NLRP12 provides a critical checkpoint for osteoclast differentiation

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The alternative or noncanonical nuclear factor kappa B (NF-κB) pathway regulates the osteoclast (OC) response to receptor activator of nuclear factor kappa B ligand (RANKL) and thus bone metabolism. Although several lines of evidence support the emerging concept that nucleotide-binding leucine-rich repeat and pyrin domain-containing receptor 12 (NLRP12) impedes alternative NF-κB activation in innate immune cells, a functional role for NLRP12 outside an inflammatory disease model has yet to be reported. Our study demonstrates that NLRP12 has a protective role in bone via suppression of alternative NF-κB–induced osteoclastogenesis and is down-modulated in response to osteoclastogenic stimuli. Here, we show that retroviral overexpression of NLRP12 suppressed RelB nuclear translocation and OC formation. Conversely, genetic ablation of NLRP12 promoted NIK stabilization, RelB nuclear translocation, and increased osteoclastogenesis in vitro. Using radiation chimeras, we demonstrated these in vitro observations dovetail with our in vivo findings that NLRP12 deficiency leads to enhanced OC numbers accompanied by a significant decline in bone mass under physiological conditions. Consistent with the basal bone phenotype, we also observed an enhanced osteolytic response following RANKL injection over the calvaria of NLRP12-deficient chimeric mice compared with wild-type control mice. Thus, modulation of NLRP12 levels controls alternative NF-κB signaling in OC precursors, altering bone homeostasis and osteolytic responses.

NLRP12 | osteoclast | bone | alternative NF-κB

The unique capacity of osteoclasts (OCs) to resorb bone, in both physiological and pathological contexts, highlights the importance for understanding the molecular mechanisms controlling their development and activity (1). OCs originate from myeloid/macrophage lineage cells in the bone marrow. Macrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor that not only promotes proliferation and survival of myeloid progenitors but also induces expression of receptor activator of NF-κB (RANK), the receptor for RANKL. RANKL, a tumor necrosis factor (TNF) superfamily member, works in concert with M-CSF to instruct myeloid precursor cells to undergo differentiation into OCs and orchestrates the activation of several essential downstream signaling pathways, including both classical and alternative nuclear factor-κB (NF-κB).

In addition to its well-characterized role in host defense and modulating inflammatory responses in immune cells, NF-κB has a critical utility in controlling skeletal mass via its regulatory role in OC development. In the classical pathway, RANK ligation triggers the degradation of NF-κB–associated IκBs. Consequently, the pathway is activated by lifting this cytoplasmic restraint to allow the nuclear translocation of several NF-κB complexes, mainly the p50/p65 (RelA) heterodimer, to induce transcription of target genes. Using a combination of genetic and biochemical approaches, we previously demonstrated that the NF-κB subunit p65 blocks the RANKL-associated cell death pathway during OC differentiation (2). In this respect, p65 behaves as a prosurvival factor that supports OC development indirectly by preventing JNK-mediated RANKL-induced apoptosis. The alternative NF-κB pathway regulates OC differentiation independently of controlling prosurvival pathways and uses a different repertoire of adaptors and kinases to transduce signal. Intracellular accumulation and stability of NF-κB–inducing kinase (NIK) is an essential prerequisite for instigating alternative NF-κB signaling. In this regard, NIK behaves as a regulatory switch because its RANKL-induced accumulation leads to activation of inhibitor of κB kinase alpha (IKKα). This kinase targets p100, leading to its processing to p52, and promotes the subsequent translocation of the p52/RelB NF-κB heterodimer into the nucleus to drive gene expression. Genetic ablation of NIK or RelB severely impairs OC formation in vitro (3, 4). Intriguingly, both NIK and RelB-deficient mice exhibit relatively normal OC function in vivo under physiological conditions; however, they are very resistant to multiple forms of pathological bone loss (3, 4). Moreover, the differentiation defect in $Nik^{-/-}$ or $RelB^{-/-}$ OC precursors is rescued by virally reconstituting the expression of RelB, the key signaling component downstream of NIK, thus demonstrating that both NIK and RelB are critical components of alternative NF-κB–mediated osteoclastogenesis.

In the absence of receptor ligation, de novo NIK does not detectably accumulate and undergoes constitutive degradation as a consequence of its direct association with TNFR-associated

Significance

Members of the nucleotide-binding leucine-rich repeat-containing receptor (NLR) family are generally thought of as initiators of inflammation and are important in a number of inflammatory diseases. However, recent evidence has started to emerge that several NLRs can serve as checkpoint proteins against specific inflammatory pathways. Although checkpoint proteins are well accepted for their importance in adaptive immunity, their roles in innate immunity are still nascent. Receptor activator of nuclear factor kappa B ligand (RANKL), a tumor necrosis factor family cytokine responsible for basal and most forms of pathologic osteoclastogenesis, sends important differentiation signals through the alternative nuclear factor kappa B pathway. This report shows that an NLR member, nucleotide-binding leucine-rich repeat and pyrin domain-containing receptor 12, provides a brake on the activity of RANKL even in noninflammatory settings, extending the role for this type of NLR beyond inflammation-related disease.

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factor 3 (TRAF3), an intrinsic negative regulatory component of the E3 ligase complex (TRAF2:TRAF3:cIAP1/2) (5–8). Recruitment by TRAF3 brings NIK into close molecular proximity with other complex members, TRAF2 and cellular inhibitor of apoptosis 1/2 (cIAP1/2), the latter of which ubiquitylates NIK, leading to its proteasomal degradation. Hence, recruitment of NIK by the E3 ligase regulatory complex is a molecular restraint that renders the alternative NF-κB pathway inactive under basal conditions.

NLRP12, a recently identified nucleotide-binding leucine-rich repeat and pyrin domain-containing receptor (NLR), possesses a characteristic NLR tripartite domain architecture that facilitates the assembly of multimeric complexes encompassing a wide spectrum of recruited adaptors and kinases. NLRs are intracellular pattern-recognition receptors that function as signaling platforms of the innate immune system in response to internal danger signals such as microbial challenge and environmental stressors. Historically, most NLRs have been ascribed a proinflammatory role, with most possessing inflammasome-activating potential. However, NLRP12 has been assigned to an emerging class of inhibitory or checkpoint NLRs (9–11), although it can exhibit inflammasome function with specific stimuli (12, 13). NLRP12 is predominately expressed in immune cells, specifically cells of the myeloid/monocytic lineage, including macrophages, granulocytes, and immature dendritic cells (14–17). Intriguingly, NLRP12 expression is induced by nitric oxide but transcriptionally down-regulated in response to pathogens, their products, and proinflammatory molecules, thus engendering the notion that NLRP12 functions to restrain inputs that promote innate immune responses. Indeed, NLRP12 has been reported to negate the function of several important effector molecules downstream of TNFR and toll-like receptor (TLR) signaling such as NIK and IRAK-1 in human monocytes (11, 14, 16, 18). Of note, all of the presently known inhibitory NLRs function to attenuate classical NF-κB signal transduction, but NLRP12 has uniquely been demonstrated to also impede alternative NF-κB activation (10, 14). Mechanistically, NLRP12 blocks the hyperphosphorylation of IRAK-1, ultimately limiting downstream activation of the classical NF-κB pathway, and NLRP12 deficiency potentiates phosphorylation of p105 and nuclear trafficking of p65 in response to TLR ligation (15, 19). NLRP12 was also shown to antagonize alternative NF-κB in monocytes by directly associating with TRAF3 and NIK, thus limiting basal activation of the pathway by preventing the degradation of TRAF3 and instigating the proteasomal degradation of NIK (11, 14, 16, 18, 20, 21).

Here we show that NLRP12 is transcriptionally down-regulated in response to osteoclastogenic cytokines M-CSF and RANKL and functions as a critical checkpoint protein of alternative NF-κB–mediated osteoclastogenesis. We also provide genetic evidence that NLRP12 has a fundamental physiological and pathophysiological role in bone turnover as NLRP12 deficiency promotes OC formation and decreases bone mass in vivo. Together, these data clearly demonstrate a role for NLRP12 outside the context of controlling innate immunity.

Results

NLRP12 Deficiency Potentiates Osteoclast Formation. Because NLRP12 regulates NF-κB pathways in myeloid lineage cells, we tested whether NLRP12 deficiency modulates OC formation, a process dependent on NF-κB. First, wild-type (*Wt*) and *Nlrp12*$^{-/-}$ unfractionated whole bone-marrow cells were cultured in vitro

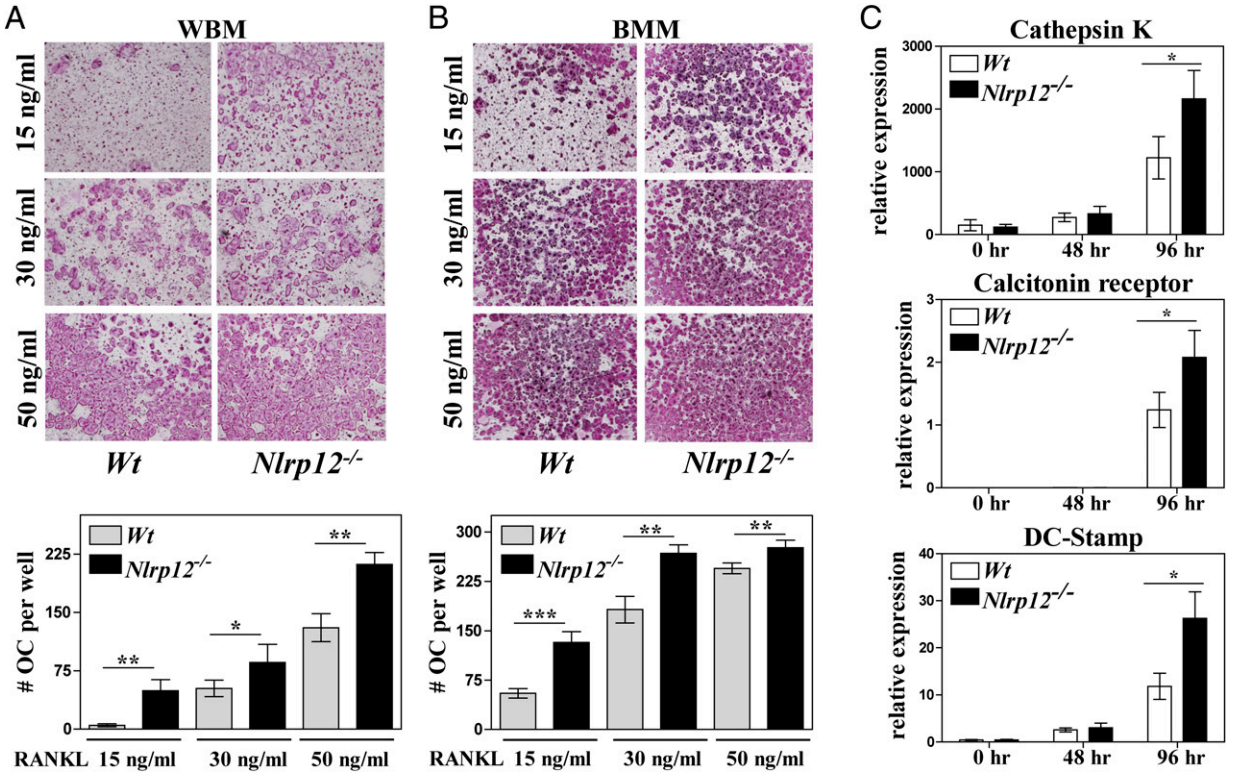

Fig. 1. NLRP12 deficiency augments OC formation and expression of OC-specific genes in vitro. *Wt* and *Nlrp12*$^{-/-}$ unfractionated whole bone marrow (WBM) (*A*) or BMM (*B*) cells were cultured under osteoclastogenic conditions for 4–5 d. Differentiation was assessed by quantitating the number of multinucleated (more than three nuclei per cell) TRAP-positive cells. (*C*) BMMs induced to differentiate into OCs in the presence of M-CSF and RANKL (30 ng/mL) were analyzed by quantitative RT-PCR for relative expression of cathepsinK, calcitonin receptor, and DC-Stamp, normalized to cyclophilin. Data show mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$.

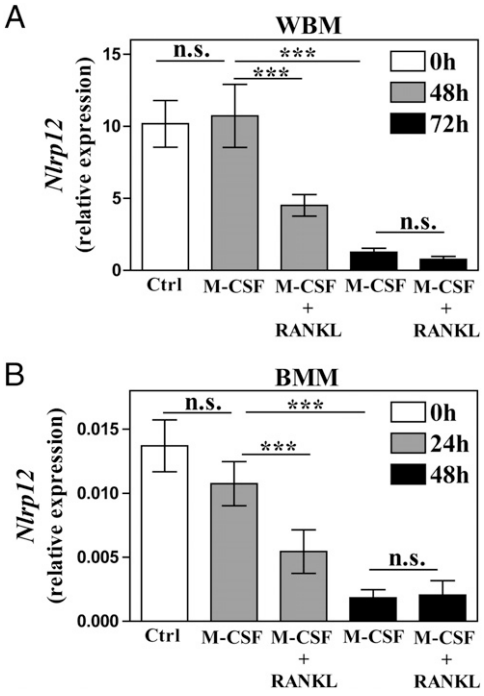

Fig. 2. *Nlrp12* expression is suppressed during osteoclastogenesis in vitro. (*A*) WBM cells (Ctrl, 0 h) were cultured with M-CSF alone or M-CSF and RANKL (30 ng/mL) for 48 and 72 h, then analyzed by quantitative RT-PCR for *Nlrp12* expression, normalized to cyclophilin. (*B*) BMM cells (Ctrl, 0 h), generated by culturing in M-CSF for 4 d, were further cultured in M-CSF with or without RANKL (30 ng/mL) for 24 and 48 h, then analyzed as in *A*. Data show mean ± SD of three independent experiments. ***$P < 0.001$.

with M-CSF and RANKL for 6 d to generate OCs. As shown in Fig. 1*A*, NLRP12 deficiency increases the formation of multinuclear tartrate-resistant acid phosphatase (TRAP)-positive cells (three or more nuclei) relative to *Wt* over a range of RANKL doses. To determine whether this result could be due to an expansion in the number of OC precursors, we performed flow cytometry on unfractionated whole bone marrow and found the proportion of cells in CD11b/GR1 fractions to be unchanged, as was the level of CD117 in these fractions (Fig. S1). We then differentiated OCs from M-CSF–expanded bone marrow macrophages (BMMs) and observed a similar enhancement of OC formation in NLRP12-deficient cultures compared with *Wt* controls (Fig. 1*B*). Reflecting a greater number of mature OCs in *Nlrp12*$^{-/-}$ cultures, transcript levels of OC differentiation markers cathepsin K, DC-Stamp, and calcitonin receptor were all significantly elevated in the absence of the NLRP12 at a low dose of RANKL (Fig. 1*C*). However, proliferation and RANK expression in response to M-CSF and RANKL were comparable between *Wt* and NLRP12-deficient BMMs (Fig. S2). Taken together, these results suggest that NLRP12 provides a checkpoint for osteoclastogenesis by reducing sensitivity of OC precursors to RANKL rather than changing the number of precursors available for differentiation.

NLRP12 Is Down-Regulated by M-CSF and RANKL. Because NLRP12 deficiency promoted OC differentiation from both the early precursors present in whole bone marrow as well as expanded BMMs, we expected to find NLRP12 down-regulation in response to RANKL. To examine *Nlrp12* expression in response to osteoclastogenic stimuli, we exposed unfractionated whole bone-marrow cells to M-CSF in vitro for 48–72 h, with or without RANKL. As expected, RANKL treatment decreased *Nlrp12* transcript levels compared with M-CSF alone, as determined by quantitative RT-PCR (Fig. 2*A*). Surprisingly, we also observed a dramatic (10-fold) decrease in *Nlrp12* expression between 48 and 72 h in M-CSF alone. Next, we performed a similar experiment, this time starting with BMMs that had been expanded from whole marrow for 4 d in M-CSF (control) and treated with M-CSF, with or without RANKL, for an additional 24–48 h. Again, RANKL decreased *Nlrp12* levels compared with M-CSF alone at 24 h, but by 48 h, levels were very low and not different between conditions (Fig. 2*B*). Furthermore, *Nlrp12* levels remain low in mature OCs (Fig. S3). These results demonstrate that *Nlrp12* transcription is down-regulated during M-CSF–driven differentiation of murine myeloid progenitors and is further reduced by costimulation with RANKL. Given that *Nlrp12*$^{-/-}$ cultures showed enhanced osteoclastogenesis from both early and late precursor populations (corresponding to those examined for *Nlrp12* expression here), it appears that NLRP12 maintains its checkpoint function over a wide range of expression levels.

NLRP12 Suppresses Alternative NF-κB Activation in Osteoclast Precursors. Because NF-κB drives OC differentiation in response to RANKL and several reports demonstrate that NLRP12 attenuates NF-κB activation to control inflammatory responses in myeloid cells, we sought to investigate whether the absence of NLRP12 in OC precursors would lead to the hyperactivation of NF-κB. To clarify whether NLRP12 impacts the classical NF-κB pathway leading to p65 activation, we examined the levels of p65 protein in the nuclear and cytoplasmic fractions of *Wt* and NLRP12-deficient BMMs in response to RANKL. As shown in Fig. 3*A*, the nuclear translocation of p65 was comparable between *Wt* and NLRP12-deficient BMMs

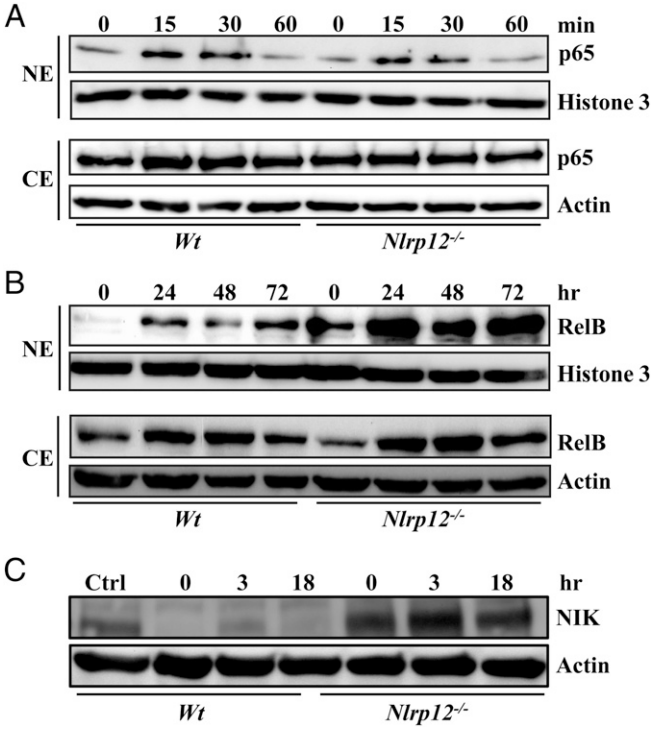

Fig. 3. NLRP12 deficiency enhances alternative NF-κB activation. *Wt* and *Nlrp12*$^{-/-}$ BMMs were stimulated with RANKL (30 ng/mL) for the times indicated. Extracts from the nuclear (*Top* NE) and cytoplasmic (*Bottom* CE) fractions were analyzed by immunoblot for p65 (*A*) or RelB (*B*). (*C*) NIK was assessed by immunoblot analysis from whole cell lysates of *Wt* and NLRP12-deficient BMMs stimulated with RANKL (30 ng/mL) for the times indicated. Control in *C* (Ctrl) is *Wt* BMMs treated with MG-132 (10 μM) for 4 hr. Data are representative of three independent experiments.

for all time points observed, suggesting that the suppressive effect of NLRP12 on OC formation is likely not mediated by p65 inhibition.

To address whether loss of NLRP12 affects the alternative NF-κB pathway in OC precursors, we stimulated *Wt* and *Nlrp12*$^{-/-}$ BMMs with RANKL and monitored levels of RelB, a key alternative pathway subunit, in the nuclear and cytoplasmic fractions (Fig. 3*B*). NLRP12 deficiency significantly enhanced the translocation of RelB into the nuclear compartment, including in unstimulated BMMs and with 24–72 h of RANKL stimulation, compared with *Wt* controls. This activation of alternative NF-κB occurred upstream at the level of NIK stabilization, as we observed increased levels of NIK protein in BMMs, with or without RANKL treatment in the absence of NLRP12 (Fig. 3*C*).

Because *Nlrp12* expression is rapidly down-regulated by RANKL, we asked if overexpression of this molecule could inhibit OC differentiation. To this end, we cloned a FLAG-tagged *Nlrp12* cDNA into the pMX retroviral vector and transduced *Wt* BMMs with this retrovirus. Following blasticidin selection, transduced cells were cultured in osteoclastogenic conditions. OC numbers were significantly reduced in cultures overexpressing NLRP12, compared with those transduced with empty vector (Fig. 4*A*). Supporting a role for NLRP12 in inhibiting alternative NF-κB, RANKL-induced RelB nuclear translocation was significantly reduced by NLRP12 overexpression (Fig. 4*B*). These data indicate that NLRP12 alters the cellular response of myeloid precursors to osteoclast-inducing stimuli by limiting activation of the alternative NF-κB pathway.

NLRP12 Limits Osteoclastogenesis and Bone Resorption in Vivo. Next, we sought to characterize the physiological role of NLRP12 on OC development and bone resorption in vivo. Although NLRP12 expression is primarily found in cells of myeloid lineage, we generated radiation chimeras to exclude any modulatory effects of NLRP12 in osteoblasts. Eight weeks after bone marrow transplantation into wild-type recipients, micro-CT analysis of tibiae revealed that the trabecular bone volume fraction (BV/TV) was significantly reduced in recipients of NLRP12-deficient marrow compared with *Wt* controls (Fig. 5*A*). Likewise, NLRP12 deficiency coincided with a marked decrease in bone mineral density accompanied by significant trabecular thinning (Fig. 5 *B* and *C*). In support of these findings, histomorphometric analysis demonstrated a significant

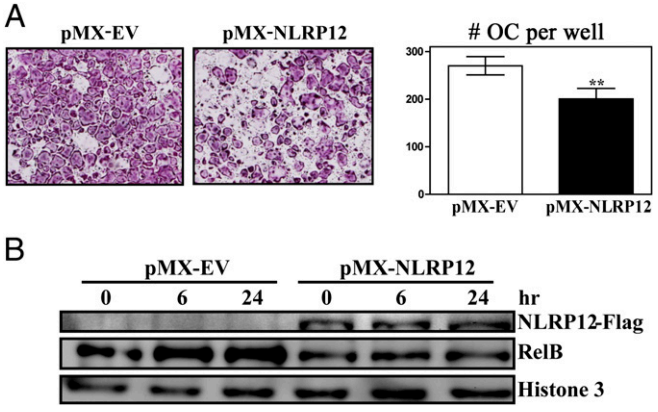

Fig. 4. Overexpression of NLRP12 blunts OC formation and attenuates nuclear translocation of RelB. *Wt* BMMs were retrovirally infected with full-length NLRP12 or empty vector (control), followed by M-CSF and RANKL (30 ng/mL) stimulation. (*A*) OC formation was determined via enumeration of TRAP-positive cells containing more than three nuclei. Numbers are mean ± SD of multinuclear cells (**$P < 0.01$; $n = 3$). Data are representative of three independent experiments. (*B*) Nuclear translocation of RelB was evaluated by immunoblot analysis of nuclear fractions for the times indicated.

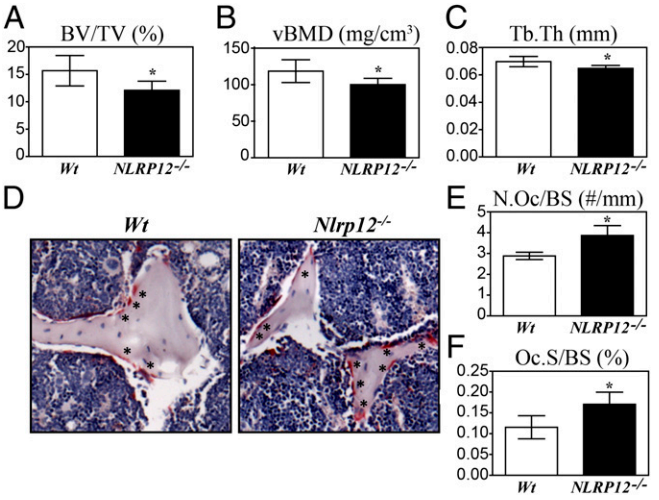

Fig. 5. NLRP12-deficient chimeric mice exhibit significantly lower bone mass and enhanced osteoclast formation under basal conditions in vivo. Wild-type mice were irradiated and reconstituted with either *Wt* or *Nlrp12*$^{-/-}$ bone marrow cells. Eight weeks postreconstitution, tibias were scanned by micro-CT and trabecular bone parameters were analyzed. (*A*) Bone volume per total volume (BV/TV). (*B*) Bone mineral density (vBMD). (*C*) Trabecular thickness (Tb.Th). (*D*) TRAP staining of tibial sections of mice reconstituted with *Wt* or *Nlrp12*$^{-/-}$ marrow; OCs are stained red (annotated by asterisks). Histomorphometric analysis was used to determine (*E*) osteoclast number per bone surface (N.Oc/BS) and (*F*) osteoclast surface per bone surface (Oc.S/BS). All data are mean ± SD *$P < 0.05$ compared with *Wt*; $n = 10$ per group.

increase in OC number and surface (Fig. 5 *D*–*F*) in radiation chimeras bearing NLRP12-deficient bone marrow relative to *Wt*.

We and others have previously shown that genetic ablation of critical components of alternative NF-κB signaling render mice severely impaired in their ability to form OCs in vitro and recalcitrant to multiple forms of pathological bone loss in vivo (3, 22–26). Therefore, we next wanted to assess the protective potential of NLRP12 under pathological, yet noninflammatory conditions. To address this question, we turned to a RANKL-induced bone loss model, in which RANKL was injected daily over the periosteum of mouse calvaria for 5 d. Echoing the basal bone phenotype, we observed an enhanced osteolytic response of NLRP12 chimeric mice relative to controls, as assessed by micro-CT (Fig. 6*A*). Quantitation of volumetric bone mineral density for the crown of the calvaria, relative to PBS controls, demonstrates that NLRP12-deficient mice exhibit a 50% increase in bone resorption in response to RANKL injection compared with *Wt* controls (Fig. 6*B*). Collectively, the findings indicate that NLRP12 has a protective role in bone by limiting bone resorption under both homeostatic and pathological conditions, in vivo, independent of its reported role in controlling inflammatory responses in myeloid progenitors.

Discussion

Alternative NF-κB activation is critical for mediating OC formation in vitro and for pathological osteolysis in vivo. Here, we demonstrate that NLRP12 functions as an important checkpoint protein that restrains alternative NF-κB–driven osteoclastogenesis and functions to control skeletal turnover, both basally and under conditions of RANKL-induced osteolysis. Our study reveals that NLRP12 has a protective role in bone and is to our knowledge the first reported example of an NLR to suppress osteoclastogenesis, thus expanding its biological importance beyond attenuating NF-κB–mediated inflammatory responses.

OC formation is dictated by external cues including M-CSF and RANKL, which signal through their respective receptors and initiate

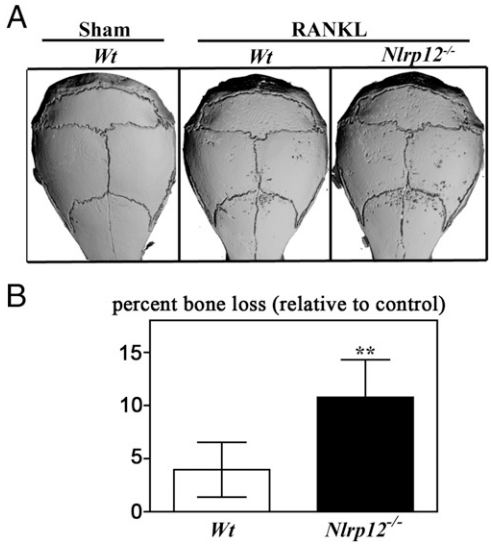

Fig. 6. RANKL-induced osteolysis is significantly enhanced in NLRP12-deficient chimeric mice in vivo. RANKL (1.5 mg/kg body weight) or PBS (sham control) was injected over the calvaria of *Wt* or NLRP12-deficient chimeric mice once per day for 5 d. (*A*) Micro-CT was used to determine the volumetric bone density and it was expressed as percent bone loss relative to respective PBS controls (*B*). $^{**}P < 0.01$ compared with *Wt*; $n = 10$ per group.

activation of the alternative NF-κB pathway in myeloid progenitors in the bone marrow (27). We observed that NLRP12 is transcriptionally down-regulated during myeloid differentiation under conditions of prolonged M-CSF exposure, in line with previous reports of GM-CSF differentiation-associated and inflammatory-driven NLRP12 suppression. Strikingly, costimulation with M-CSF and RANKL significantly augmented the repression in unfractionated bone marrow precursors as well as BMMs, compared with M-CSF alone. *Nlrp12* expression remained extremely low in mature OCs, suggesting the NLRP12 checkpoint function likely occurs at the level of differentiation as wild-type osteoclasts are unlikely to contain NLRP12 at the mature, bone-resorbing stage.

A recent study identified that B lymphocyte-induced maturation protein 1 (Blimp1) binds the NLRP12 promoter, leading to a partial inhibition of NLRP12 transcription in human monocytes (28). However, it is unlikely that Blimp1 plays a significant role in the suppression of NLRP12 in murine OC precursors, as NLRP12 is down-regulated before RANKL-induced Blimp1 induction in our culture conditions (Fig. S4). Further studies are needed to elucidate the molecular basis of the observed M-CSF and RANKL-mediated transcriptional repression of NLRP12.

It is intriguing that we found significant enhancement of osteoclastogenesis in *Nlrp12*$^{-/-}$ precursors whether the differentiation was initiated from whole bone marrow or expanded BMMs, given that the latter population starts with 10-fold lower *Nlrp12* transcript levels. Although it is possible that NLRP12 protein is long-lived, and that its loss lags behind that of mRNA, it seems unlikely that BMMs differentiated for several days retain similar protein levels as early progenitors. Rather, it appears that NLRP12 can regulate alternative NF-κB signaling over a wide dynamic range. Unfortunately, antibody reagents are not available at this time to address this question directly. NIK levels also appear to be exquisitely regulated, as this protein is extremely difficult to detect in M-CSF–expanded BMMs, and remains at the lower limits of detection by immunoblot even after RANKL stimulation, which should stabilize it, and when downstream events in the alternative NF-κB pathway are readily detectable. In fact, we found that absence of NLRP12 leads to increased NIK stability in BMMs with or without RANKL. Congruently, we found that nuclear accumulation of RelB was augmented in untreated *Nlrp12*$^{-/-}$ BMMs and after exposure to RANKL. Because both NIK and RelB are critical for osteoclastogenesis in vitro, the regulation of these alternative NF-κB proteins is the likely mechanism by which NLRP12 limits OC differentiation.

Whereas mice lacking either NIK or RelB are resistant to pathological bone loss in vivo, deficiency of NLRP12 has the opposite effect, sensitizing mice to osteoclastogenic stimuli. Using radiation chimeras, we found that loss of NLRP12 in bone marrow cells disrupts bone homeostasis and leads to a decrease in bone mass associated with an increase in OC numbers. Similarly, we find that NLRP12 deficiency leads to an increased susceptibility to bone loss in response to supracalvarial injection of RANKL, a noninflammatory model of focal osteolysis that allowed us to disengage the protective role of NLRP12 in bone from its established role in attenuation of inflammatory responses. Because elevation of RANKL is a final common event that occurs in most forms of pathological osteolysis, both inflammatory and not, and initiates critical signals through the alternative NF-κB pathway, it is likely that NLRP12 plays an important regulatory role in limiting most forms of bone loss.

Numerous reports have shown that NLRP12 impedes alternative NF-κB signaling to attenuate inflammatory responses within the myeloid lineage (11, 15, 16, 18). NLRP12 interacts not only with NIK, but also TRAF3, which is directly involved in proteasome-mediated degradation of NIK, thus safeguarding against alternative pathway activation in innate immune cells under basal conditions. Our studies support and extend those findings, indicating a role for NLRP12 in negatively regulating OC development via down-regulation of the alternative NF-κB pathway. Using human monocytes, one study demonstrated NLRP12-mediated inhibition of classical NF-κB in response to TLR ligation (16). However, we observed that RANKL-induced nuclear translocation of the p65 subunit was comparable between *Wt* and NLRP12-deficient BMMs, suggesting that NLRP12 does not modulate osteoclastogenesis via the classical NF-κB pathway. Thus, it appears that the molecular events in which NLRP12 participates are dependent on cell type and stimulus. Furthermore, NLRP12 is an adaptor molecule and its scaffolding activity (i.e., ability to bring together other signaling molecules such as NIK and TRAF3) could be regulated by ligands in a fashion similar to other members of the NLR family. More work is needed to elucidate the exact signals and interactions that determine the effects of NLRP12.

Nlrp12 mutations in humans are linked to autoimmune hereditary periodic fevers that result from a loss of regulatory control over either inflammasome formation or NF-κB activation (17, 29, 30). Musculoskeletal manifestations with associated joint pain have been reported, but no thorough clinical evaluation of bone phenotype has been conducted. By demonstrating that NLRP12 restrains bone resorption, even in the absence of overt inflammation, we have established a role for NLRP12 outside of controlling immune responses to inflammatory stimuli. Our study positions NLRP12 as a previously unidentified member of the ever-growing list of molecules shared by bone and the immune system that define the field of osteoimmunology, and improved understanding of its role broadens our understanding of the complex regulatory pathways at play.

Materials and Methods

Reagents. FBS, penicillin, and streptomycin were from Gibco-BRL. α-MEM and MG-132 were obtained from Sigma-Aldrich. M-CSF in the form of CMG 14–12 supernatant and GST–RANKL were prepared as previously described (4). Antibodies for immunoblotting were from the following vendors: anti-RelB (no. 4954), anti-p65 NF-κB (no. 3034), anti-NIK (4994S), anti-histone H3 (96C10) (no. 3638) from Cell Signaling Technology and anti–β-actin (A2228) and anti-FLAG (F3165) from Sigma-Aldrich.

Mice. All animal procedures were approved by Washington University and University of North Carolina Institutional Animal Care and Use Committees, in compliance with established federal and state policies. C57BL/6J mice (*Wt*) were purchased from The Jackson Laboratory. Age and sex-matched NLRP12-deficient bone marrow used in vitro and to generate chimeric mice was obtained from long bones shipped overnight on ice. *Nlrp12*$^{-/-}$ mice were generated by homologous recombination and backcrossed for nine generations to C57BL/6J as previously described (31). For chimeric mice, recipient 6-wk-old C57BL/6J mice were lethally irradiated (10 Gy total body γ-irradiation) 8 h before injection of bone marrow cells (5×10^6) from *Wt* or *Nlrp12*$^{-/-}$ donors via tail vein. Recipients were allowed to reconstitute for 6–8 wk before use.

OC Culture. To generate OCs from unfractionated bone marrow cells, bone marrow was harvested from the long bones of 10- to 12-wk-old mice and cells (5×10^4) were cultured in α-MEM plus 10% (vol/vol) FBS containing a 1:50 dilution of CMG 14–12 cell supernatant (30) (containing equivalent of 20 ng/mL of M-CSF) and GST–RANKL at indicated doses in 96-well tissue-cultured–treated plates with media changes every other day. To obtain OCs from enriched BMMs, bone marrow cells were cultured in α-MEM plus 10% (vol/vol) FBS and a 1:10 dilution of CMG 14–12 cell supernatant (containing equivalent of 100 ng/mL of M-CSF) for 4 d to expand BMMs. Nonadherent cells were removed by several washes in PBS and adherent BMMs were detached with trypsin-EDTA, plated at 8×10^3 per well in a 96-well plate and cultured as described above. For TRAP staining, cells were fixed in 4% (vol/vol) ultrapure formaldehyde (Polysciences) plus 0.1% Triton X-100 in PBS for 10 min at room temperature, rinsed gently with water, and incubated with TRAP staining solution according to manufacturer protocol (Sigma-Aldrich). Multinucleated (more than three nuclei) TRAP-positive OCs were enumerated in triplicate wells and counted by imaging the entire well (encompassed in a single 2× field) using an Olympus BX41 microscope.

Quantitative Real-Time RT-PCR. Methods and primer sequences can be found in *SI Materials and Methods*.

Immunoblotting and Subcellular Fractionation. Cells were lysed and processed by standard methods detailed in *SI Materials and Methods*.

NLRP12 Retroviral Overexpression. Full-length human NLRP12 was PCR amplified with an in-frame sequence encoding a FLAG tag at the N terminus to facilitate detection, subcloned into a pMX retroviral vector, and sequence verified before use (16). The generated NLRP12–pMX construct was transiently transfected into Plat-E packaging cells via the calcium phosphate-precipitation method. Virus was harvested 48 h posttransfection and used to infect BMMs for 48 h in the presence of 20 ng/mL M-CSF equivalent and 4 μg/mL polybrene, followed by selection in blasticidin for 3 d before culture with 20 ng/mL M-CSF equivalent and GST–RANKL (30 ng/mL).

Microcomputed Tomography and Bone Histomorphometric Analysis. Calvariae and tibias were scanned using a microcomputed tomography 40 (μCT40) or VivaCT40 scanners (Scanco), respectively, according to guidelines for assessment of bone microarchitecture in rodents (32), and data were analyzed by a blinded observer. Further details can be found in *SI Materials and Methods*. Tibias were fixed in 10% (vol/vol) neutral buffered formalin for 24 h, processed through a series of graded alcohols, decalcified, and embedded in paraffin for sectioning. Sections for histomorphometry were stained for TRAP. Histomorphometric measurements were conducted by a blinded observer according to the American Society for Bone and Mineral Research guidelines using BIOQUANT OSTEO 2012 software (BIOQUANT Image Analysis) and standard parameters (33).

Statistical Analysis. Data reported graphically for *Nlrp12*, *Rank*, and *Blimp1* expression were evaluated by ANOVA and the Tukey–Kramer multiple comparisons test (GraphPad InStat). For all other data, unpaired one-tailed Student's *t* tests were performed and $P < 0.05$ was taken as the level of significance.

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1. Novack DV, Teitelbaum SL (2008) The osteoclast: Friend or foe? *Annu Rev Pathol* 3:457–484.
2. Vaira S, et al. (2008) RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest* 118(6):2088–2097.
3. Vaira S, et al. (2008) RelB is the NF-κB subunit downstream of NIK responsible for osteoclast differentiation. *Proc Natl Acad Sci USA* 105(10):3897–3902.
4. Novack DV, et al. (2003) The IkappaB function of NF-κB2 p100 controls stimulated osteoclastogenesis. *J Exp Med* 198(5):771–781.
5. Razani B, et al. (2010) Negative feedback in noncanonical NF-κB signaling modulates NIK stability through IKKα-mediated phosphorylation. *Sci Signal* 3(123):ra41.
6. Zarnegar B, Yamazaki S, He JQ, Cheng G (2008) Control of canonical NF-κB activation through the NIK-IKK complex pathway. *Proc Natl Acad Sci USA* 105(9):3503–3508.
7. Xiao G, Fong A, Sun SC (2004) Induction of p100 processing by NF-κB-inducing kinase involves docking IκB kinase α (IKKα) to p100 and IKKα-mediated phosphorylation. *J Biol Chem* 279(29):30099–30105.
8. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: The control of NF-κB activity. *Annu Rev Immunol* 18:621–663.
9. Zhong Y, Kinio A, Saleh M (2013) Functions of NOD-like receptors in human diseases. *Front Immunol* 4:333.
10. Lupfer C, Kanneganti TD (2013) Unsolved mysteries in NLR biology. *Front Immunol* 4:285.
11. Ye Z, et al. (2008) ATP binding by monarch-1/NLRP12 is critical for its inhibitory function. *Mol Cell Biol* 28(5):1841–1850.
12. Watari K, et al. (2000) Identification of a melanoma antigen, PRAME, as a BCR/ABL-inducible gene. *FEBS Lett* 466(2-3):367–371.
13. Vladimer GI, et al. (2012) The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity* 37(1):96–107.
14. Allen IC, et al. (2012) NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-κB signaling. *Immunity* 36(5): 742–754.
15. Zaki MH, et al. (2011) The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* 20(5):649–660.
16. Williams KL, et al. (2005) The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor α-, and Mycobacterium tuberculosis-induced pro-inflammatory signals. *J Biol Chem* 280(48):39914–39924.
17. Arthur JC, et al. (2010) Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *J Immunol* 185(8):4515–4519.
18. Lich JD, et al. (2007) Monarch-1 suppresses non-canonical NF-κB activation and p52-dependent chemokine expression in monocytes. *J Immunol* 178(3):1256–1260.
19. Zaki MH, Man SM, Vogel P, Lamkanfi M, Kanneganti TD (2014) Salmonella exploits NLRP12-dependent innate immune signaling to suppress host defenses during infection. *Proc Natl Acad Sci USA* 111(1):385–390.
20. Ting JP, Duncan JA, Lei Y (2010) How the noninflammasome NLRs function in the innate immune system. *Science* 327(5963):286–290.
21. Lich JD, Ting JP (2007) Monarch-1/PYPAF7 and other CATERPILLER (CLR, NOD, NLR) proteins with negative regulatory functions. *Microbes Infect* 9(5):672–676.
22. Soysa NS, Alles N, Takahashi M, Aoki K, Ohya K (2011) Defective nuclear factor-κB-inducing kinase in aly/aly mice prevents bone resorption induced by local injection of lipopolysaccharide. *J Periodontal Res* 46(2):280–284.
23. Aya K, et al. (2005) NF-κB-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. *J Clin Invest* 115(7):1848–1854.
24. Yang C, et al. (2010) NIK stabilization in osteoclasts results in osteoporosis and enhanced inflammatory osteolysis. *PLoS One* 5(11):e15383.
25. Chaisson ML, et al. (2004) Osteoclast differentiation is impaired in the absence of inhibitor of kappa B kinase α. *J Biol Chem* 279(52):54841–54848.
26. Maruyama T, et al. (2010) Processing of the NF-κ B2 precursor p100 to p52 is critical for RANKL-induced osteoclast differentiation. *J Bone Miner Res* 25(5):1058–1067.
27. Teitelbaum SL (2000) Bone resorption by osteoclasts. *Science* 289(5484):1504–1508.
28. Lord CA, et al. (2009) Blimp-1/PRDM1 mediates transcriptional suppression of the NLR gene NLRP12/Monarch-1. *J Immunol* 182(5):2948–2958.
29. Arimura T, et al. (2001) Identification, characterization, and functional analysis of heart-specific myosin light chain phosphatase small subunit. *J Biol Chem* 276(9): 6073–6082.
30. Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J Bone Miner Res* 15(8):1477–1488.
31. Allen IC, et al. (2013) Characterization of NLRP12 during the in vivo host immune response to Klebsiella pneumoniae and Mycobacterium tuberculosis. *PLoS One* 8(4): e60842.
32. Bouxsein ML, et al. (2010) Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 25(7):1468–1486.
33. Dempster DW, et al. (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: A 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 28(1):2–17.