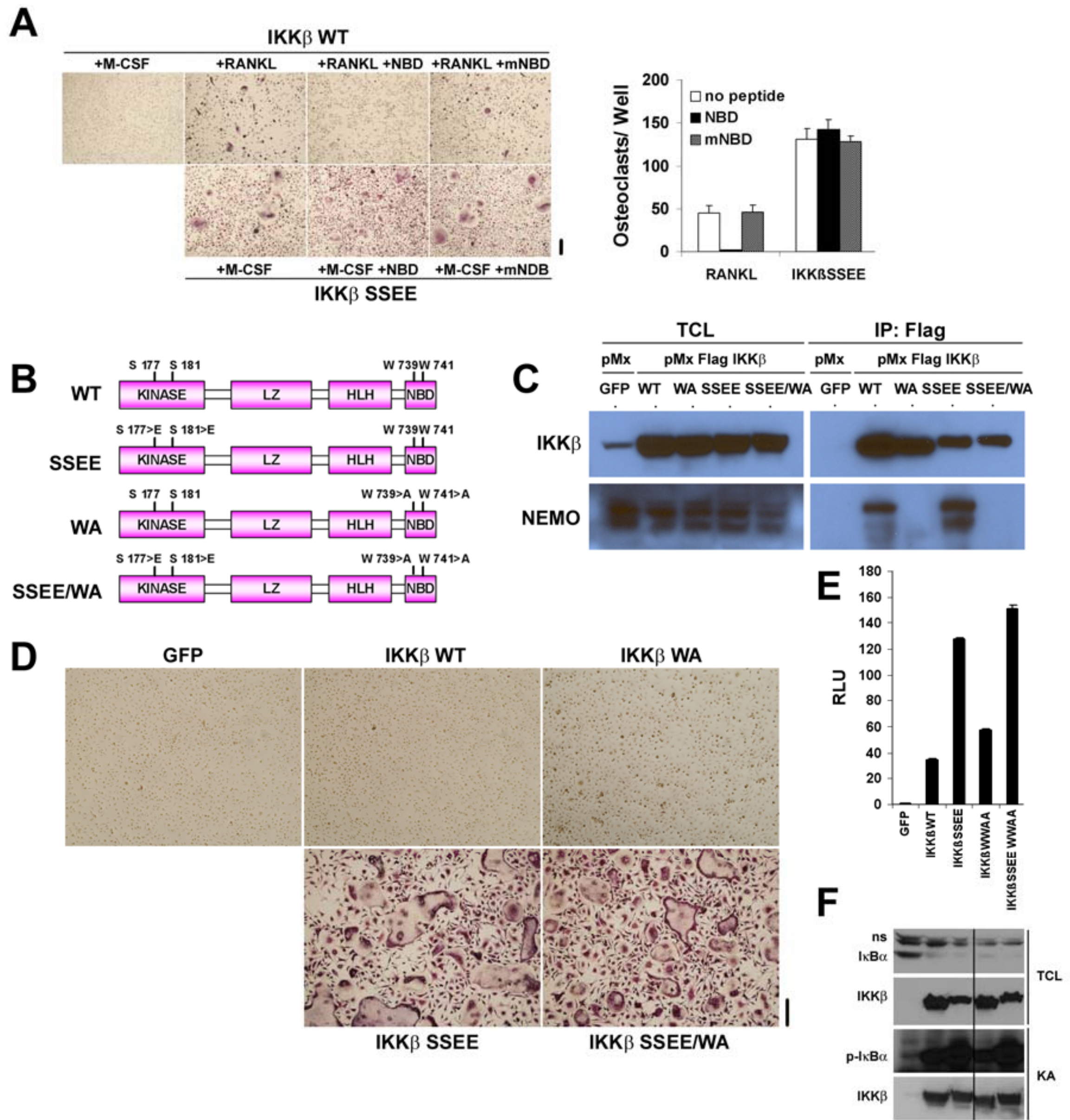


Figure 4

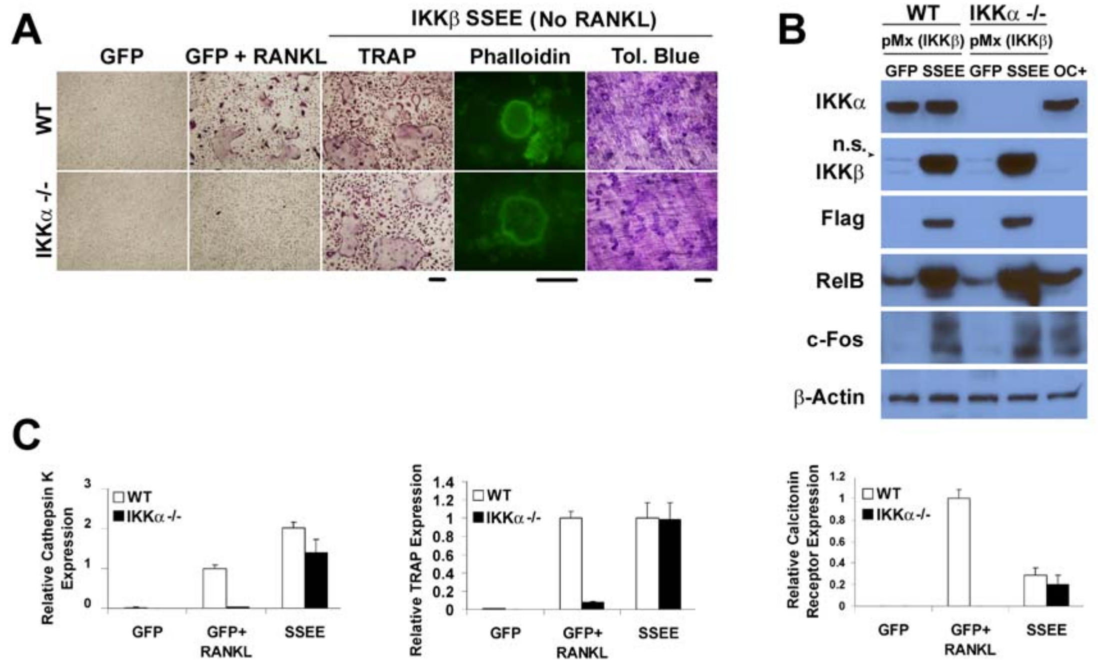


Figure 5

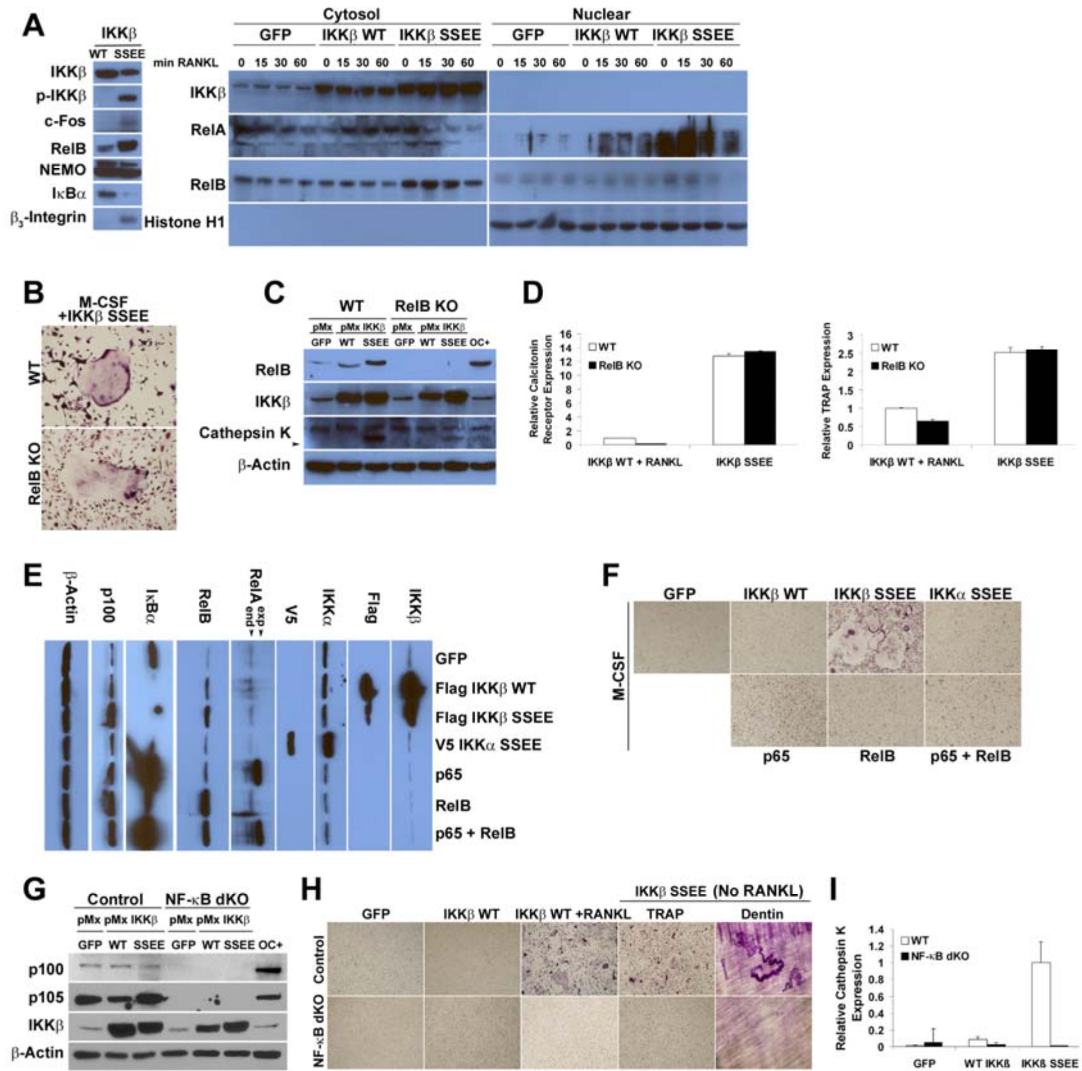
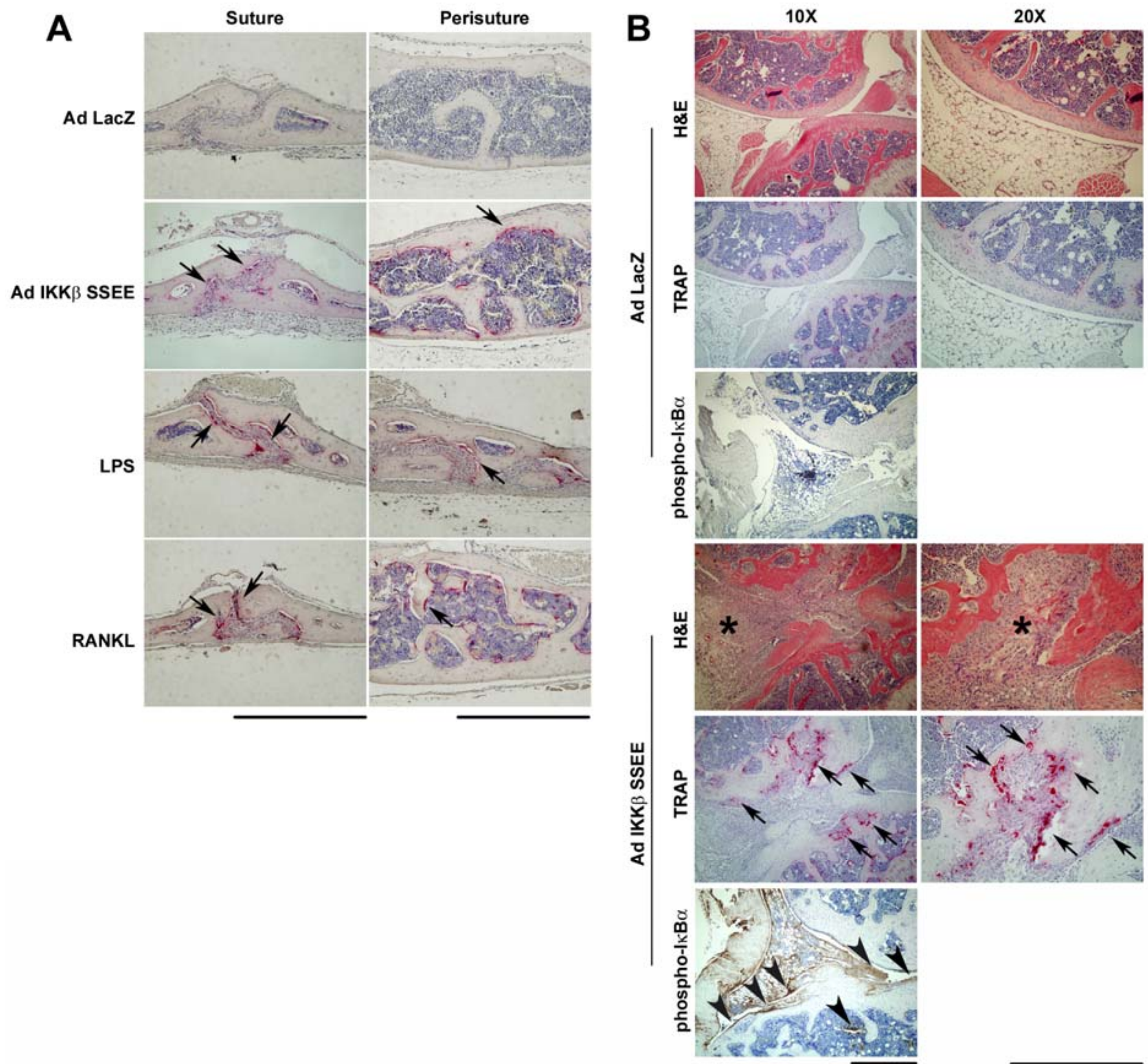


Figure 6



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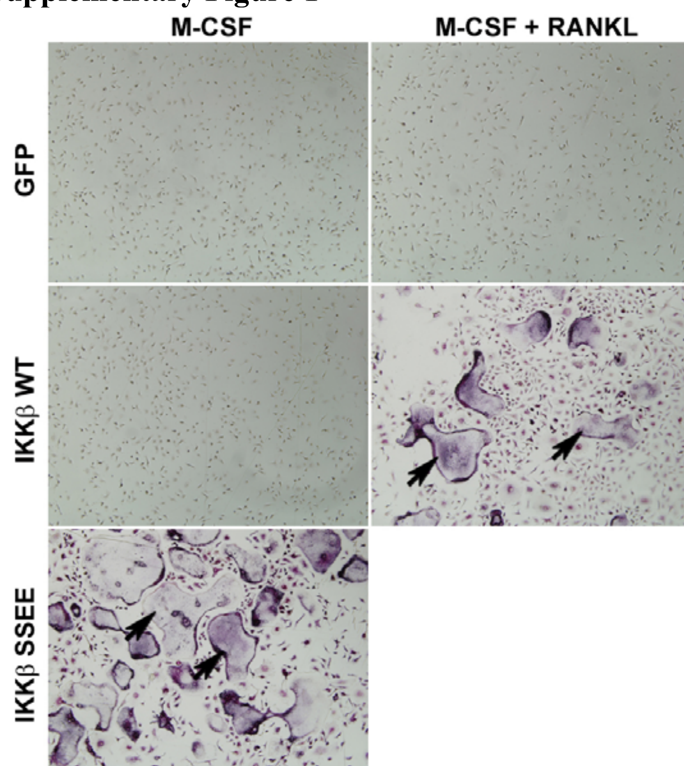

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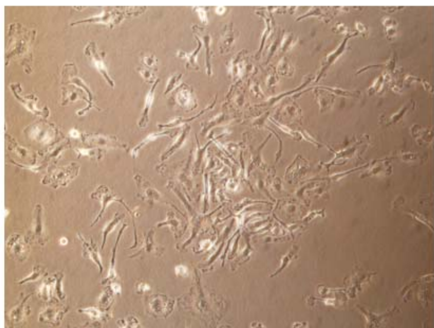
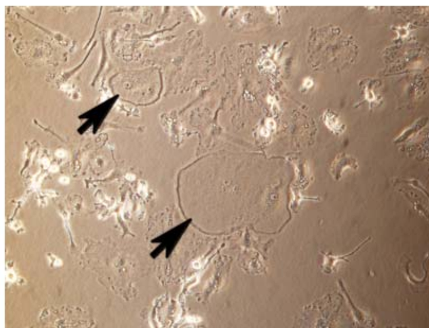
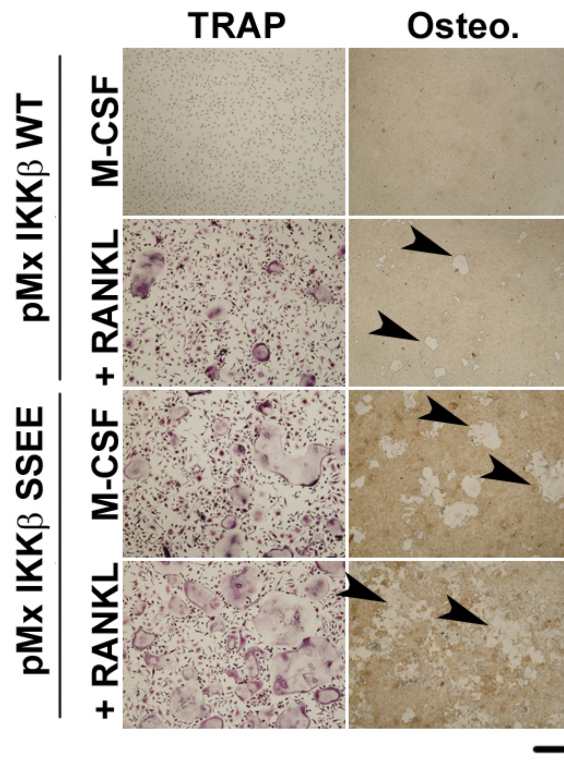




Supplementary Figure 3



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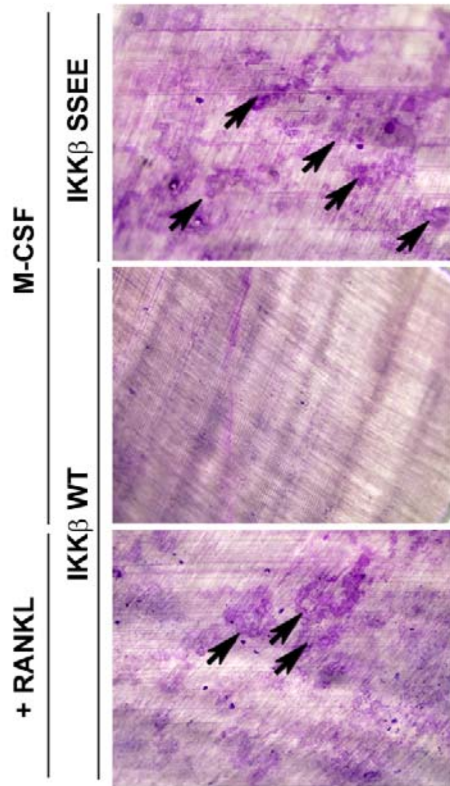
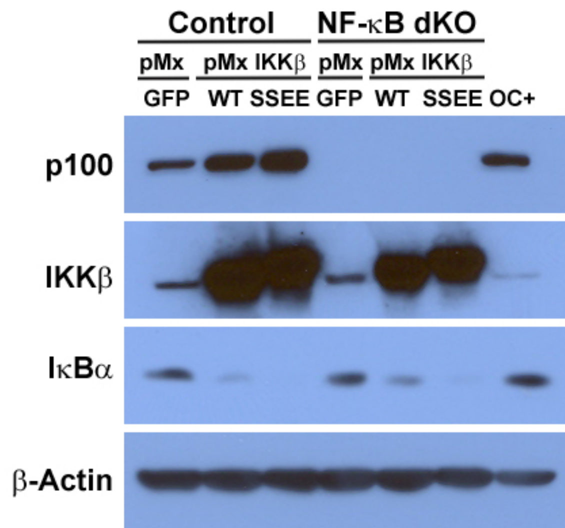
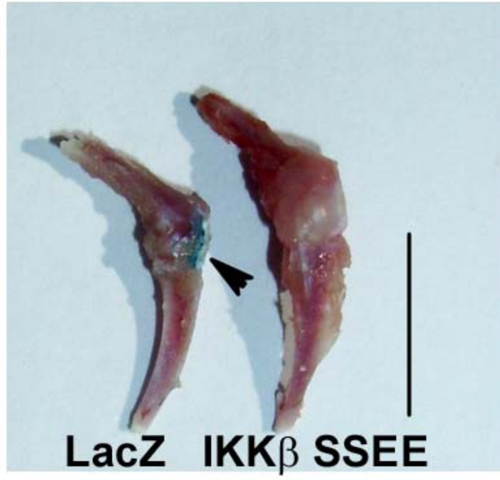


Supplementary Figure 5



Supplementary Figure 6



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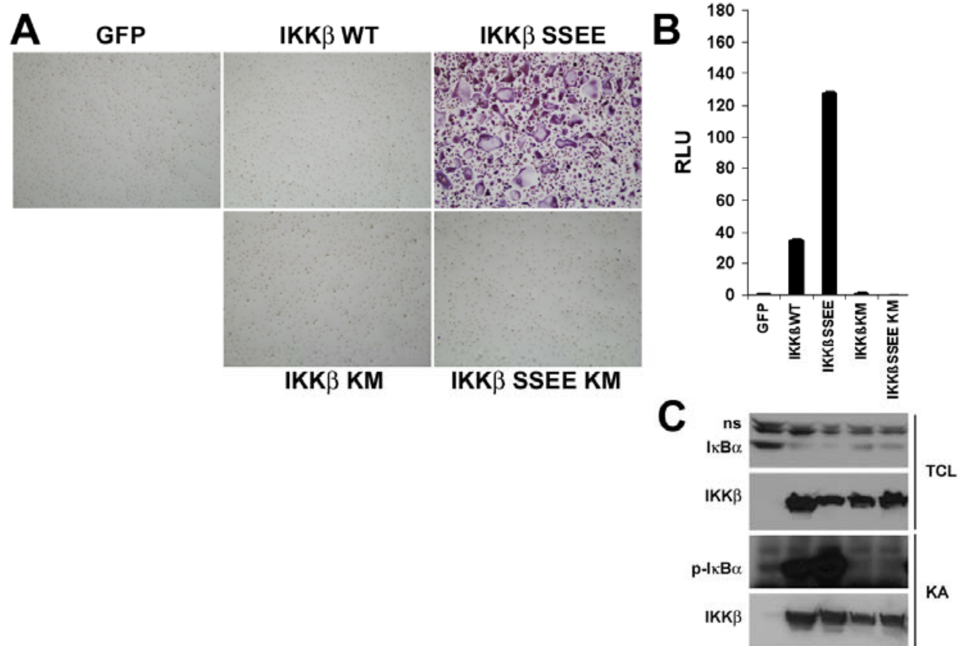

CHAPTER 4

INFLAMMATORY CYTOKINE SECRETION AND OSETOCLAST DIFFERENTIATION INDUCED BY ACTIVATION OF IKKβ IN MACROPHAGES ARE UNCOUPLED EVENTS

ABSTRACT

We have previously demonstrated that constitutively active IKKβ (IKKβ^{SSEE}) induces a program for differentiation of fully functional osteoclasts in the absence of RANK signaling. This finding, coupled with the fact that IKKβ is required for normal osteoclast differentiation in response to RANKL treatment of macrophages, argues that IKKβ activation is necessary for osteoclast differentiation and constitutive activation of IKKβ is sufficient. It is known that IKKβ activates NF-κB which is an inducer of both osteoclastogenic and inflammatory signals. We sought to determine whether inflammatory signals contribute to the osteoclastogenic effect of constitutively active IKKβ in macrophages. We confirmed that, in conjunction with induction of osteoclastogenesis, IKKβ^{SSEE} induces expression of both TNFα and IL-1β, two pro-inflammatory cytokines linked to osteoclastogenesis and inflammatory osteolysis. However, using macrophages genetically deficient in TNFα or the IL-1 type 1 receptor, we determined that neither TNFα nor IL-1β are required for osteoclast differentiation induced by IKKβ^{SSEE}. These results confirm that *in vitro*, osteoclast differentiation and the cellular inflammatory response, with respect to TNFα and IL-1 are uncoupled. Furthermore, we confirm that pure activation IKKβ is capable of inducing osteoclastogenesis from macrophages in an autocrine stimulation-independent, cell-autonomous manner.

INTRODUCTION

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joints resulting in joint pain, swelling, immobility, and deformity. Although the initiating event responsible for RA is unknown, several factors are believed to play a role in the pathogenesis of the disease. It is known that several cell types interact in the inflamed joint and contribute to the progression of RA including synovial fibroblasts and lymphocytes, which secrete pro-inflammatory factors, and osteoclasts which degrade bone in response to these factors [1]. The role of cytokines in progression of RA is well established [2]. For example, synovial mononuclear cell cultures from patients affected by RA were demonstrated to produce the pro-inflammatory cytokine, TNFα [3], which was later localized to the inflamed synovial membrane in tissue sections of RA patients[4]. Further, mice engineered to express high levels of human TNFα develop spontaneous arthritis with cartilage and bone destruction [5], and neutralizing antibodies against TNFα significantly reduced the clinical severity of arthritis in a murine model for the disease [6]. It is believed that TNFα contributes to the bone destruction mediated by osteoclasts in arthritis [7]. Whether TNF itself is sufficient for osteoclast differentiation [8,9] or whether TNF enhances established osteoclastogenesis [10] is controversial, but it is clear that osteoclasts are necessary for TNF-mediated bone destruction [11]. IL-1 is another cytokine known to contribute to the pathogenesis of arthritis as IL-1 gene expression has been demonstrated in the synovium of patients with RA [12], and IL-1 blockade ameliorates the joint destruction associated with experimental arthritis in mice [13]. The role of IL-1 in

mediating bone destruction in arthritis has been suggested based on the observation that IL-1 knockout mice were resistant to TNF-mediated bone destruction [14] and that IL-1 is essential for TNF-mediated osteoclastogenesis *in vitro* [15]. Factors that influence TNF and IL-1 expression in the setting of RA are candidate targets in the treatment of the bone destruction associated with the disease.

NF-κB is a family of transcription factors that plays a critical role in the inflammatory response [16]. It is known that TNF and IL-1 are gene targets for NF-κB [17,18] and that these cytokines also activate NF-κB [19,20] creating a positive feedback loop which may contribute to chronic inflammatory diseases. It has been shown in mice that disruption of NF-κB or upstream activators of NF-κB are resistant to different models for inflammatory arthritis and bone destruction [21-25]. Given the therapeutic value of TNF and IL-1 blockade in arthritis [2], it is likely that NF-κB mediated activation of TNF and IL-1 contributes to the inflammation and bone destruction associated with arthritis. We wondered whether osteoclastogenesis driven by constitutive activation of NF-κB (reported in Chapter 3 of this thesis) was a result of continuous secretion and therefore autocrine stimulation of monocytes by TNF or IL-1.

We used retrovirus mediated delivery of constitutively activated IKKβ (IKKβ^{SSEE}) into monocytes to activate NF-κB, and we demonstrated that in response to IKKβ^{SSEE}, TNFα and IL-1β are upregulated at both mRNA and protein levels. However, TNFα and IL-1R knockout monocytes were stimulated to become osteoclasts in response to IKKβ^{SSEE},

which demonstrates that pro-inflammatory autocrine signaling does not contribute to spontaneous osteoclast differentiation driven by constitutively active IKKβ. These observations suggest that IKKβ-driven transcriptional activity within IKKβ^{SSEE} –expressing monocytes leads to osteoclast differentiation in a cell-autonomous manner, which provides strong evidence that osteoclast differentiation can be stimulated by a single signaling pathway downstream of RANK.

RESULTS

Constitutively Active IKKβ Induces both Pro-Inflammatory and Osteoclastogenic Programs

NF-κB is an important stimulator of the production of the inflammatory cytokines, TNF and IL-1 [17,18]. We have shown that constitutively active IKKβ (IKKβ^{SSEE}), which is an upstream activator of NF-κB, triggers osteoclast differentiation from monocytes in the absence of RANKL. Since TNF and IL-1 have been demonstrated to induce osteoclastogenesis in certain settings independent from the RANKL/RANK signaling pathway [9], we sought to determine whether IKKβ^{SSEE} induces production of TNF and IL-1 in macrophages and, further, whether these molecules are responsible for triggering spontaneous osteoclast differentiation promoted by IKKβ^{SSEE}. We confirmed by Western blot that IKKβ^{SSEE} but not WT IKKβ induced nuclear translocation of p65/RelA in macrophages, which demonstrates the ability of phosphomimmetic Serine to Glutamic

Acid mutations in the t-loop of IKKβ to activate NF-κB in macrophages (**Fig. 1**). In a culture system in which wild-type bone marrow macrophages were induced by $IKK\beta^{SSEE}$ to differentiate into mature osteoclasts (**Fig. 2A**), we measured production of the mRNA for the osteoclastogenic cytokine, RANKL, and its receptor RANK. We determined that RANKL mRNA was not detectible in our culture system in GFP, WT IKKβ, or $IKK\beta^{SSEE}$ expressing cells (**Fig. 2B**), suggesting that $IKK\beta^{SSEE}$ induces osteoclastogenesis without positive feedback by RANKL. Furthermore, mRNA for the receptor RANK was not induced by $IKK\beta^{SSEE}$ (**Fig. 2B**), which argued against the possibility that $IKK\beta^{SSEE}$ – expressing cells may be more sensitive to undetectable levels of RANKL in the culture through receptor upregulation. This result is consistent with our previous findings in which we show that the osteoclastogenic effect of $IKK\beta^{SSEE}$ is a RANK/RANKL-independent event. Next, to determine whether activation of IKKβ in macrophages results in production of pro-inflammatory cytokines, we measured mRNA levels for the pro-inflammatory cytokines, TNFα and IL-1β. We determined that $IKK\beta^{SSEE}$ induces increased expression of mRNA for both TNFα and IL-1β compared with GFP and WT IKKβ – expressing macrophages (**Fig. 2C**). Interestingly, TNFα mRNA level was only induced 3 fold, while mRNA for IL-1β was increased by more than 1000 fold (**Fig. 2C**). Nevertheless, the level of TNFα protein secreted into the culture media measured by ELISA was 7.8 fold greater in $IKK\beta^{SSEE}$ – expressing cells compared with GFP expressing cells ($p<0.005$), and IL-1β protein measured in the media of $IKK\beta^{SSEE}$ – expressing cells was 22.6 fold greater than in the media of GFP – expressing cells ($p<0.005$) (**Fig. 2D**). WT IKKβ did not induce increased TNFα or IL-1β protein secretion

from macrophages compared with GFP. These results demonstrate that in macrophages, constitutively active IKKβ induces secretion of pro-inflammatory cytokines, which have been shown to contribute to osteoclast differentiation [8-10,15].

TNFα is Dispensable for Induction of Spontaneous Osteoclast Formation by $IKK\beta^{SSEE}$

Whether TNFα or IL-1β was a causative factor for osteoclastogenesis in response to activation of IKKβ was unclear. To address this question, we tested the ability of $IKK\beta^{SSEE}$ to induce osteoclastogenesis from bone marrow macrophages isolated from TNFα knockout mice, which are defective in TNFα production [26]. We retrovirally expressed either GFP, WT IKKβ, or $IKK\beta^{SSEE}$ in WT and TNFα KO macrophages. Western blot revealed equivalent expression of IKKβ constructs (**Fig. 3A**). However, although $IKK\beta^{SSEE}$ induced secretion of TNFα from WT macrophages, this induction did not occur in TNFα KO macrophages (**Fig. 3B**). Nevertheless, TNFα KO macrophages were induced by $IKK\beta^{SSEE}$ to become osteoclasts to the same extent as WT macrophages (**Fig. 3C**), indicating that $IKK\beta^{SSEE}$ – induced TNFα autocrine stimulation of macrophages is not responsible for the spontaneous osteoclast differentiation induced by constitutive activation of IKKβ. Interestingly, we observed reduced IL-1β secretion by TNFα KO compared with WT macrophages expressing $IKK\beta^{SSEE}$ ($p<0.005$) (**Fig. 3D**). Therefore, active IKKβ partially relies on TNFα for maximal IL-1β production, but reduced IL-1β production in the TNFα – null setting does not result in impaired spontaneous osteoclast differentiation.

IL-1 Does Not Mediate the Osteoclastogenic Effect of Constitutively Active IKKβ

To determine whether constitutively active IKKβ requires IL-1β autocrine stimulation to induce osteoclastogenesis, we tested the ability of $IKK\beta^{SSEE}$ to induce osteoclasts from macrophages lacking the IL-1α/β type 1 receptor [27]. IL-1 signaling is required for osteoclast bone erosion in response to systemic inflammation [14], so the possibility exists that $IKK\beta^{SSEE}$ may induce osteoclast differentiation through production of IL-1. We observed that although IL-1β failed to enhance RANKL-mediated osteoclastogenesis from IL-1 receptor knockout macrophages (**Fig 4 A. B**), these cells were not defective in osteoclastogenesis induced by expression of $IKK\beta^{SSEE}$(**Fig 4 B**), despite equivalent expression of IKKβ constructs in WT and IL-1 receptor knockout macrophages (**Fig 4 C**). These results indicate that IL-1 does not mediate the osteoclastogenic effect of constitutive activation of IKKβ.

DISCUSSION

Osteoclastogenesis in the setting of inflammation has been widely documented [28-30], and it is known that NF-κB is a critical mediator of both inflammatory as well as osteoclastogenic signaling [31]. We discovered that constitutively activating NF-κB by expressing active IKKβ ($IKK\beta^{SSEE}$) in monocytes induces osteoclast differentiation without upregulating RANKL or its receptor, RANK. We therefore hypothesized that

constitutively active IKKβ induced osteoclast differentiation either from within the monocyte in a cell-autonomous fashion or through secretion of and autocrine stimulation by pro-inflammatory mediators. Although RANKL, the most important osteoclastogenic cytokine, is required for inflammatory osteoclastogenesis in response to certain *in vivo* models of arthritis [32], it has been proposed that the inflammatory cytokines TNF and IL-1 may compensate for RANKL to induce osteoclast differentiation from isolated monocytes [8,9]. Since activation of NF-κB is associated with production of TNF and IL-1 [17,18], we sought to determine whether $IKK\beta^{SSEE}$ induced osteoclast differentiation through these pro-inflammatory cytokines. In addition to stimulation of osteoclast differentiation, we observed induction of both TNFα and IL-1β secretion by macrophages expressing constitutively active IKKβ. However, genetic disruption of TNF or the IL-1 type 1 receptor had no effect on the osteoclastogenic phenotype induced by $IKK\beta^{SSEE}$. Therefore, we have demonstrated that there are separate and exclusive programs for osteoclast differentiation and inflammation induced downstream of IKKβ activation. Furthermore, the fact that autocrine stimulation by TNFα and IL-1β does not mediate osteoclast differentiation induced by constitutively active IKKβ strongly suggests that the program for osteoclastogenesis can be encoded entirely through NF-κB downstream of IKKβ. This finding, in addition to the fact that IKKβ is required for osteoclast differentiation induced by RANKL [33], supports the notion that IKKβ is a critical target in treating inflammatory bone erosion and other osteoclast – mediated diseases.

METHODS

Reagents: Antibodies against IKKβ, Actin, and RelA as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against Flag epitope was purchased from Sigma (St. Louis, MO). Cytokines were purchased from R&D Systems (Minneapolis, MN). Enhanced Chemiluminescence kit was purchased from Pierce Biotechnology, Inc (Rockford, IL). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Animals: All mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed in a controlled barrier facility at Washington University (St. Louis, MO).

Plasmids: pMxs retroviral expression plasmid was a generous gift from Dr. Toshio Kitamura (University of Tokyo, Japan). IKKβ cDNA was purchased from ATCC, I.M.A.G.E. Clone ID #4482634. All expression constructs were subcloned into pMxs using standard techniques. The following mutations were generated using the QuickChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer pairs in parentheses: IKKβ^{SSEE} (IKKβ_S177_181E_f, GAGCTGGATCAGGGCGAACTGTGCACGGAATTTGTGGGGACTCTGC, and IKKβ_S177_181E_r, GCAGAGTCCCCACAAATTCCGTGCACAGTTCGCCCTGATCCAGCTC). Note that

the constitutive activating effect of this mutation of IKKβ has been established previously [34,35].

Macrophage Culture: Whole marrow was flushed from long bones into α- Minimum Essential Medium (MEM). Spleens were crushed into cell suspensions in α-MEM and were centrifuged at 453 rcf. Cell pellets were resuspended in whole media (α-MEM with 1X penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS)). Monocytes/ macrophages were produced by growing cell suspensions in the presence of 10 ng/ml M-CSF. Monocytes/ macrophages were allowed to proliferate for 3 days at 37 ^{o}C in 5% CO_2 at which point they were infected with retrovirus. See below.

Infection of Macrophages: The use of Plat-E retrovirus packaging cells stably expressing retroviral structural proteins gag-pol and env for transient production of high titer retrovirus was described previously [36]. Briefly, 8μg pMx vectors expressing our gene of interest were transfected into 5 million plat-E cells (grown in DMEM supplemented with 10% FBS, 10ng/ml M-CSF, and penicillin/streptomycin) using Fugene 6 (Roche, Palo Alto, CA) according to manufacturer's instructions. Twenty-four hrs post transfection, media was exchanged to remove transfection reagent. Twenty-four and 48 hrs post media exchange, supernatant was collected and pooled. In parallel, monocyte/macrophage cultures from bone marrow, spleen, or fetal liver were developed on petri dishes, washed with PBS, and infected with retrovirus in infection mix (50% virus supernatant, 50% αMEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 4μg/ml hexadimethrine bromide). Twenty-four hrs post infection, cells were selected in αMEM containing 10% FBS, 10ng/ml M-CSF,

penicillin/streptomycin, and 2μg/ml puromycin for 72 hrs, at which point selection media was removed, cells were washed, and grown for 24 additional hrs without puromycin. At this point, cells were lifted, counted, and plated for downstream experiments.

Osteoclast Differentiation: IKKβ^{SSEE} expressing cells were cultured in 10ng/ml M-CSF, while GFP and IKKβ^{WT}-expressing cells were cultured in 10ng/ml M-CSF plus 100ng/ml RANKL for 4 days. IKKβ^{SSEE} induced spontaneous osteoclastogenesis in the absence of RANKL, while RANKL was required for osteoclasts to form from monocyte/macrophages expressing GFP or IKKβ^{WT}. At this point, cells were fixed and TRAP stained using the Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO). TRAP-positive cells with three or more nuclei were scored as osteoclasts.

Western Blot: Equivalent amounts of total cell protein were boiled in the presence of an equal volume of 2X SDS sample buffer consisting of (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, 3% β-Mercaptoethanol (v/v), and distilled water) for 5 min. and subjected to electrophoresis on 8–10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad, Hercules, CA) 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 PBS/Tween buffer and exposed to primary antibodies (16 h at 4 ^{o}C), washed (4 x 15 min.), and incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (1 h at room temperature). The membranes

were washed extensively (4 X 15 min.), and an ECL detection assay was performed following the manufacturer's instructions.

Enzyme Linked Immunosorbent Assay: Supernatants were collected from 6-well plates of $1x10^6$ cells expressing various constructs and were rapidly frozen at -80°C. TNFα or IL-1β concentrations were measured in thawed supernatants by ELISA using Quantikine kits from R&D Systems (Minneapolis, MN).

Isolation of RNA and cDNA Production: RNA was isolated from macrophage or osteoclast cultures using the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. Reverse transcription was performed as follows: 1.0 μg RNA and 1.0 μg random hexamer primer in 10 μl nuclease-free deionized H_2O in PCR tubes were heated to 70 °C for 5 minutes, 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 μl: 1x AMV RT buffer (Roche, Palo Alto, CA), 40U RNAseIn (Promega, San Luis Obispo, CA), 1.25 mM dNTPs, 5 mM Sodium Pyruvate, 5 U Reverse Transcriptase Enzyme, AMV (Roche). To produce cDNA, tubes were placed in a thermocycler programmed as follows: 42 °C for 60 minutes, 50 °C for 10 minutes, and 95 °C for 5 minutes.

Quantitative Real-Time PCR: Triplicate samples of 4 μl cDNA product (5X diluted), 10 μl Sybr Green PCR Master Mix (Applied Biosystems, Inc. Foster City, CA), 0.1 μl each of 10 μM forward and reverse primer stocks, and 6 μL nuclease-free deionized H_2O were subjected to real-time PCR according to the following program in an ABI 7300 Real Time PCR System: 50 ^oC for 2 minutes, 95 ^oC for 10 minutes, (95 ^oC for 15 seconds, 60 ^oC for 1 minute) x 40 cycles. Results were analyzed using AB RQ Study Software. Real Time PCR primers were designed using Primer Express Software (Applied Biosystems, Inc.) mouse RANK Forward 5'-CTGCCTCCTGGGCTTCTTCT-3', mouse RANK Reverse 5'-CCCCTGGTGTGCTTCTAGCT-3'; mouse RANKL Forward 5'-CCTGAGGCCCAGCCATTT-3' mouse RANKL Reverse 5'-AGCCTCGATCGTGGTACCAA-3'; mouse TNFα Forward 5'-GACACCATGAGCACAGAAAGCATGATCCGC-3' mouse TNFα Reverse 5'-CGAAGTTCAGTAGACAGAAGAGCGTGGTGG-3'; mouse IL-1β Forward 5'-GCTTCCTTGTGCAAGTGTCTGA-3' mouse IL-1β Reverse 5'-TCAAAAGGTGGCATTTCACAGT-3'.

Microscopy: Cells were imaged under white light on an inverted microscope (Olympus IX-51). Digital images were captured using a CCD camera (Olympus DP70, 12 mega-pixel resolution).

Statistics: Students two-tailed T-test for comparison between means was used for all analyses.

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FIGURE LEGENDS

Figure 1: Constitutively Active IKKβ Induces NF-κB Nuclear Translocation

WT macrophages were infected (infx) with retroviruses expressing GFP or flag-tagged WT IKKβ or IKKβ^{SSEE} for either 0, 1, 2, 4, or 6 days. Nuclei were extracted and blotted for p65/RelA (lower two panels). Cytosolic fraction was blotted with flag antibody to detect retrovirally expressed IKKβ (upper pannel). ns, non-specific antibody reactivity; N, nucleus; C, cytosol.

Figure 2: Induction of Osteoclastogenesis and Inflammatory Cytokine Secretion by IKKβ^{SSEE}

WT macrophages were infected with retroviruses expressing GFP, WT IKKβ (WT), or IKKβ^{SSEE} (SSEE). (A) Cells were plated for osteoclastogenesis. GFP, WT IKKβ, and IKKβ^{SSEE} expressing cells were cultured in M-CSF alone. Where indicated, WT IKKβ –expressing cells were treated with RANKL to induce osteoclastogenesis. Cells were TRAP-stained to visualize osteoclasts. (B) mRNA was collected from cells, and RANK and RANKL gene expression were measured by quantitative real-time PCR. MC3T3, mouse bone stromal cell line. Values are expressed as mean quantitation plus standard error of the mean. (C) TNFα and IL-1β gene expression levels were measured by quantitative real-time PCR. Values are expressed as mean quantitation plus standard error of the mean. (D) Media supernatants were harvested from cultures of macrophages (or osteoclasts in the case with IKKβ^{SSEE}-expressing cells) expressing the indicated

constructs. TNFα and IL-1β protein concentrations were measured by ELISA. (*), $p<0.005$.

Figure 3: TNFα Autocrine Stimulation is not Responsible for $IKK\beta^{SSEE}$ – Induced Osteoclastogenesis. (A) Macrophages from TNF KO and WT littermates were collected and infected with retroviruses expressing either GFP, WT IKKβ, or $IKK\beta^{SSEE}$. Cells were lysed and total IKKβ protein was detected by Western blot. Actin was blotted as a loading control. (B) Media supernatant was collected from cultures of cells expressing either GFP, WT IKKβ, or $IKK\beta^{SSEE}$. TNFα protein concentration in media was measured by ELISA. (**), $p<0.001$. (C) Cells were grown in media supplemented with M-CSF (without RANKL) and were TRAP-stained to visualize osteoclasts. (D) Media supernatant was collected from cultures of WT and TNFα KO cells expressing either GFP, WT IKKβ, or $IKK\beta^{SSEE}$. IL-1β protein concentration in media was measured by ELISA. (*), $p<0.005$

Figure 4: IL-1 Receptor Autocrine Stimulation is not an Essential Mediator of the Osteoclastogenic Effect of Constitutively Active IKKβ. (A) Macrophages were isolated from WT or IL-1 receptor knockout mice and were infected with retroviruses expressing either GFP, WT IKKβ (WT), or $IKK\beta^{SSEE}$ (SSEE). Cells were grown in the presence M-CSF (without RANKL) or were supplemented with RANKL or RANKL and IL-1β as

indicated. Cells were TRAP-stained to visualize osteoclasts. (B) Quantification of (A). (C) Expression of IKKβ constructs in cells from (A).

FIGURES

Figure 1

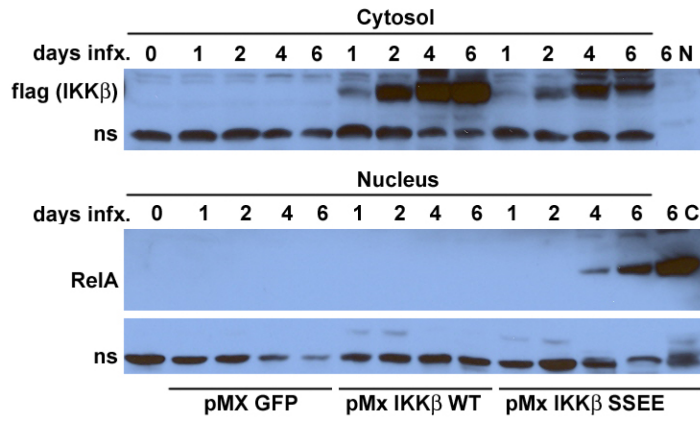


Figure 2

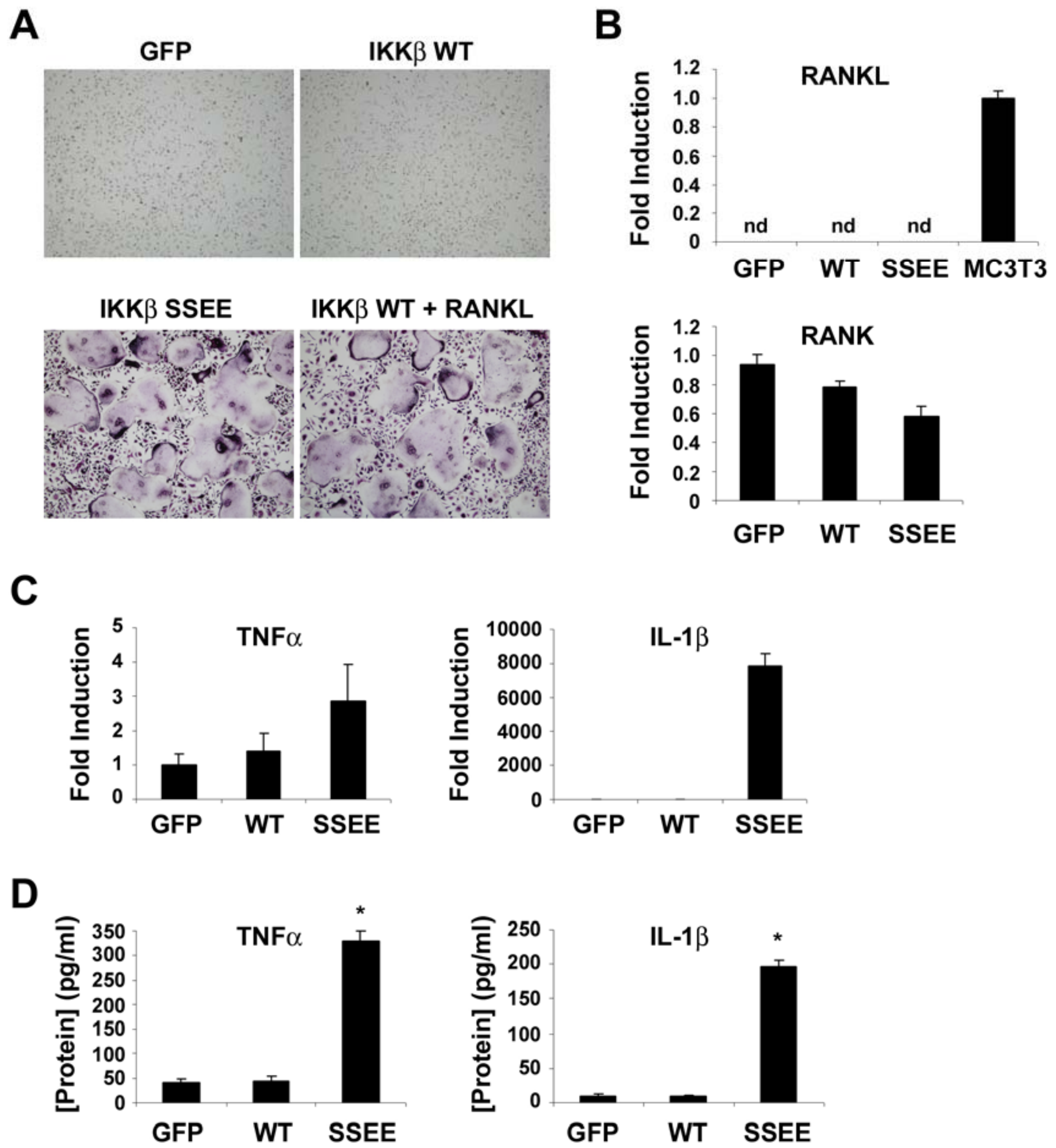


Figure 3

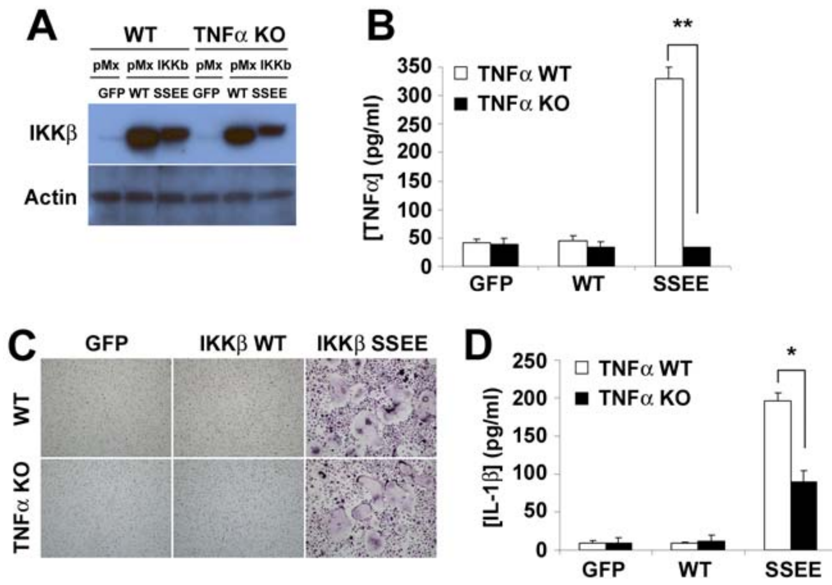
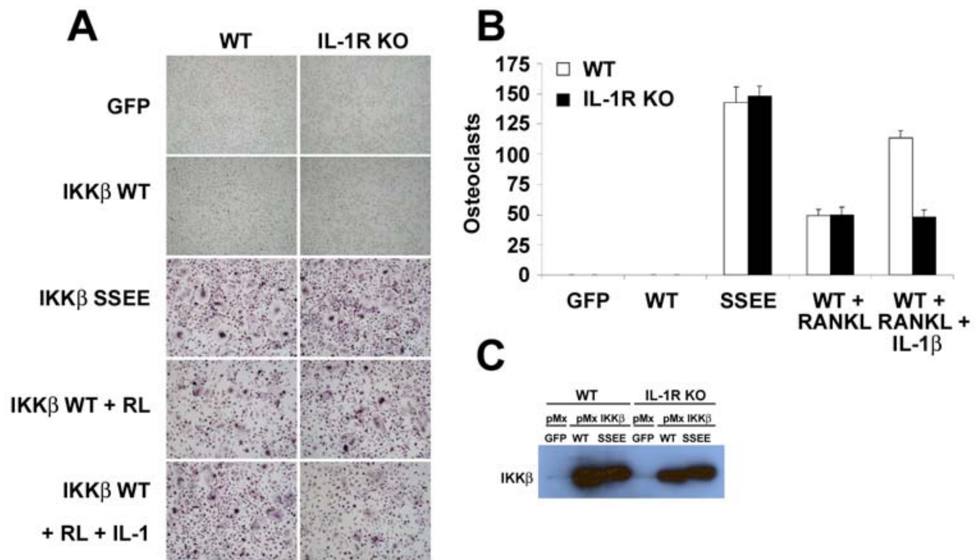


Figure 4



CHAPTER 5

ACTIVATION OF THE ALTERNATIVE NF-κB PATHWAY BY CONSTITUTIVELY ACTIVE IKKβ

INTRODUCTION

NF-κB is a family of transcription factors which serves diverse functions in development, immunity, and bone metabolism. Traditionally, NF-κB signaling is divided into two pathways, classical (canonical) and alternative (non-canonical) [1,2]. In classical NF-κB signaling, extracellular signals such as TNF or IL-1, activate an IKK complex comprising IKKα, IKKβ, and IKKγ (NEMO). IKK then phosphorylates IκBα, which holds NF-κB in the cytoplasm in an inactive state. Upon phosphorylation, IκBα becomes ubiquitinated and rapidly degraded by the proteasome. Classical NF-κB heterodimers consisting of p50 and p65/RelA are released from cytoplasmic sequestration, enter the nucleus, and activate gene transcription. In the alternative signaling pathway, signals from cytokines such as BAFF and lymphotoxin β lead to activation of IKKα which phosphorylates the C-terminal IκB portion of NF-κB p100. This event results in ubiquitination and proteosomal processing of p100 to p52 leading to nuclear translocation of the p52/RelB heterodimer and gene transcription [3].

The ability of the two NF-κB pathways to interact or be coordinated at multiple levels has been suggested. For example, RANKL, the pro-osteoclastogenic cytokine, is known to activate both classical and alternative NF-κB [4,5]. In addition, the kinase, NIK, has been shown to activate both IKKβ [6] and IKKα [7,8], responsible for activation of classical and alternative NF-κB signaling, respectively. Furthermore, the level and activity of RelB, the alternative NF-κB signaling pathway transactivator, are set by activity of the classical

pathway [9]. The inability of these two pathways to compensate for each other in development highlights the notion that they are indeed separate signaling modules [10], but whether they are coordinated in disease states is unknown. Interestingly, constitutive activation of classical NF-κB has been demonstrated to compensate for absence of alternative signaling through BAFFR in B-cell development [11], raising the possibility that activation of IKKβ may regulate alternative signaling in certain settings.

We have shown that constitutively active IKKβ is capable of inducing osteoclastogenesis from monocytes in the absence of IKKα, a setting in which alternative NF-κB signaling induced by RANKL is inhibited. We now demonstrate that constitutively active IKKβ ($IKK\beta^{SSEE}$) induces nuclear translocation of RelB in monocytes. This event is likely a result of processing of p100 to p52, which we show can be induced by $IKK\beta^{SSEE}$ in the absence of IKKα. Whether the activation of RelB by $IKK\beta^{SSEE}$ contributes to the osteoclast differentiation observed in the absence of IKKα is unclear. Further, it is uncertain whether alternative NF-κB can be activated by IKKβ in pathological settings. It will be important to identify the mechanism by which constitutive activation of IKKβ leads to RelB nuclear translocation, as this will lead to a better understanding of NF-κB regulation in the setting of normal physiology and disease.

RESULTS

Constitutively Active IKKβ Induces RelB Nuclear Translocation

A signature of RANKL-induced osteoclast differentiation is activation of both Classical and Alternative NF-κB signaling [4,5,12]. We have shown that constitutive activation of NF-κB by IKKβ^{SSEE} results in differentiation of osteoclasts. Although this phenomenon was also observed in the absence of RelB, we noted that RelB protein levels were constitutively increased in cells expressing IKKβ^{SSEE}, but not WT IKKβ. We sought to determine whether in addition to increased RelB expression, IKKβ^{SSEE} also induced RelB nuclear translocation. We performed nuclear/cytosolic fractionation in monocytes, which we infected with retroviruses expressing GFP, WT IKKβ, or IKKβ^{SSEE}. We observed that indeed, RelB was induced to enter the nucleus after 4 days of infection with IKKβ^{SSEE}, but not WT IKKβ or GFP (**Fig. 1**). This indicates that constitutive activation of IKKβ, normally considered an activator of the Classical NF-κB pathway, can also activate the Alternative NF-κB pathway in monocytes.

Constitutively Active IKKβ Induces p100 Processing to p52

We sought to determine the mechanism responsible for IKKβ^{SSEE} – induced RelB nuclear translocation. It is known that p100 processing to p52 is required for activation of the Alternative NF-κB pathway [2,8]. We hypothesized that IKKβ^{SSEE} may induce processing

of p100 to p52 resulting in RelB activation. We expressed GFP, WT IKKβ, and IKKβ^{SSEE} in WT and NF-κB1/2 double knockout (DKO) monocytes. We observed that IKKβ^{SSEE}, but not WT IKKβ or GFP, induced production of p52 in WT but not NF-κB1/2 DKO monocytes (**Fig. 2**). The absence of p52 in cells lacking the precursor, p100, suggests the authenticity of the p52 product. The ability of WT IKKβ and IKKβ^{SSEE} to induce processing of IκBα even in the absence of *NF-κB1* and *NF-κB2* demonstrates that NF-κB is not required for IKKβ activity.

Production of p52 by IKKβ^{SSEE} does not require IKKα

It is known that NIK activates the alternative NF-κB pathway through IKKα [13]. We wondered whether IKKα is required for IKKβ^{SSEE} mediated p52 processing. We expressed GFP or IKKβ^{SSEE} in WT and IKKα KO fetal liver – derived monocytes and detected p52 expression by Western blot. We observed that even in the absence of IKKα, IKKβ^{SSEE} induced the processing of p100 to p52 (**Fig. 3**). This result suggests that active IKKβ may act on p100 directly to produce p52 and activate the alternative NF-κB pathway. It will be important to determine whether this crosstalk between classical and alternative NF-κB signaling has functional significance in the setting of bone pathology.

METHODS

Reagents: Antibodies against IKKβ, Actin, IκBα, and RelB as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against Flag epitope was purchased from Sigma (St. Louis, MO). Antibodies against IKKα and p100/p52 were from Cell Signaling Technologies, Inc (Danvers, MA). Cytokines were purchased from R&D Systems (Minneapolis, MN). Enhanced Chemiluminescence kit was purchased from Pierce Biotechnology, Inc (Rockford, IL). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Animals and Cells: All mice were housed in a controlled barrier facility at Washington University (St. Louis, MO). IKKα heterozygous mice [12] were obtained from Dr. Akira (Osaka University, Japan). RelB KO [5] and control bone marrow was obtained from Dr. Novack (Washington University, St. Louis, MO). NF-κB double KO [14] and control spleens were a generous gift from Dr. Lianping Xing (University of Rochester Medical Center, N.Y.)

Plasmids: pMxs retroviral expression plasmid was a generous gift from Dr. Toshio Kitamura (University of Tokyo, Japan). IKKβ cDNA was purchased from ATCC, I.M.A.G.E. Clone ID #4482634. All expression constructs were subcloned into pMxs using standard techniques. The following mutations were generated using the

QuickChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer pairs in parentheses: IKKβ^{SSEE} (IKKβ_S177_181E_f, GAGCTGGATCAGGGCGAACTGTGCACGGAATTTGTGGGGACTCTGC, and IKKβ_S177_181E_r, GCAGAGTCCCCACAAATTCCGTGCACAGTTCGCCCTGATCCAGCTC). Note that the constitutive activating effect of this mutation of IKKβ has been established previously [6,15].

Macrophage Culture: Whole marrow was flushed from long bones into α- Minimum Essential Medium (MEM). Spleens and Fetal livers were crushed into cell suspension in α-MEM and were centrifuged at 453 rcf. Cell pellets were resuspended in whole media (α-MEM with 1X penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS)). Monocytes/ macrophages were produced by growing cell suspensions in the presence of 10 ng/ml M-CSF. Monocytes/ macrophages were allowed to proliferate for 3 days at 37 ^{o}C in 5% CO_2 at which point they were infected with retrovirus. See below.

Infection of Macrophages: The use of Plat-E retrovirus packaging cells stably expressing retroviral structural proteins gag-pol and env for transient production of high titer retrovirus was described previously [16]. Briefly, 8μg pMx vectors expressing our gene of interest were transfected into 5 million plat-E cells (grown in DMEM supplemented with 10% FBS, 10ng/ml M-CSF, and penicillin/streptomycin) using Fugene 6 (Roche, Palo Alto, CA) according to manufacturer's instructions. Twenty-four hrs post transfection, media was

exchanged to remove transfection reagent. Twenty-four and 48 hrs post media exchange, supernatant was collected and pooled. In parallel, monocyte/macrophage cultures from bone marrow, spleen, or fetal liver were developed on petri dishes, washed with PBS, and infected with retrovirus in infection mix (50% virus supernatant, 50% αMEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 4μg/ml hexadimethrine bromide). Twenty-four hrs post infection, cells were selected in αMEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 2μg/ml puromycin for 72 hrs, at which point selection media was removed, cells were washed, and grown for 24 additional hrs without puromycin. At this point, cells were lifted, counted, and plated for downstream experiments.

Western Blot: Equivalent amounts of total cell protein were boiled in the presence of an equal volume of 2X SDS sample buffer consisting of (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, 3% β-Mercaptoethanol (v/v), and distilled water) for 5 min. and subjected to electrophoresis on 8–10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad, Hercules, CA) 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 PBS/Tween buffer and exposed to primary antibodies (16 h at 4 ^{o}C), washed (4 x 15 min.), and incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (1 h at room temperature). The membranes were washed extensively (4 X 15 min.), and an ECL detection assay was performed following the manufacturer's instructions.

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FIGURE LEGENDS

Figure 1. Expression of IKKβ^{SSEE} in Monocytes results in RelB Activation

Wild-type monocytes were infected with retroviruses expressing either GFP or flag-tagged WT IKKβ or IKKβ^{SSEE} for either 0, 1, 2, 4, or 6 days. Nuclear fractions were separated from cytosolic fractions. Retroviral IKKβ was detected in the cytosolic fraction by Western blot for flag. RelB was detected in nuclear fraction by Western blot. N, nuclear. C, cytosolic. ns, non-specific background band.

Figure 2. IKKβ^{SSEE} induces p100 processing to p52

Wildtype and NF-κB1/2 double knockout (DKO) monocytes were infected with retroviruses expressing either GFP or flag-tagged WT IKKβ or IKKβ^{SSEE}. Expression of indicated proteins was detected in total cell lysates by Western Blot. OC+, osteoclast positive control total cell lysate.

Figure 3. IKKα – independent p52 production by IKKβ^{SSEE}

WT and IKKα KO monocytes were infected with retroviruses expressing either GFP or flag-tagged IKKβ^{SSEE}. Expression of the indicated proteins was detected by Western blot. OC+, osteoclast positive control total cell lysate.

FIGURES

Figure 1

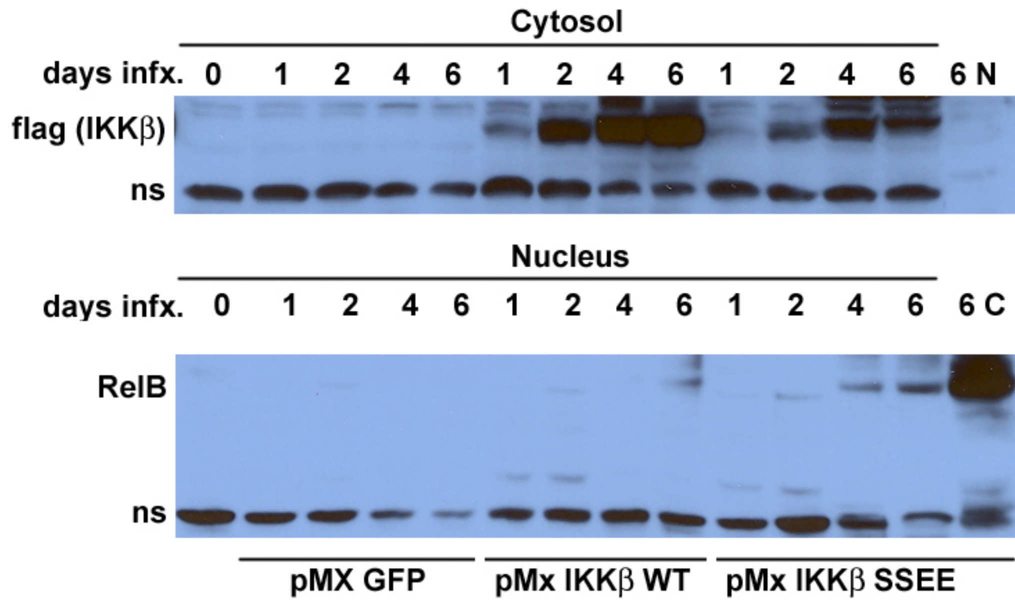


Figure 2

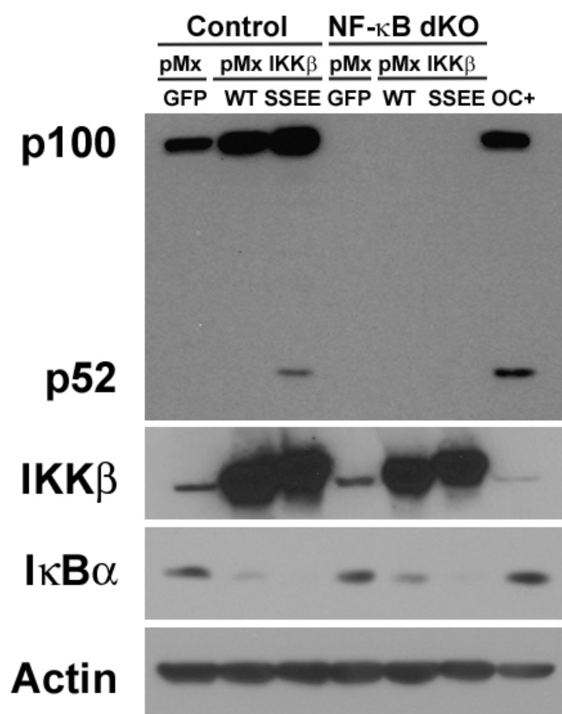
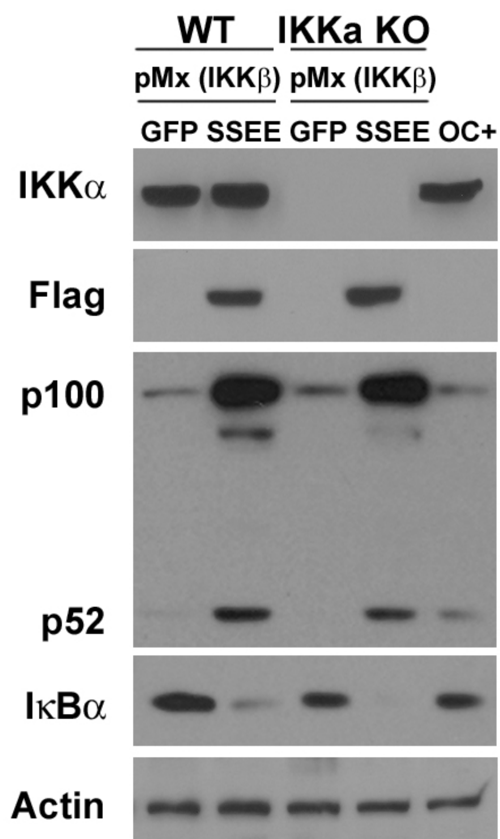


Figure 3



CHAPTER 6

CONCLUSION

SUMMARY OF RESULTS

IKKβ is Necessary for Osteoclast Differentiation and Prevents JNK-Mediated Apoptosis in Differentiating Pre-Osteoclasts

We began with the hypothesis that since NF-κB activity is absolutely critical for osteoclast differentiation [1], then upstream activators of NF-κB would play an important role in osteoclastogenesis. We focused on IKKβ to build on previous work in our laboratory which demonstrated that the NEMO – IKKβ interaction is important for osteoclast differentiation [2]. Further, an osteoclast defect resulting in resistance to inflammatory osteolysis was noted in mice lacking IKKβ in hematopoietic tissues [3]. This fact, coupled with the observation that IKKα activation is dispensable for osteoclastogenesis [3] led us to investigate the role IKKβ plays in response to RANKL in the setting of osteoclastogenesis.

To this end, we used Cre/loxP technology to produce a mouse lacking IKKβ specifically in osteoclast precursors (OCPs) by crossing mice possessing homozygous floxed IKKβ alleles [4] with mice transgenically expressing Cre recombinase under the control of the CD11b promoter [5]. Initial observation of Cre-expressing, homozygous floxed IKKβ ($OCP^{\Delta IKK\beta}$) mice revealed a defect in bone metabolism, since $OCP^{\Delta IKK\beta}$ demonstrated shorter and more radio-dense bones on x-ray. Macrophages from these mice isolated from both spleen and bone marrow showed decreased IKKβ expression at both mRNA

and protein levels. This confirmed that our genetic targeting of IKKβ in osteoclast precursors was successful and resulted in defects in bone metabolism.

To determine whether deletion of IKKβ in OCPs affects osteoclast differentiation, we cultured control and $OCP^{\Delta IKK\beta}$ cells in the presence of M-CSF and RANKL to induce osteoclast differentiation *in vitro*. Differentiation of $OCP^{\Delta IKK\beta}$ cells into osteoclasts *in vitro* was severely impaired, and long bones of $OCP^{\Delta IKK\beta}$ mice possessed significantly fewer osteoclasts than control littermates. We demonstrated a defect in RelA nuclear translocation in response to RANKL in $OCP^{\Delta IKK\beta}$ cells as well, confirming that IKKβ mediated NF-κB activation in response to RANKL is important for osteoclastogenesis. In order to determine the cause for impaired osteoclastogenesis in $OCP^{\Delta IKK\beta}$ cells, we performed quantitative real-time PCR for osteoclast-specific gene expression after treatment of WT and $OCP^{\Delta IKK\beta}$ cells with M-CSF and RANKL, we noted significantly reduced induction of osteoclast genes in $OCP^{\Delta IKK\beta}$ cells compared with control cells. This finding correlated well with our observation that absence of IKKβ results in defective differentiation of monocytes into osteoclasts. We also determined by real-time PCR that $OCP^{\Delta IKK\beta}$ cells showed reduced expression of NF-κB-controlled anti-apoptotic proteins. We hypothesized that perhaps RANKL induced apoptosis of $OCP^{\Delta IKK\beta}$ cells as a result. Indeed, RANKL treatment of $OCP^{\Delta IKK\beta}$ but not control cells for 4 hrs resulted in PARP cleavage, an indicator of apoptosis. Furthermore, $OCP^{\Delta IKK\beta}$ mice possessed many apoptotic osteoclasts associated with bony trabeculae in long bones, suggesting that osteoclast lineage cells in $OCP^{\Delta IKK\beta}$ mice were susceptible to apoptosis *in vivo*.

Analysis of kinase signaling downstream of RANK stimulation in $OCP^{\Delta IKK\beta}$ cells revealed a gain-of-function of JNK compared with WT cells. Since JNK hyperactivity has been linked to apoptosis in response to RANKL in differentiating osteoclasts [6], we hypothesized that inhibition of JNK may rescue the osteoclast defect in $OCP^{\Delta IKK\beta}$ cells. Indeed, treatment of $OCP^{\Delta IKK\beta}$ monocytes with low doses of a cell-permeable peptide inhibitor of JNK restored osteoclastogenesis in $OCP^{\Delta IKK\beta}$. This rescue phenomenon was only observed with incomplete deletion of IKKβ, whereas OCPs with more efficient deletion of IKKβ (with less IKKβ mRNA expression) could not be differentiated into osteoclasts even with JNK inhibition. We concluded that IKKβ serves two functions which are important for osteoclastogenesis. First, IKKβ inhibits JNK-mediated apoptosis in response to RANKL. Second, IKKβ facilitates the osteoclast differentiation program downstream of RANKL.

Constitutive Activation of IKKβ is Sufficient for Osteoclastogenesis

With an established genetic model for deficiency of IKKβ in osteoclast precursors in hand, our next step was to identify the residues and domains of IKKβ which are important for its function in the setting of osteoclast differentiation. We took the approach of retroviral re-introduction of WT IKKβ or mutant forms of IKKβ into $OCP^{\Delta IKK\beta}$ cells to attempt to rescue osteoclastogenesis. We chose K44M as a negative control, since this molecule lacks kinase activity [7]. Also, in order to test phosphorylation – dependent loss-of-function and gain-of-function, we tested S177/181A ($IKK\beta^{SSAA}$) and

S177/181E (IKKβ$^{SSEE)}$, respectively. Mutation of T-loop Serines to Alanine renders IKKβ refractory to signal-induced activation, while mutation to Glutamic Acid creates a constitutively active form of IKKβ [8]. In response to RANKL, WT IKKβ restored osteoclastogenesis in OCP$^{\Delta IKK\beta}$. IKKβ^{K44M} and IKKβ^{SSAA} failed to rescue osteoclastogenesis, indicating that RANKL-induced serine phosphorylation of IKKβ is required for osteoclast differentiation. IKKβ^{SSEE} also rescued osteoclastogenesis from OCP$^{\Delta IKK\beta}$, and upon closer examination, we made the surprising discovery that IKKβ^{SSEE} induced osteoclast differentiation in the absence of RANKL administration.

This finding led to the hypothesis that phosphorylation of IKKβ at Serines 177 and 181 in response to RANK stimulation by RANKL represents a molecular switch that activates the osteoclast differentiation program. Our subsequent investigation was focused on challenging this hypothesis. First, we characterized the osteoclasts induced by IKKβ^{SSEE} to determine if they met the criteria for authentic osteoclasts derived by RANKL stimulation of M-CSF-dependent monocytes. Our examination revealed that IKKβ^{SSEE} –induced osteoclasts expressed all markers of mature osteoclasts, formed actin rings, and resorbed bone. We determined that, indeed, constitutively active IKKβ induces differentiation of *bona fide* osteoclasts.

We further demonstrated that IKKβ^{SSEE} induced osteoclasts in the absence of the receptor, RANK, and in the presence of inhibitory concentrations of OPG-fc (which

interferes with RANKL stimulation of monocytes). These results led us to conclude that IKKβ^{SSEE} induces differentiation of osteoclasts in a RANK – independent manner.

Next, we desired to dissect the molecular environment surrounding IKKβ in the monocyte. Since IKKβ is a member of the IKK complex [7], and all the members of this complex play an essential role in osteoclastogenesis [2,9-11], we wondered if the integrity of the IKK complex was critical for IKKβ^{SSEE} to induce osteoclastogenesis. We found that constitutively active IKKβ could induce osteoclastogenesis in the absence of endogenous IKKβ and IKKα and in conditions in which NEMO- IKKβ association were inhibited pharmacologically and genetically. We concluded that IKKβ^{SSEE} acts independently and autonomously with respect to the IKK complex to induce osteoclastogenesis. This result suggests that the function of the IKK complex downstream of RANKL is to allow for phosphorylation and activation of IKKβ, which then triggers osteoclast differentiation.

We looked downstream to determine which factors were necessary for IKKβ^{SSEE} to induce RANK-independent osteoclastogenesis. Nuclear/cytosol fractionation experiments revealed that IKKβ^{SSEE} induces nuclear translocation of NF-κB subunits RelA and RelB and also stimulates increased production of total RelB. Although RelB plays a role in RANKL-induced osteoclast differentiation [12], IKKβ^{SSEE} induced bone resorbing osteoclasts from RelB knockout spleen monocytes. Importantly, *NF-κB1/NF-κB2* double knockout macrophages did not become osteoclasts when expressing IKKβ^{SSEE}, demonstrating a pivotal role for NF-κB in this phenomenon. These results suggested that

constitutive activation of NF-κB by IKKβ^{SSEE} is sufficient for RANK-independent osteoclast differentiation. Overexpression of IKKα^{SSEE}, RelB, or RelA failed to induce osteoclast differentiation, highlighting the specificity of IKKβ as an inducer of osteoclastogenesis.

Importantly, kinase activity of IKKβ^{SSEE} was critical for mediating its osteoclastogenic effect, since mutation of Lysine 44 to Methionine in the presence of S177/181E mutations abrogated the effect. IKKβ^{SSEE} demonstrated some qualities which were surprising. For example, it induced processing of p100 to p52 in monocytes, a function which is thought to be mediated by IKKα. The role of this phenomenon in inducing osteoclasts in response to IKKβ^{SSEE} is unclear at this point.

We wished to determine whether activation of IKKβ in an *in vivo* setting possesses pathological significance. Indeed, adenoviral gene transfer of IKKβ^{SSEE} into knees of mice results in massive inflammation and bone erosion, mimicking the findings in rheumatoid arthritis.

These data provide evidence that IKKβ is a specific inducer of osteoclast differentiation and that gain-of-function in IKKβ may contribute to the pathogenesis of inflammatory osteolysis. Therefore, therapy targeting the activation of IKKβ may represent a successful approach to ameliorate pathological bone loss.

Inflammation and Osteoclastogenesis Induced by Constitutively Active IKKβ are Uncoupled

It is well known that NF-κB is a crucial mediator of osteoclastogenic [1,13] as well as inflammatory signaling [14-16]. Inflammatory inducers such as TNFα and IL-1β have been shown to contribute to osteoclast differentiation [17,18], so we wondered whether IKKβ^{SSEE} – mediated induction of osteoclastogenesis required these inflammatory modulators. We demonstrated by quantitative real-time PCR and ELISA that in addition to stimulating osteoclastogenesis, IKKβ^{SSEE} induced expression of the pro-inflammatory cytokines, TNFα and IL-1β, by monocytes. Since these two cytokines have been shown to induce RANK-independent osteoclast differentiation from monocytes in the presence of TGF-β [19], we wondered if TNFα and IL-1β were responsible for inducing osteoclast differentiation in our system. We demonstrated in TNFα knockout and IL-1 receptor knockout monocytes that IKKβ^{SSEE} was still capable of inducing osteoclast differentiation *in vitro*. Whether the inflammatory component downstream of IKKβ^{SSEE} plays a role in the arthritis pathogenesis in the adenoviral IKKβ^{SSEE} *in vivo* model is still to be investigated, but it is absolutely clear that constitutively active IKKβ can drive osteoclastogenesis in a cell-autonomous, NF-κB-dependent, inflammatory cytokine-independent manner.

Altogether, our work has identified IKKβ as a central regulator of the osteoclast program. It is essential for normal differentiation of osteoclasts in response to RANKL, and gain-of-function results in spontaneous osteoclast formation in the absence of RANKL. It is

likely that IKKβ gain-of-function – whether mutational or signal-induced - plays a role in osteolysis in patients with inflammatory diseases, and our work leads us to strongly support targeting of IKKβ for amelioration of these conditions.

FUTURE DIRECTIONS

Screening Patients with Inflammatory Bone Loss for IKKβ Gain-of-Function Mutations

Our results strongly suggest that gain-of-function in IKKβ results in spontaneous osteoclastogenesis that occurs in the absence of upstream signals. Since IKKβ is an essential downstream component in RANKL-induced osteoclast differentiation [10], and since gain-of-function in the RANKL/RANK pathway results in pathological conditions of heightened bone turnover [20,21], it is likely that gain-of-function mutations in IKKβ could cause osteolysis in human patients. Currently, genetic diagnosis of many of these conditions remains elusive [22,23]. A diagnostic approach which involves screening for IKKβ mutations may lend insight into the pathogenesis of these and other inflammatory conditions involving heightened bone turnover.

Identification of Substrates of IKKβ Important in Osteoclastogenesis

IKKβ has recently been shown to phosphorylate several proteins in addition to its canonical target, IκBα [24]. When we overexpressed p65/RelA in monocytes, mimicking the outcome of phosphorylation-dependent degradation of IκBα, we did not observe

induction of osteoclastogenesis. However, given that IKKβ^{SSEE} induces osteoclastogenesis in a kinase-dependent manner, it is likely that there are additional targets of IKKβ in the monocyte which mediate this effect. We propose a high-power approach to screen multiple targets of IKKβ^{SSEE} with the use of phosphoprotein detection by mass spectrometry [25]. Identification of these molecules will lead to a better understanding of the mechanisms through which IKKβ activates the osteoclast program. Furthermore, identification of these molecules will lend insight into potential modes of therapy for inflammatory conditions.

Structure Function Analysis of IKKβ for Therapeutic Peptide Design

We have demonstrated that constitutively active IKKβ is an extremely specific inducer of osteoclast differentiation. Constitutively active IKKα, although possessing more than 50% sequence conservation with IKKβ [7], fails to induce osteoclastogenesis. We rationalize that we can take advantage of the differences between IKKβ and IKKα in order to learn which unique regions in IKKβ confer its osteoclast – inducing properties. This can be accomplished through domain swapping by PCR to generate chimeric molecules. Also, structurally unique regions in IKKβ can be sequentially mutated to determine the minimum essential structure which preserves IKKβ function. Important regions may be further investigated biochemically to determine how they regulate IKKβ activity, and they can be targeted or mimicked molecularly to interfere with IKKβ function. This approach will lead to novel therapeutic modalities in inflammatory diseases.

Amelioration of Osteopetrosis

Osteopetrosis is a condition of dense brittle bone owing mostly to defective osteoclast function or differentiation [26]. Most forms of clinical and experimental osteopetrosis are amenable to bone marrow transplantation, which means that correction of a defect in the hematopoietic compartment rescues the osteoclast defect. We have identified a potential approach using viral gene transfer of constitutively active IKKβ to correct an osteoclast differentiation defect in cells. Theoretically, using this approach, osteoclast precursors from an osteopetrotic mouse or congenic mouse, and perhaps a patient whose osteopetrosis results from defective osteoclast differentiation, may be infected with a virus expressing active IKKβ and then reintroduced locally into bone. IKKβ would trigger osteoclast differentiation in progenitors and would correct the osteopetrosis. There are potential pitfalls of this approach related to NF-κB-mediated inflammation. Nevertheless, exploring new means to cure this disease may prove to be therapeutically beneficial in the future.

PERSPECTIVES

Given the wide range of pathological conditions in which NF-κB activity is believed to play a role, an intense effort to develop drugs which target the NF-κB signaling pathway is logical and justified. Our data provide evidence that these drugs may prove to be beneficial in reducing bone – destructive effects of inflammatory conditions. Exciting progress has been made toward discovery of selective small-molecule inhibitors of IKKβ, which are now in the pre-clinical phase of testing [27]. Given the central role for IKKβ in differentiation of the osteoclast, the continued effort to target this molecule provides hope for effective therapy in patients with conditions of inflammatory osteolysis and other conditions of pathologically heightened bone turnover.

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