INTRODUCTION

Nucleotide-binding oligomerization domain-like receptors (NLRs) and Toll-like receptors (TLRs) are subsets of germline-encoded pattern recognition receptors (PPRs). NLRs and TLRs are engaged not only by pathogen-associated molecular patterns (PAMPs) but also by sterile exogenous and endogenous danger-associated molecular patterns (DAMPs).1,2 As a result, these PRRs participate in host immunity and homeostasis.3-5
the actions of TLRs in bone metabolism have been extensively studied in humans and animal models, the role that NLRs play in the pathophysiology of this tissue is not well known.

Unlike TLRs, which are present on the plasma membranes and endosomes, NLRs are intracellular proteins, which upon sensing or recognition of PAMPs or DAMPs, trigger the assembly of protein complexes called inflammasomes. The NLR family pyrin domain containing 3 (NLRP3) and NLR family CARD domain containing 4 (NLRC4) inflammasomes are among the most studied family members. They are implicated in various physiological and pathological responses including infections, neuroinflammation, cryopyrin-associated periodic syndromes, and macrophage-activation syndrome owing to their stimulation not only by various PAMPs and DAMPs but also by NLRP3 or NLRC4 gain-of-function mutations. Irrespective of the activation mechanisms, these inflammasomes process pro-interleukin (IL)-1β, pro-IL-18, and gasdermin D (GSDMD) into IL-1β, IL-18, and GSDMD fragments, respectively. GSDMD N-terminal fragments ultimately form pores within the membranes of intracellular organelles such as mitochondria, and within the plasma membrane, through which IL-1β and IL-18 are secreted to the extracellular environment. It is now recognized that extensive pore formation due to excessive stimulation of the inflammasomes causes the inflammatory cell death, pyroptosis, which is characterized by cell lysis, hence, by the uncontrolled release of not only IL-1β and IL-18 but also other numerous inflammatory factors including IL-1α and high mobility group box 1. The adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC) is required for the assembly of the NLRP3 inflammasome, and NLRC4 inflammasome to a lesser extent, whereas the catalytic activity of caspase-1 is central to the function of both inflammasomes. Thus, these inflammasomes compete not only for the substrates (pro-IL-1β, pro-IL-18, and GSDMD) but also for downstream effectors such as caspase-1. Functional redundancy and physical interactions have also been reported between NLRP3 and NLRC4 as they recognize different Salmonella typhimurium PAMPs, some of which induce the recruitment of NLRP3 to NLRC4-nucleated complexes. In addition, NLRP3 and NLRC4 play overlapping roles in sterile neuroinflammation caused by lysophosphatidylcholine. Finally, signaling by these inflammasomes is influenced by other effector molecules, including caspase-8, which is recruited to inflammasome complexes. Caspase-8 is believed to process pro-IL-1β, pro-IL-18, and GSDMD, and regulate necroptosis, pyroptosis, and apoptosis. Thus, NLRP3 and NLRC4 inflammasomes interact not only with each other but also with other inflammatory and death pathways.

Despite advances in the biology of the inflammasomes, only a few studies have explored these pathways in skeletal homeostasis. The NLRP3 inflammasome, the focus of most of these studies, is expressed by bone-forming cells, the osteoblasts, but to a lesser extent compared to macrophages, the precursors of bone-resorbing cells, the osteoclasts. Both cell types influence bone remodeling and repair, interacting with each other and with stromal and immune cells through growth factor and cytokine pathways. NLRP3 harboring activating mutations causes arthropathies in humans, and a severe low bone mass phenotype correlated with a massive expansion of the osteoclast precursors and exaggerated osteoclastogenesis in mice. Thus, a need for extensive research to reveal the functions of the inflammasomes in the bone.

Here, we tested the hypothesis that the NLRP3 and NLRC4 inflammasome pathways play an important role in bone metabolism. We found that loss of NLRP3 or NLRC4 to a lesser extent caused higher baseline bone mass and protected against LPS-induced inflammatory osteolysis. Unexpectedly, the phenotype of WT mice and Nlrp3−/− knockout mice was comparable, suggesting that bone appears to function normally upon compound loss of NLRP3 and NLRC4.

2 | MATERIALS AND METHODS

2.1 | Animals and in vivo experiments

Nlrp3 null (Nlrp3−/−) mice were purchased from Jackson Laboratory. Nlrc4 null (Nlrc4−/−) mice were generously given by Dr. V. M. Dixit (Genentech, South San Francisco, CA, United States of America). All mice were on C57BL6 background, and mouse genotyping was performed by PCR. Nlrp3−/− mice were crossed with Nlrc4−/− mice to generate Nlrp3+/−;Nlrc4−/− (double heterozygous, F1) mice, which were, in turn, inter-crossed to generate F2 homozygous breeders (Nlrp3+/+;Nlrc4+/+, referred here as C57BL/6, Nlrp3−/−; Nlrc4−/−, and Nlrp3−/−;Nlrc4−/−). F3 mice were used for all experiments. For LPS-induced osteolysis, 3- to 4-month-old male mice were injected once subcutaneous and supra-calvaria with 3 mg/kg of E coli LPS (Sigma Aldrich, L2630), and euthanized after 6 days. All procedures were approved by the Institutional Animal
Care and Use Committee of Washington University School of Medicine in St. Louis.

2.2 | Bone mass and microstructure

Post-mortem micro-computed tomography (µCT) system (Scanco µCT40) was performed to evaluate bone parameters in different cohorts of 3- month- and 8-month-old male mice. Briefly, femur removed from euthanized mice were cleaned from soft tissue and stabilized in 2% agarose gel. µCT was used to assess trabecular bone morphology in the distal femoral metaphysis, using 8 μm voxel resolution and 55 kEV. Scans included ~350 slices encompassing the distal epiphysis and metaphysis of the femur, including the region of the growth plate. The metaphysis was analyzed for trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N), separation (Tb.Sp), thickness (Tb.Th), and volumetric bone mineral density (vBMD). The trabecular bone region was identified manually by tracing the region of interest and the images were thresholded using an adaptive-iterative algorithm built in the Scanco analyzer software, as previously described.48

For LPS-induced osteolysis, calvariae were dissected from euthanized animals, stabilized in 2% agarose gel, and the whole calvaria was scanned. 3D reconstructions of imaged calvariae were analyzed with ImageJ software to estimate the resorption pit area versus the total area considered.

2.3 | Cell isolation and cultures

Bone marrow (BM) cells were eluted from femurs and tibias, as previously described.34,48 Briefly, BM-derived macrophages (BMMs) were obtained by culturing BM cells for 5 days in MEM-α (Gibco, 12561-049) with 10% FBS (Gibco, 26140-079), 1% Pen/Strep (Gibco, 15140-122), and a 1:25 dilution of supernatant from CMG 14-12 cells, as a source of macrophage colony-stimulating factor (M-CSF) as previously described.53 To induce OC differentiation, non-adherent cells were removed by vigorous washes with PBS, and adherent BMMs were detached with 0.05% Trypsin-EDTA (Gibco, 25300-054) and plated at 0.5-1 × 10⁶ cells/well in a 96-well plate in culture medium containing a 1:50 dilution of CMG. BMMs were then treated with 25-50 ng/mL receptor activator of NF-kB ligand (RANKL) on the next day after plating (D0) and fed every 2 days afterwards as previously described.48 Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. In some experiments, 100 ng/mL LPS from E coli (Sigma Aldrich, L4391) or 20 ng/mL recombinant mouse TNF-α (aa 80-235, R&D systems) was added on day 2 (D2) of osteoclast differentiation, and the cultures were carried out for additional 24 hours.

2.4 | Inflammasome activation

BMMs or osteoclasts were left untreated or primed with 100 ng/mL LPS. After 3 hours, cells were washed and stimulated with 15 μM nigericin (Sigma Aldrich) to activate the NLRP3 inflammasome. The NLRC4 inflammasome activation was triggered by cytosolic delivery of flagellin using the FlaTox system generously given by Dr Russel Vance (University of California, Berkeley, CA, USA). Briefly, cells were incubated with a mixture of 2 μg/mL LFn-FlaA (a recombinant Legionella pneumophila flagellin, FlaA, fused with the N-terminal domain of Bacillus anthracis lethal factor, LFn), with 4 μg/mL of anthrax protective antigen channel (PA), which mediates the cytosolic delivery of the recombinant protein through the LFn domain.54 All treatments were performed in reduced serum media (0% FBS, 1:50 CMG, with or without RANKL).

2.5 | TRAP assay

Cytochemical staining for TRAP was used to identify osteoclasts as previously described.34 Briefly, cells in a 96-well plate were fixed with 3.7% formaldehyde and 0.1% Triton X-100 for 10 minutes at room temperature. Cells were rinsed with water and incubated with the TRAP staining solution (Acid Phosphatase, Leukocyte Kit, Sigma) at room temperature for 30 minutes. Multinucleated TRAP-positive cells with ≥3 nuclei were scored as OC under light microscopy. Samples were analyzed with an inverted Nikon Eclipse Ti-U microscope and pictures were taken using a coupled QImaging digital camera and MetaMorph software.

2.6 | Resorption assays

Osteoclasts at day 3 of differentiation (D3) growing in 6-well plates were harvested with Trypsin-EDTA and plated on hydroxyapatite-coated 24-well plates (Corning Osteo Assay, 09-762-100). On the next day, LPS or cytokines were added along with RANKL, and cells were grown for additional 2 days. Culture media were discarded, and wells were treated with 10% sodium hypochlorite to remove the cells. Samples were analyzed with an inverted Nikon Eclipse Ti-U microscope, and pictures were taken using a coupled QImaging digital camera and MetaMorph software. Images were analyzed with ImageJ software to estimate the resorption area versus the total area.
2.7 | **Fluorescence microscopy**

BMMs or osteoclasts were plated in 8-well plates for microscopy (µ-Slide, tissue culture treated, iBIDI). Cells were fixed in 4% methanol-free paraformaldehyde (Fisher Scientific) in PBS for 10 minutes, washed and permeabilized with Triton 0.2% in PBS for 20 minutes. Blocking was performed in PBS with Triton 0.2%, BSA 1%, and CD61 antibody 1/1000 (Alexa Fluor 647, BD) for 30 minutes. Cells were labeled with 1/1000 dilution of anti-ASC antibody (clone 2EI-7, EMD Millipore) in blocking buffer overnight, followed by 30 minutes of incubation with secondary antibody (Alexa Fluor 488, Life Technologies). Samples were covered with mountain medium with DAPI to stain nuclei (Fluoro-Gel,
Fisher Scientific). Images were taken with a Leica inverted microscope with a TCS SPEII confocal module and processed using LAS X software.

2.8 | **Western blot analysis**

Cell extracts were prepared by lysing cells cultured in 60 mm dishes with RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% NaDOAc, 0.1% SDS, and 1.0%NP-40) plus phosphatase and protease inhibitor cocktails (Xpert P3200-001 and P3100-001, respectively, GenDEPOT). Protein concentrations were determined by the Bio-Rad method (DC Protein Assay), and equal amounts of proteins were subjected to SDS-PAGE gels (8%-15%). Culture media were collected after the treatments, precipitated with trichloroacetic acid, and washed with acetone to detect proteins released to the supernatants. Proteins were transferred onto PVDF membranes and incubated with primary antibodies overnight at 4°C, followed by a 1-hour incubation with the corresponding secondary antibody. The results were visualized using Li-Cor Odyssey Infrared Imaging System (LI-COR Biosciences). Primary and secondary antibodies used are listed in Supporting Information Table S1.

2.9 | **Immunoassays**

IL-1β levels in cell culture supernatants and serum were determined by ELISA (IL-1-beta Mouse ELISA Kit, Cat # BMS6002, Invitrogen).

2.10 | **Cell death assays**

Lytic cell death was assessed by measuring the levels of lactate dehydrogenase (LDH) in cell culture supernatants (LDH Cytotoxicity Detection Kit, cat # MK401, Takara). Cell survival was measured by MTT assay (Cell Proliferation Kit I, cat # 11465007001, Sigma).

2.11 | **Statistical analysis**

One- or two-way ANOVA analysis were followed by the appropriate multiple comparison test (described in the figure legends), using the GraphPad Prism 8 software.

3 | **RESULTS**

3.1 | **Loss of NLRP3 or NLRC4 inflammasome does not affect the function of each other**

Upon activation by ligands such as LPS and the ionophore, nigericin, NLRP3 recruits ASC, which forms polymers known as ASC specks. Using ASC speck formation as a marker of the activated state of the inflammasomes, we determined the extent to which NLRP3 deficiency impacts the NLRC4 inflammasome, and inversely. We found that the formation of ASC specks induced by LPS and nigericin was comparable between WT and Nlrc4−/− BMMs; these stimuli did not induce the specks in Nlrp3−/− and Nlrp3−/−;Nlrc4−/− cells (Figure 1A, top panel). Conversely, the NLRC4 inflammasome ligand FlaTox induced ASC polymerization comparably in WT and Nlrp3−/− BMMs, a response that was blunted in Nlrc4−/− and compound mutant cells (Figure 1A, bottom panel). The formation of ASC specks correlated with the cleavage of GSDMD and the maturation and secretion IL-1β as well as the release of lactate dehydrogenase (LDH, a marker of cell death) by LPS and nigericin (Figure 1B,C) or FlaTox (Figure 1D,E). Deficiency in certain inflammasome components such as caspase-1 or GSDMD causes a significant delay in IL-1β secretion, and ultimately, secondary necrosis or pyroptosis, responses that are driven by caspase-8 and caspase-3. We found that LPS and nigericin induced caspase-8 activation in WT and Nlrc4 null BMMs but not in Nlrp3−/− and Nlrc4−/−;Nlrc4−/− cells (Figure 1F). Nlrp3−/− cells were still capable of processing these enzymes as they were activated by Raptinal, an inducer of intrinsic apoptotic signals, or by the combination of TNF-α and the inhibitor of TGF-β-activated kinase-1 (TAK-1, Figure 1G). Collectively, these results indicate that the NLRP3 and NLRC4 inflammasome pathways are unaffected in Nlrc4−/−;Nlrc4−/− cells.
**3.2 | The NLRP3 and NLRC4 inflammasomes are active in the osteoclast lineage**

To determine the extent to which the inflammasomes are activated in osteoclasts, we treated BMMs with RANKL for 7 days to maximize osteoclast differentiation; the cultures were then exposed to LPS and nigericin or FlaTox. LPS and nigericin stimulated ASC speck formation comparably in WT and Nlrc4−/− cells but not in Nlrc3−/−;Nlrc4−/− cells (Figure 2A). Conversely, FlaTox induced ASC speck formation only in WT and Nlrc3−/− cells but not in cells lacking NLRC4 (Figure 2B). ASC speck formation correlated with GSDMD maturation and IL-1β and LDH release induced by LPS and nigericin (Figure 2C-E) or FlaTox (Figure 2F,G). Unlike in BMMs, LPS and nigericin induced the release of intracellular contents to the supernatants, including LDH (Figure 2E), caspase-8, and β-actin (Figure 2D) not only in WT and Nlrc4−/− osteoclast cultures but also to some extent, in cultures of Nlrc3−/−;Nlrc4−/− cells (Figure 2D). Thus, the NLRP3 and NLRC4 inflammasomes are active in osteoclast cultures. Determination of the extent to which the NLRP3 and NLRC4 inflammasomes are active in mature osteoclasts is an inquiry that is beyond the scope of this work.

**3.3 | Deletion of NLRP3, but not NLRC4, increases baseline bone mass, and protects against LPS-induced inflammatory osteolysis**

Previous studies showed a protective role of NLRP3 deficiency on bone loss induced by cytokine or hormonal challenges and aging.35,48,49 By contrast, NLRC4 loss was associated with increased osteolysis in an experimental periodontitis model, likely as the result of increased osteoclast activity since the number of these cells was unaffected between mice injected with dead bacteria or PBS.36 Since bone mass in C57BL/6 mice reaches a peak around 10 weeks of age and declines thereafter,56 we studied the effects of Nlrc3 and/or Nlrc4 deletion on bone homeostasis in 3- and 8-month-old WT, Nlrc3−/−, Nlrc4−/−, and double knockout mice. Micro-computed tomography (μCT) analysis of the distal femur showed a comparable volumetric bone mass (BV/TV) and bone mineral density (BMD) among 3-month-old WT, Nlrc3−/−, Nlrc4−/−, and Nlrc3−/−;Nlrc4−/− male mice (Supporting Information Figure S1A,B). By contrast, 8-month-old Nlrc3−/− mice but not Nlrc4−/− mice displayed significantly higher BV/TV and BMD compared to WT counterparts (Figure 3A,B,G). Bone mass was comparable between Nlrc3−/−;Nlrc4−/− and WT animals. Overall, BV/TV and BMD parameters correlated to some extent with the number and thickness of the trabeculae (Figure 3C-E). Taken together, these results suggest that deficiency in NLRP3 results in bone mass accrual, a response that is attenuated upon concomitant loss of NLRC4.

Bone homeostasis is regulated by chemokines and cytokines, some of which are products of inflammasome activities.57,58 Relevant to this study, we determined the impact of NLRP3 and/or NLRC4 deficiency on the concentrations of the pro-inflammatory and pro-osteoclastogenic cytokine, IL-1β, in the serum from 8-month-old mice. IL-1β levels were significantly lower in mice lacking NLRP3 or NLRC4 compared to WT controls (Figure 3F), but those of double knockout mice did not achieve statistical significance. There was a trend toward comparable levels of IL-1β between WT and Nlrc3−/−;Nlrc4−/− mice (Figure 3F), an outcome reminiscent of the bone mass phenotype of both mouse strains. Collectively, these results suggest that the NLRP3 and NLRC4 inflammasomes participate in the maintenance of physiological levels of IL-1β in mice, and that redundant mechanisms are triggered to produce this cytokine in the absence of these two pathways.

Next, we studied the role of these inflammasomes in bone resorption in a model of higher inflammation grade caused by the pro-inflammatory and osteolytic stimulus, LPS.59 For this purpose, we used 3-month-old mice given the comparable baseline bone mass phenotype among genotypes. As expected, injections of PBS onto
the calvaria of mice did not cause bone resorption as assessed by the formation of resorption pits, irrespective of the genotype (Figure 3H,I). By contrast, LPS induced the formation of calvaria resorption pits, an outcome that was associated with increased osteoclast formation; these responses were attenuated in Nlrp3−/−, Nlrc4−/−, and
Nlrp3−/−;Nlrc4−/− mice compared to WT controls (Figure 3H,I; Supporting Information Figure S1C). Although only Nlrp3-ablated mice were protected against LPS-induced body weight loss (Supporting Information Figure S1D), all genotypes developed splenomegaly, confirming that LPS was successfully injected into recipient mice (Figure 3J). These results suggest that both the NLRP3 and NLRC4 inflammasomes participate in inflammatory osteolysis, though to various extents.

3.4 The NLRP3 and NLRC4 inflammasomes regulate osteoclast differentiation and activity

Since LPS-induced bone resorption in the calvariae was significantly attenuated in Nlrp3 null mice, we determined its effects as well as those of TNF-α on osteoclast formation initiated by M-CSF and RANKL. The baseline number of osteoclasts (TRAP+ multinucleated cells) was slightly higher in WT compared to Nlrp3−/− and Nlrc4−/− cultures (Figure 4A-C). By contrast, treatment of pre-osteoclasts (cells exposed to RANKL for 2 days) with LPS or TNF-α for additional 24 hours enhanced osteoclast differentiation in WT but not Nlrp3−/− or Nlrc4−/− cells (Figure 4A-C). Decreased osteoclastogenesis of knockout cells was not caused by impaired cell growth or increased cell death because RANKL, LPS, or TNF-α did not affect the survival of WT and mutant cells (Supporting Information Figure S2A,B). We also found that the areas of osteoclast resorption pits at baseline were comparable among mutants and WT cells (Figure 4D). By contrast, pit formation induced by TNF-α was blunted in cells lacking NLRP3 or NLRC4 (Figure 4D). In general, Nlrp3−/−;Nlrc4−/− cells were more responsive to TNF-α than the individual mutants in terms of osteoclast formation and activity. Collectively, these data suggest that both the NLRP3 and NLRC4 inflammasomes are important players in the biology of the osteoclasts regulated by inflammatory stimuli. However, this cell lineage has the capacity to thrive in vitro in the absence of these inflammasomes.

4 DISCUSSION

While the bone detrimental actions of NLRP3 with a gain-of-function mutation in the monogenic autoinflammatory disorder neonatal-onset multisystem inflammatory disease (NOMID) are well established,34,37,60 the bone impact of NLRP3 loss-of-function in complex diseases remains unclear. Indeed, loss of NLRP3 attenuated trabecular bone loss caused by ovariectomy, high levels of parathyroid hormone, or RANKL,48 reduced cortical osteopenia associated with aging,35 and negated aging-linked alveolar bone deterioration in mice.36 By contrast, caspase-1 but not NLRP3 deficiency was claimed to protect alveolar bone loss caused by the administration of heat-killed Aggregatibacter actinomycetemcomitans,39 findings that conflicted with those showing preservation of alveolar bone by NLRP3 deletion or pharmacological inhibition in the ligature-induced periodontitis model.40 On the other hand, although caspase-1 deficiency was shown to protect against LPS-induced osteolysis41 the role of NLRP3 in this model has not been examined. This knowledge gap provided a strong rationale of studies described herein.

We found that adult mice lacking the NLRP3 inflammasome exhibited higher baseline trabecular bone mass compared to WT littermates. We also found that Nlrp3 knockout mice were protected against LPS-induced calvaria bone resorption and cachexia. Despite the inherent variability of baseline cytokine levels, we found that serum IL-1β levels correlated with bone outcomes. As mentioned above, others have shown that deficiency in the NLRP3 inflammasome protected against age-associated bone decline, but only cortical bone outcomes were reported.35 Therefore, our study expands early findings by revealing an active role of the NLRP3 inflammasome in the remodeling of trabecular bone, the most metabolically active compartment of the osseous tissue. They reinforce the notion that the NLRP3 inflammasome is a key player in bone resorption in various pathological conditions, including exposures to polymethylmethacrylate particles, high RANKL concentrations, radiation, estrogen deficiency, continuous administration of parathyroid hormone, and autoinflammatory and autoimmune
Although the current work was focused on bone resorption, it is worth noting that germline 4-week-old and 16-week-old Nlrp3 null male mice exhibited shorter stature and lower bone mass compared to WT controls, a phenotype explained by altered growth plate development and impaired osteogenesis as
Osteoclastogenesis was unaffected. Thus, a possible contribution of the osteoblast lineage-expressed NLRP3 to the phenotype described herein cannot be ruled out.

We anticipated higher bone mass in Nlrp3 and Nlrc4 double knockout mice compared to single mutant mice. Unexpectedly, loss of NLRC4 erased the bone-protective
effects of NLRP3 deficiency as compound mutant mice exhibited lower bone mass compared to Nlrp3 null mice. We speculate that compound loss of both receptors negatively impacts the pools of active caspase-1, thereby leading to the activation of a redundant pathway to re-establish bone homeostasis. We also surmise that caspase-8 is the likely candidate for such a compensatory mechanism because of its ability to substitute for caspase-1 in certain cell contexts. However, the latter scenario is shaky since the effects of LPS and nigericin on caspase-8 cleavage in double knock-out pre-osteoclasts are negligible despite the fact that these cells are more susceptible to death than Nlrp3-deficient pre-osteoclasts. Thus, the phenotype of Nlrp3−/−;Nlrc4−/− remains intriguing. Elucidation of the underlying mechanisms is beyond the scope of this study. Although the inducers of NLRP3 and NLRC4 interaction in bone are unknown, these receptors formed a functional complex and interacted with caspase-8 in cells exposed to Salmonella typhimurium or lysophosphatidylcholine. Although the role of NLRP3 and NLRC4 in bone formation has yet to be defined, our results suggest these inflammasomes play a role in bone resorption, and that the actions of the NLRP3 inflammasome in this process appear dominant.

Our study shows that ablation of NLRP3 or NLRC4 to a lesser extent, protects mice from bone loss associated with aging or inflammation. Although the NLRP3 inflammasome is the major regulator of pathophysiologic bone remodeling, its actions are modulated by the NLRC4 inflammasome.

ACKNOWLEDGMENTS
This work was supported by NIH/NIAMS AR068972 and AR076758 grants to GM. YA-A was supported by NIH grants AR049192, AR072623, and #85160 grant from the Shriners Hospital for Children. The histology & morphometry and structure & strength cores at Washington University Musculoskeletal Research Center are supported by P30 AR074992 grant. We thank Dr V. M. Dixo (Genentech, South San Francisco, CA) and Dr Dr Russel Vance (University of California, Berkeley, CA).

CONFLICT OF INTEREST
Dr Gabriel Mbalaviele is a consultant for Aclaris Therapeutics, Inc. He owns stocks for this company. Other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Y. Alippe: Conceptualization, Methodology, Data Acquisition, Reviewing, Writing—Original draft preparation, Editing; D. Kress, B. Ricci, K. Sun, T. Yang, C. Wang, J. Xiao: Methodology, Data Acquisition, Editing; Y. Abu-Amer: Reviewing and Editing; G. Mbalaviele: Conceptualization, Reviewing, Writing—Original draft preparation, Editing.

REFERENCES
1. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010;10(12):826-837.
2. Rubartelli A, Lotze MT, Latz E, Manfredi AA. Mechanisms of sterile inflammation. Front Immunol. 2013;4:398.
3. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-435.
4. Chovatiya R, Medzhitov R. Stress, inflammation, and defense of homeostasis. Mol Cell. 2014;54(2):281-288.
5. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. Cell. 2015;160(5):816-827.
6. Kikuchi T, Matsuuchi T, Tsuboi N, et al. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol. 2001;166(5):3574-3579.
7. Takami M, Terry V, Petruzelli L. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. J Immunol. 2002;168(9):4559-4566.
8. Sato N, et al. MyD88 but not TRIF is essential for osteoclastogenesis induced by lipopolysaccharide, diacyl peptidolipid, and IL-1alpha. J Exp Med. 2004;200(5):601-611.
9. Zou W, Bar-Shavit Z. Dual modulation of osteoclast differentiation by lipopolysaccharide. J Bone Miner Res. 2002;17(7):1211-1218.
10. Aksentijevich I, Nowak M, Mallah M, et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): A new member of the expanding family of pyrin-associated autoinflammatory diseases. Arthritis Rheum. 2002;46(12):3340-3348.
11. Bernard NJ. Autoinflammation: NLRC4 mutation causes rare autoinflammatory disease. Nat Rev Rheumatol. 2014;10(11):635.
12. Canna SW, de Jesus AA, Gouni S, et al. An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. Nat Genet. 2014;46(10):1140-1146.
13. Freeman L, Guo H, David CN, et al. NLR members NLRC4 and NLRP3 mediate sterile inflammasome activation in microglia and astrocytes. J Exp Med. 2017;214(5):1351-1370.
14. Li Y, Ling J, Jiang Q. Inflammasomes in alveolar bone loss. Front Immunol. 2021;12:691013.
15. Rathinam VA, Fitzgerald KA. Inflammasome complexes: emerging mechanisms and effector functions. Cell. 2016;165(4):792-800.
16. Heilig R, et al. The Gasdermin-D pore acts as a conduit for IL-1beta secretion in mice. Eur J Immunol. 2018;48(4):584-592.
17. Place DE, Kanneganti TD. Recent advances in inflammasome biology. Curr Opin Immunol. 2018;50:32-38.
18. Evavold CL, Ruan J, Tan Y, et al. The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. Immunity. 2018;48(1):35-44.e6.
19. He WT, Wan H, Hu L, et al. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. Cell Res. 2015;25(12):1285-1298.
20. Liu X, Zhang Z, Ruan J, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016;535(7610):153-158.

21. Aglietti RA, Dueber EC. Recent insights into the molecular mechanisms underlying pyroptosis and gasdermin family functions. Trends Immunol. 2017;38(4):261-271.

22. Franchi L, Eigenbrod T, Muñoz-Planillo R, et al. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol. 2009;10(3):241-247.

23. Proell M, Gerlic M, Mace P, et al. The CARD plays a critical role in ASC foci formation and inflammasome signalling. Biochem J. 2013;449(3):613-621.

24. Lechtenberg BC, Mace PD, Riedl SJ. Structural mechanisms in NLR inflammasome signaling. Curr Opin Struct Biol. 2014;29:17-25.

25. Ming MS, Rajendra K, Thirumala-Devi K. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunol Rev. 2017;277(1):61-75.

26. Broz P, Newton K, Lamkanfi M, et al. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. J Exp Med. 2010;207(8):1745-1755.

27. Man SM, Hopkins LJ, Nugent E, et al. Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. Proc Natl Acad Sci U S A. 2014;111(20):7403-7408.

28. Man SM, et al. Salmonella infection induces recruitment of Caspase-8 to the inflammasome to modulate IL-1beta production. J Immunol. 2013;191(10):5239-5246.

29. Philip NH, et al. Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF-kB and MAPK signaling. Proc Natl Acad Sci U S A. 2014;111(20):7385-7390.

30. Weng D, Marty-Roix R, Ganesan S, et al. Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death. Proc Natl Acad Sci U S A. 2014;111(20):7391-7396.

31. Fritsch M, Günther SD, Schwarzer R, et al. Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis. Nature. 2019;575(7784):683-687.

32. Newton K, Wickliffe KE, Dugger DL, et al. Cleavage of RIPK1 by caspase-8 is crucial for limiting apoptosis and necroptosis. Nature. 2019;574(7778):428-431.

33. Malireddi RKS, Gurung P, Kesavardhana S, et al. Innate immune priming in the absence of TAK1 drives RIPK1 kinase activity-independent pyroptosis, apoptosis, necroptosis, and inflammmatory disease. J Exp Med. 2020;217(3). https://doi.org/10.1084/jem.20191644

34. Bonar SL, Brydges DS, Mueller JL, et al. Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. PLoS ONE. 2012;7(4):e35979.

35. Youn Y-H, Grant R, McCabe L, et al. Canonical Nlrp3 inflammasome links systemic low-grade inflammation to functional decline in aging. Cell Metab. 2013;18(4):519-532.

36. Rocha FRG, Delitto AE, de Souza JAC, et al. NLRC4 inflammasome has a protective role on inflammatory bone resorption in a murine model of periodontal disease. Immunobiology. 2020;225(1):151855.

37. Qu C, Bonar SL, Hickman-Brecks CL, et al. NLRP3 mediates osteolysis through inflammation dependent and -independent mechanisms. FASEB J. 2015;29(4):1269-1279.

38. Zang Y, Song JH, Oh SH, et al. Targeting NLRP3 inflammasome reduces age-related experimental alveolar bone loss. J Dent Res. 2020;99(11):1287-1295.

39. Rocha FRG, Delitto AE, de Souza JAC, et al. Relevance of caspase-1 and NLRP3 inflammasome on inflammatory bone resorption in a murine model of periodontitis. Sci Rep. 2020;10(1):7823.

40. Chen Y, Yang Q, Lv C, et al. NLRP3 regulates alveolar bone loss in ligature-induced periodontitis by promoting osteoclastic differentiation. Cell Proteom. 2021;54(2):e12973.

41. Burton L, Paget D, Binder NB, et al. Orthopedic wear debris mediated inflammatory osteolysis is mediated in part by NALP3 inflammasome activation. J Orthop Res. 2013;31(1):73-80.

42. Xiao J, Wang C, Yao J-C, et al. Radiation causes tissue damage by dysregulating inflammasome-gasdermin D signaling in both host and transplanted cells. PLoS Biol. 2020;18(8):e3000807.

43. McCall SH, Sahraei M, Young AB, et al. Osteoblasts express NLRC3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. J Bone Miner Res. 2008;23(1):30-40.

44. Sims NA, Gooi JH. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Semin Cell Dev Biol. 2008;19(5):444-451.

45. Mbalaviele G, Novack DV, Schett G, et al. Inflammatory osteolysis: a conspiracy against bone. J Clin Invest. 2017;127(6):2030-2039.

46. Hill SC, Namde M, Dwyer A, et al. Arthropathy of neonatal onset multisystem inflammatory disease (NOMID/CINCA). Pediatr Radiol. 2007;37(2):145-152.

47. Wang C, Xu C-X, Alippe Y, et al. Chronic inflammation triggered by the NLRP3 inflammasome in myeloid cells promotes growth plate dysplasia by mesenchymal cells. Sci Rep. 2017;7(1):4880.

48. Alippe Y, Wang C, Ricci B, et al. Bone matrix components activate the NLRP3 inflammasome and promote osteoclast differentiation. Sci Rep. 2017;7(1):6630.

49. Furman D, Chang J, Lartigue L, et al. Expression of specific inflammasome gene modules stratifies older individuals into two extreme clinical and immunological states. Nat Med. 2017;23(2):174-184.

50. Kitamura A, Sasaki Y, Abe T, et al. An inherited mutation in NLRC4 causes autoinflammation in human and mice. J Exp Med. 2014;211(12):2385-2396.

51. Baum R, Sharma S, Carpenter S, et al. Cutting edge: AIM2 and endosomal TLRs differentially regulate arthritis and autoantibody production in DNase II-deficient mice. J Immunol. 2015;194(3):873-877.

52. Jakobs C, Ferner S, Hornung V. AIM2 drives joint inflammation in a self-DNA triggered model of chronic polyarthritis. PLoS ONE. 2015;10(6):e0131702.

53. Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res. 2000;15(8):1477-1488.

54. von Molkte J, Trinidad NJ, Moayeri M, et al. Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature. 2012;490(7418):107-111.
55. Taabazuing CY, Okondo MC, Bachovchin DA. Pyroptosis and apoptosis pathways engage in bidirectional crosstalk in monocytes and macrophages. Cell Chem Biol. 2017;24(4):507-514 e4.

56. Halloran BP, Ferguson VL, Simske SJ, et al. Changes in bone structure and mass with advancing age in the male C57BL/6J mouse. J Bone Miner Res. 2002;17(6):1044-1050.

57. Novack DV, Mbalaviele G. Osteoclasts-key players in skeletal health and disease. Microbiol Spectr. 2016;4(3). https://doi.org/10.1128/microbiolspec.MCHD-0011-2015

58. Alippe Y, Mbalaviele G. Omnipresence of inflammasome activities in inflammatory bone diseases. Semin Immunopathol. 2019;41(5):607-618.

59. Hussain Mian A, Saito H, Alles N, Shimokawa H, Aoki K, Ohya K. Lipopolysaccharide-induced bone resorption is increased in TNF type 2 receptor-deficient mice in vivo. J Bone Miner Metab. 2008;26(5):469-477.

60. Snouwaert JN, Nguyen MyTrang, Repenning PW, et al. An NLRP3 mutation causes arthropathy and osteoporosis in humanized mice. Cell Rep. 2016;17(11):3077-3088.

61. Haneklaus M, O’Neill LAJ. NLRP3 at the interface of metabolism and inflammation. ImmunoL Rev. 2015;265:53-62.

62. Zhang Y, Zheng Y, Li H. NLRP3 inflammasome plays an important role in the pathogenesis of collagen-induced arthritis. Mediators Inflamm. 2016;2016:9656270.

63. So AK, Martinon F. Inflammation in gout: mechanisms and therapeutic targets. Nat Rev Rheumatol. 2017;13(11):639-647.

64. Detzen L, Cheat B, Besbes A, et al. NLRP3 is involved in long bone edification and the maturation of osteogenic cells. J Cell Physiol. 2021;236(6):4455-4469.

65. Qu Y, Misaghi S, Newton K, et al. NLRP3 recruitment by NLRC4 during Salmonella infection. J Exp Med. 2016;213(6):877-885.

66. Mascarenhas DPA, Cerqueira DM, Pereira MSF, et al. Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome. PLoS Pathog. 2017;13(8):e1006502.

67. Lee BL, Mirrashidi KM, Stowe IB, et al. ASC- and caspase-8-dependent apoptotic pathway diverges from the NLRC4 inflammasome in macrophages. Sci Rep. 2018;8(1):3788.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Alippe Y, Kress D, Ricci B, et al. Actions of the NLRP3 and NLRC4 inflammasomes overlap in bone resorption. FASEB J. 2021;35:e21837. https://doi.org/10.1096/fj.20210767RR