A distinctive role of the leukotriene B₄ receptor BLT1 in osteoclastic activity during bone loss

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Although leukotriene B₄ (LTB₄) is produced in various inflammatory diseases, its functions in bone metabolism remain unknown. Using mice deficient in the high-affinity LTB₄ receptor BLT1, we evaluated the roles of BLT1 in the development of two bone resorption models, namely bone loss induced by ovariectomy and lipopolysaccharide. Through observations of bone mineral contents and bone morphometric parameters, we found that bone resorption in both models was significantly attenuated in BLT1-deficient mice. Furthermore, osteoclasts from BLT1-deficient mice showed reduced calcium resorption activities compared with wild-type osteoclasts. Osteoclasts expressed BLT1, but not the low-affinity LTB₄ receptor BLT2, and produced LTB₄. LTB₄ changed the cell morphology of osteoclasts through the BLT1-Gi protein-Rac1 signaling pathway. Given the causal relationship between osteoclast morphology and osteoclastic activity, these findings suggest that autocrine/paracrine LTB₄ increases the osteoclastic activity through the BLT1-Gi protein-Rac1 signaling pathway. Inhibition of BLT1 functions may represent a strategy for preventing bone resorption diseases.

Bone remodeling | G protein-coupled receptor | knockout mice | lipid mediator | osteoporosis |

leukotriene B₄ (LTB₄), a metabolite of arachidonic acid, is a potent lipid mediator with various biological activities toward neutrophils and differentiated T cells, including chemotaxis, degranulation, and production of superoxide anions (1, 2). These actions of LTB₄ are mediated by specific cell surface receptors (BLTs). We previously cloned two distinct BLTs, BLT1 and BLT2 (3, 4). BLT1 is a high-affinity receptor that mediates the inhibition of adenylate cyclase and calcium entry by coupling with the Gi- and Gq-classes of G proteins (5). BLT2 transduces comparable intracellular signals but has a lower affinity to LTB₄ (5). Although several hydroxyeicosatetraenoic acids were found to activate BLT2 (6), we recently identified 12(S)-hydroxyeicosatetraenoic acid, 8E, 10E-trienoic acid (12-HHT) as a very potent endogenous ligand for BLT2 (7). LTB₄ is produced in inflammatory diseases such as psoriasis (8), bronchial asthma (9), ulcerative colitis (10), postischemic tissue injuries (11), and rheumatoid arthritis (12–15). Bone remodeling consists of old bone resorption by osteoclasts and new bone deposition by osteoblasts. Osteoclasts and osteoblasts participate in bone remodeling under the control of many hormones, cytokines (16, 17), and autacoids, including lipid mediators (18). The effects of LTB₄ on bone resorption were investigated using organ cultures of mouse calvariae (19, 20). LTB₄ enhanced calcium efflux from the mouse calvariae, suggesting that LTB₄ stimulates bone resorption (19). LTB₄ increased the formation of resorption pits by osteoclasts in rat bone tissues (20), suggesting that LTB₄ modulates bone resorption by increasing the number and/or activity of osteoclasts. However, few reports have provided definitive biochemical information about the mRNA/protein expression and intracellular signaling pathways of BLTs in osteoclasts as well as in vivo roles of LTB₄/BLTs in osteoclastic bone resorption.

The clinically important hard-tissue diseases are inflammatory joint diseases and metabolic bone diseases (18). Inflammatory joint diseases include rheumatoid arthritis characterized by leukocyte infiltration and synovitis accompanied by erosions of cartilage and subchondral bone (21). In bone resorption diseases, such as osteoporosis, an imbalance of bone remodeling in which the rate of resorption exceeds the rate of formation causes the reduction in bone mass (22). Recently, BLT1-KO mice were shown to be resistant to inflammatory arthritis (23, 24). Similar phenotypes were observed with mouse strains deficient in LTB₄-synthesizing enzymes [i.e., cytosolic phospholipase A₂α (cPLA₂α), 5-lipoxygenase, and LTA₄ hydrolase] (25, 26). A pharmacological study with the BLT1 antagonist CP105696 (27) also revealed a critical role of BLT1 in arthritis (15). Despite these important findings in this inflammatory joint disease, the roles of BLT1 in bone resorption diseases are still unknown.

In the present study, we identified critical roles for BLT1 in osteoclastic bone resorption through analyses of BLT1-KO mice affected with two bone resorption diseases, namely bone loss induced by ovariectomy and LPS. Several lines of in vitro data consistently demonstrated that LTB₄ increased osteoclastic activity through autocrine/paracrine signaling mediated by BLT1. Our findings suggest that BLT1 is a potential therapeutic target for bone resorption diseases.

Results

BLT1-KO Mice Are Resistant to Bone Loss Induced by Ovariectomy. Bone mineral content. We examined whether mice lacking BLT1 develop ovariectomy-induced bone loss to elucidate the roles of BLT1 in bone resorption. Using dual X-ray absorptiometry (DXA), the areal bone mineral density (BMD; bone mineral content divided by the coronal area of the bone tissue measured) of the metaphyseal region of the femur from ovariectomized female mice was compared with that from sham-operated mice. The difference in BMD observed by the DXA measurements was significant between the ovariectomized and sham-operated WT mice (Fig. 1A Left). On the other hand, the DXA analysis revealed that the femoral BMD of ovariectomized BLT1-KO mice was comparable to that of sham-operated BLT1-KO mice. Consistent results were obtained by microcomputed tomography (microCT) analysis, which selectively detects the trabecular bone mineral content. This characteristic is in contrast to the

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DXA analysis, which evaluates the bone mineral content of both the cortical and trabecular bones. It is of note that trabecular bone is more profoundly affected in bone resorption diseases than cortical bone (28). In WT mice, the trabecular bone mineral content per tissue volume (BMC/TV) in the metaphyseal region of the femur was significantly lower in the ovariectomized mice than in the sham-operated mice (Fig. 1 B Left and C Left). “Tissue volume” means the volume of the total bone tissue including the trabecular bone and bone marrow but not the cortical bone. Furthermore, in ovariectomized mice, BMC/TV was significantly lower in WT mice than in BLT1-KO mice (Fig. 1 B Left and C Left). The difference between the BMC/TV values of ovariectomized and sham-operated BLT1-KO mice was not significant (Fig. 1 B Left and C Left). To confirm the role of BLT1 in the ovariectomy-induced bone loss, we examined the effect of the BLT1 antagonist CP105696 on ovariectomized mice. In accordance with the phenotypes of BLT1-KO mice, we observed that BMD and BMC/TV were significantly higher in CP105696-treated mice than vehicle-treated mice (Fig. S1 A–C).

Bone mass. The microCT analysis revealed that the trabecular bone volume per tissue volume (BV/TV) in the metaphyseal region of the femur was significantly reduced in ovariectomized WT mice, but not in ovariectomized BLT1-KO mice, compared with sham-operated mice (Fig. 2 A). Two other indices related to BV/TV, the trabecular number (Tb.N; linear density of trabecular bone) and trabecular separation (Tb.Sp; distance between the edges of trabecular bone), also indicated that the bone volume of ovariectomized BLT1-KO mice was similar to that of sham-operated BLT1-KO mice (Fig. 2 A). BV/TV was significantly increased in CP105696-treated ovariectomized mice compared with vehicle-treated mice (Fig. S1 D). Tb.N and Tb.Sp values also indicated the amelioration of bone volume of ovariectomized mice by treatment with CP105696 (Fig. S1 D).

BLT1-KO Mice Are Resistant to Bone Loss Induced by LPS Injection. Bone mineral content. LPS, a key component of the outer wall of Gram-negative bacteria, has been proposed to be a potent stimulator of bone resorption (29–31). Similar to the DXA data obtained for the female ovariectomized mice, WT male mice exhibited a significantly decreased BMD after LPS injection, while the BMD of BLT1-KO mice was unchanged (Fig. 1 A Right).

A microCT analysis of the metaphyseal region of the femur consistently showed that, in WT mice, the trabecular BMC/TV was significantly lower in the LPS-injected mice than in the saline-injected mice (Fig. 1 B Right and C Right). The trabecular BMC/TV value was significantly lower in WT mice than in BLT1-KO mice after LPS injection (Fig. 1 B Right and C Right). The LPS-injected BLT1-KO mice displayed a similar BMC/TV value to the saline-injected BLT1-KO mice (Fig. 1 B Right and C Right).

Bone mass. The microCT analysis also demonstrated that the trabecular BV/TV in the metaphyseal region of the femur was...
reduced in LPS-injected WT mice, but unaltered in LPS-injected BLT1-KO mice, compared with the saline-injected mice (Fig. 2B). In accordance with these observations, both the Tb.N and Tb.Sp values were similar between LPS- and saline-injected BLT1-KO mice (Fig. 2B). Histologically, deep Howship’s lacunae (i.e., bone hollows) were commonly observed in LPS-injected WT mice as a result of active bone resorption (Fig. S2A). Osteoclasts lay within these distinctive Howship’s lacunae (Fig. S2B). Compared with LPS-injected WT mice, the Howship’s lacunae were shallower in saline-injected WT mice. In contrast to WT mice, LPS-induced Howship’s lacuna formation was unremarkable in BLT1-KO mice (Fig. S2A and B).

mRNAs of BLT1, 5-Lipoxygenase and LTA4 Hydrolase Are Expressed and LTB4 Is Produced in Osteoclasts. We analyzed the mRNA expression profile of LTB4-related genes in primary osteoclasts, which were differentiated from bone marrow cells in the presence of receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF). BLT1 mRNA was expressed in the primary osteoclasts, whereas BLT2 mRNA was not detected under our experimental conditions (Fig. 3A). These results strongly suggest that LTB4 acts on osteoclasts mainly through BLT1. Osteoclasts also expressed mRNAs for 5-lipoxygenase and LTA4 hydrolase (Fig. 3B). Western blot analysis further revealed that osteoclasts expressed 5-lipoxygenase protein (Fig. 3C). In accordance with these results, we observed that osteoclasts produced LTB4 upon calcium-ionophore stimulation (Fig. 3D). These results suggest that LTB4 represents a paracrine/autocrine factor in the regulation of osteoclasts.

**LTB4 Changes the Morphology of Osteoclasts.** WT osteoclasts changed their cell morphology from round shapes to irregular shapes after 30 min of LTB4 treatment (Fig. 4A). In contrast, BLT1-KO osteoclasts exhibited no changes in their contours. Consistent with the data for BLT1-KO osteoclasts, pretreatment with CP105696 almost completely suppressed the LTB4-induced morphological changes of WT osteoclasts (Fig. 4B). Furthermore, pertussis toxin (PTX), a Gi-specific inhibitor, also inhibited the morphological changes of osteoclasts (Fig. 4B). Rac proteins (Rac1, 2, and 3) are a subfamily of the Rho family of small GTPases engaged in many functions such as changes in morphology and motility (32, 33). The Rac1 inhibitor NSC23766 is a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1 GDP/GTP exchange activity by interfering with Rac1 interactions with Rac-specific guanine nucleotide exchange factors (34). Again, the LTB4-induced morphological changes of osteoclasts were suppressed by this Rac1 inhibitor (Fig. 4B). Upon LTB4 treatment, the level of active GTP-bound Rac was significantly increased in WT osteoclasts, but not in BLT1-KO osteoclasts (Fig. S3). These results support the notion that Rac1 is involved in LTB4-induced osteoclast activation. Taken together, these results suggest that the BLT1-Gi protein-Rac1 signaling pathway is involved in the observed morphological changes of osteoclasts.

**Calcium Resorption by Osteoclasts from BLT1-KO Mice Is Impaired.** The regulation of the morphological changes of osteoclasts is deeply related to the function of osteoclasts (35, 36). WT osteoclasts showed more advanced calcium resorption than BLT1-KO osteoclasts in vitro (Fig. 5A). CP105696 inhibited the calcium resorption by WT osteoclasts to the levels resorbed by
BLT1-KO osteoclasts in the presence or absence of CP105696 (Fig. 5A). These results suggest that BLT1 deficiency suppresses calcium resorption by osteoclasts. PTX reduced the calcium resorption by WT osteoclasts to the levels resorbed by BLT1-KO osteoclasts in the presence or absence of PTX (Fig. 5A). Therefore, the BLT1-Gi protein signaling pathway probably plays an important role in calcium resorption by osteoclasts.

BLT1 gene ablation, CP105696, and PTX had no effects on the osteoclast numbers, which were determined after the differentiation of bone marrow cells in the presence of RANKL and M-CSF (Fig. 5B). These results indicate that BLT1 regulates the osteoclastic activity without changing the number of osteoclasts. The activity of individual osteoclasts seems to be enhanced through BLT1 and Gi.

The Rac1 inhibitor reduced the calcium resorption by WT osteoclasts but not that by BLT1-KO osteoclasts, consistent with the results for BLT1 and Gi (Fig. 5A). However, the Rac1 inhibitor NSC23766 reduced the numbers of both WT and BLT1-KO osteoclasts (Fig. 5B). Instead, this compound increased the number of preosteoclasts, which were defined as tartrate-resistant acid phosphatase (TRAP)-positive cells with more than three nuclei (Fig. S4). Since Rac1 lies at the convergence of various signaling pathways, this molecule probably regulates the osteoclastogenesis independently of BLT1.

**Discussion**

This study demonstrates the critical effects of BLT1 in murine models of bone resorption induced by ovariectomy and LPS injection. Ovariectomy, a model of postmenopausal osteoporosis, gives rise to bone resorption conditions by acute decreases in the serum estrogen levels (28). Estrogen deficiency increases the production of inflammatory cytokines (37, 38), causes a bone-remodeling imbalance in which bone resorption exceeds bone formation, and consequently induces bone resorption by activated osteoclasts (39). LPS injection also causes severe bone resorption (31). LPS activates lymphocytes and macrophages to produce inflammatory cytokines, increases osteoclastic activity, and subsequently stimulates bone resorption (40). Osteoclasts are the principal cells involved in bone loss (35) and thus appear to play important roles in these bone resorption diseases. We found that LTB₄ was produced in osteoclasts and activated BLT1 via an autocrine/paracrine mechanism and that BLT1-KO osteoclasts lost the ability to resorb calcium. These findings may account for the ameliorated bone resorption in BLT1-KO mice after ovariectomy and LPS injection.

Osteoclasts are considered to resorb bone under the control of osteoblasts or bone marrow cells (41). The autocrine/paracrine control of osteoclasts was unknown until we identified the stimulatory effects of platelet-activating factor (PAF) on cell survival and bone resorption of osteoclasts (42). In the present study, the primary osteoclasts expressed BLT1 mRNA but not BLT2 mRNA. In addition, we detected 5-lipoxygenase and LTA₄ hydrolase mRNAs as well as 5-lipoxygenase protein in osteoclasts. LTB₄ production by osteoclasts was observed. Thus, we propose that LTB₄ is the second autocrine/paracrine factor of osteoclasts after PAF (42).

Among phospholipase A₂ (PLA₂) enzymes, cPLA₂α plays a dominant role in arachidonic acid release (18, 43). Arachidonic acid is metabolized to eicosanoids including LTB₄ and prostaglandin E₂ (PGE₂). By analyzing cPLA₂α-KO mice, we observed previously that this enzyme plays a key role in LPS-induced bone resorption (31). In that study, it was shown that LPS-induced production of the bone resorption mediator PGE₂ by osteoclasts was impaired in cPLA₂α-KO mice, but the effects of cPLA₂α deficiency on the actions of osteoclasts were not examined. Because osteoclasts express higher amounts of cPLA₂α than primary osteoblasts (42), cPLA₂α in osteoclasts may exert a significant effect on bone resorption by enhancing LTB₄ production.

Estrogen withdrawal after ovariectomy or natural menopause is associated with increased production of TNF-α and IL-1 (37, 38), both of which increase LTB₄ production in neutrophils (44) and macrophages (45). LPS was also reported to increase LTB₄ production in both cell types (46, 47). Therefore, neutrophils and macrophages may be other sources of LTB₄ in the bone marrow of mice suffering from bone resorption diseases. Nevertheless, no in vivo studies have demonstrated enhanced levels of LTB₄ in the bone marrow of these mice.

In granulocytes and some cell lines, BLT1 was shown to transduce intracellular signals through the Gi- and G₁₆-classes of G proteins, which inhibit cAMP production and raise the intracellular calcium concentration (5). However, we previously revealed that this enzyme plays a key role in LPS-induced production of the bone resorption mediator PGE₂ by osteoclasts in osteoclasts (data not shown), we identified critical roles for BLT1 in osteoclastic activity through the PTX-sensitive Gi protein and Rac1. These findings are consistent with previous reports showing that Gi and its downstream effector Rac1 are related to osteoclast functions (48–50). The number of osteoclasts was reduced by ~75% by treatment with a Rac1 inhibitor (Fig. S5), whereas only an ~50% reduction in calcium resorption was seen under the same experimental conditions (Fig. 5A). This apparent discrepancy was probably due to impaired cell–cell fusion of osteoclasts. Rac deficiency reportedly inhibited the fusion of osteoclasts (50). Indeed, preosteoclasts have been shown to exhibit potency to resorb dentin (51). In the present study, we defined osteoclasts as TRAP-positive cells with more than...
three nuclei. Given the significant increase in the number of preosteoclasts by a Rac inhibitor (Fig. S4), the number of osteoclasts shown in Fig. 5B may not necessarily reflect the total osteoclastic activity.

Considering the causal relationship between osteoclast morphology and osteoclastic activity (35, 36), it is reasonable that exogenous LTB₄ changes the shape of osteoclasts through the BLT₁-Gi protein-Rac1 pathway. The morphological changes and migration of osteoclasts are closely related to one another (52, 53). The migration of osteoclasts is enhanced in a Gi-dependent manner (49). Rac1 is generally thought to play a role in cell motility (33) and was indeed reported to be involved in the motility of osteoclasts (48, 50). The functional cycle of bone resorption by osteoclasts consists of bone adherence, bone degradation, bone detachment, and movement to a new site of resorption (35, 54). Therefore, increased osteoclast motility is closely associated with enhanced bone resorption. LTB₄ may increase osteoclast bone resorption, at least in part, by enhancing osteoclast motility.

It has recently been reported that lipoxin and resolvin E1 with anti-inflammatory and proresorptive activities blocked the inflammation-induced bone loss in periodontal diseases (55, 56). The inhibition of bone resorption by resolvin E1 appeared to involve BLT₁ antagonism in osteoclasts (57). These reports are in alignment with our current findings.

In conclusion, we propose a model for BLT₁ actions in bone resorption. LTB₄ is produced in osteoclasts and activates BLT₁ in an autocrine/paracrine manner. This mechanism is responsible for maintaining homeostatic bone remodeling by affecting the cell morphology and bone resorption activity of osteoclasts. In bone resorption diseases, osteoclasts are activated and enhanced bone resorption with a large contribution by LTB₄/BLT₁. The markedly ameliorated bone resorption observed in BLT₁-KO mice after ovariectomy or LPS injection suggests comprehensive roles of BLT₁ in a variety of bone resorption diseases. Many therapeutic agents are being investigated to prevent bone resorption diseases (58). Our findings suggest that BLT₁ antagonists would be one of the candidate agents for therapeutic use for bone resorption diseases.

Materials and Methods

Mice. All animal studies were conducted in accordance with the guidelines for animal research at The University of Tokyo and were approved by The University of Tokyo Ethics Committee for Animal Experiments. BLT₁-KO mice were established using a gene targeting strategy (59). BLT₁-KO and WT male mice (7- to 8-week-old) were i.p. injected with sodium pentobarbital (Somnopentyl; 50 mg/kg body weight; Kyoritsu). Mice were killed at 4 weeks after the surgical procedure. The body weights of the BLT₁-KO female mice (ovariectomized group, 21.2 ± 1.2 g [n = 8]); sham-operated group, 20.9 ± 1.1 g [n = 8]) were indistinguishable from those of the WT female mice (ovariectomized group, 20.7 ± 1.2 g [n = 8]; sham-operated group, 20.4 ± 1.3 g [n = 8]).

Ovariectomy-Induced Bone Loss. BLT₁-KO and WT female mice (10-week-old) underwent a bilateral ovariectomy or a sham procedure in which the bilateral ovaries were exteriorized but not removed, under anesthesia by an i.p. injection of sodium pentobarbital (Somnopentyl; 50 mg/kg body weight; Kyoritsu). Mice were killed at 4 weeks after the surgical procedure. The body weights of the BLT₁-KO female mice [ovariectomized group, 21.2 ± 1.2 g (n = 8); sham-operated group, 20.9 ± 1.1 g (n = 8)] were indistinguishable from those of the WT female mice [ovariectomized group, 20.7 ± 1.2 g (n = 8); sham-operated group, 20.4 ± 1.3 g (n = 8)].

LPS-Induced Bone Loss. BLT₁-KO and WT male mice (7- to 8-week-old) were i.p. injected with LPS from Salmonella enterica (1.25 mg/kg of body weight; Sigma) dissolved in saline on days 0 and 2. On day 7, the femurs were collected. Mice in the control group were injected with saline only. The body weights of the BLT₁-KO male mice [LPS-injected group, 23.2 ± 2.1 g (n = 9); saline-injected group, 24.7 ± 1.4 g (n = 10)] were indistinguishable from those of the WT male mice [LPS-injected group, 23.5 ± 1.4 g (n = 9); saline-injected group, 24.9 ± 2.0 g (n = 10)].

Analysis of Bone Phenotypes. Mouse hindlimb bones were subjected to radiographic and morphometric examinations. The femurs were dissected and stored in 70% ethanol. The BMDs of the femurs were measured by DXA (D-CAT; Aloka). microCT (inspeXio SMX-90CT; Shimadzu) was used to assess the bone mineral content and bone mass of the trabecular bone in the distal femoral metaphysis using a 12-µm isotropic voxel size with 40 kV of tube voltage and 100 µA of tube current. Three-dimensional CT images were reconstructed and analyzed using a TRI system (Ratoc).

Osteoclast Culture. Bone marrow was flushed from the femurs and tibias of 6- to 8-week-old male mice. Osteoclasts were differentiated from hematopoietic cell lineages in bone marrow cultures by stimulation with RANKL and M-CSF (60, 61). Briefly, bone marrow cells were cultured in a modified Eagle’s medium (αMEM; Invitrogen) containing 10% FBS (JRH) with soluble RANKL (30 ng/mL; PeproTech) and M-CSF (50 ng/mL; R&D Systems) for 5 days. Osteoclasts were stained with 0.01% naphthol AS-MX phosphate (Sigma) in the presence of 100 mM L(+/-)-tartaric acid (pH 5.0; Wako) to detect TRAP activity. TRAP-positive cells with more than three nuclei were counted as viable osteoclasts.

RT-PCR Analysis. cDNA was synthesized from total RNA of cultured primary osteoclasts. The resultant cDNA was amplified by PCR. The details are described in SI Materials and Methods.

Western Blot Analysis. Proteins were extracted from cultured osteoclasts and separated by polyacrylamide gel electrophoresis for Western blot analysis as described in SI Materials and Methods.

ELISA for LTB₄. After washing with PBS, osteoclasts were stimulated with 1 µM A23187 for 15 min at 37 °C. TB₄ production by osteoclasts was determined with a LTB₄ ELISA kit (Cayman Chemical) according to the manufacturer’s instructions.

Confocal Microscopy. Bone marrow cells were seeded onto 35-mm polyDlysine-coated glass-bottomed dishes (Iwaki) in αMEM containing 10% FBS with soluble RANKL (30 ng/mL) and M-CSF (50 ng/mL). On day 5, osteoclasts were stimulated with 100 nM LTB₄ for 30 min following pretreatment with CP105696 (1 µM for 5 min; a kind gift from Pfizer), PTX (10 ng/mL for 2 h; List Biological Laboratories) or NSC23766 (50 µM for 10 min; Merck). Then, osteoclasts were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 5 min. For actin labeling, osteoclasts were incubated with 0.03% rhodamine-phalloidin and 0.1% Triton X-100 in PBS for 40 min. The stained cells were observed using a confocal laser scanning microscope (LSM510; Carl Zeiss).

Calcium Resorption Assay. Bone marrow cells were cultured in αMEM containing 10% FBS with soluble RANKL (30 ng/mL) and M-CSF (50 ng/mL) for 6 days on calcium phosphate-coated dishes (BioCoat Osteologic bone culture system; BD Biosciences). The medium was changed every 2 days. In some experiments, 1 µM CP105696 or 10 ng/mL PTX were added to the replaced medium. The Rac1 inhibitor NSC23766 was supplemented to the medium at 50 µM every day. After removal of the cells with a bleach solution (6% NaOCl and 5.2% NaCl), the dishes were washed with water and photographed under a light microscope (BH-2; Olympus). The area of the calcium phosphate-resorbed pits was measured using the image-processing application software ImageJ (National Institutes of Health; NIH).

Statistical Analysis. All values are expressed as means ± SD. The means of multiple groups were compared by ANOVA (Prism; GraphPad Software). The statistical significance of differences was determined by Tukey’s multiple comparison test (for parametric analyses) or Dunnnett’s multiple comparison test (for nonparametric analyses). Values of P < 0.05 were considered to indicate statistical significance.

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Supporting Information

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SI Materials and Methods

RT-PCR Analysis. cDNA was synthesized from 0.8 μg total RNA extracted from cultured primary osteoclasts by oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen). The resultant cDNA was amplified by PCR with the following primers: Mouse BLT1, 5’-ATGGCTGCAAACACTACATCTCTC-3’ and 5’-CAGTGCGCATATGCTATTCCAC-3’; mouse BLT2, 5’-ACAGCCTTGGCTTTCTTCAG-3’ and 5’-TGCCCATTTCTTGAGCT-3’; mouse 5-lipoxygenase, 5’-CTGGTACCTGAAGTACATCACACTG-3’ and 5’-AACAAAGTCCACTCTTTTTCACTA-3’; mouse LTA4 hydrolase, 5’-CAGGAAGATTTACAGATTCAACCAG-3’ and 5’-GAAAATTCATTAGCAGATTTCTCCA-3’; and mouse β-actin, 5’-GCTGTGCTATGTTGCTCTAGACTT-3’ and 5’-AATTGAATGTAGTTTCATGGATGC-3’.

Western Blot Analysis. Proteins were extracted from cultured osteoclasts using M-PER (Pierce) with 1х Complete protease inhibitor mixture (Roche). The protein concentrations of the cell lysates were measured using a BCA Protein Assay kit (Pierce) with BSA (fraction V, fatty acid-free; Sigma) as a standard. Aliquots (15 μg protein) were separated by 10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% skim milk, the membrane was sequentially incubated with a rabbit polyclonal antibody against 5-lipoxygenase (1:500 dilution; Cayman Chemical) and an HRP-conjugated anti-rabbit IgG antibody (1:2,000 dilution; GE Healthcare). β-actin was evaluated as an internal control for protein loading, by sequentially incubating the membrane with a mouse monoclonal antibody against β-actin (1:4,000 dilution; Sigma) and an HRP-conjugated anti-mouse IgG antibody (1:2,000 dilution; Sigma). Immunoreactive proteins were visualized by the ECL chemiluminescence reaction (Amersham Biosciences) following the manufacturer’s instructions.

Administration of CP105696 to Ovariectomized Mice. CP-105696 at a dose of 10 mg/kg body weight or vehicle (0.5% methylcellulose) was administered orally to C57BL/6N female mice (10-week-old) daily. Treatment commenced the day before a bilateral ovariectomy. These mice were killed 10 days after the surgical procedure.

Histology. The tibias of mice after LPS or saline injection were stained with Villanueva bone stain solution (2) for 7 days, dehydrated in ascending grades of ethanol, and embedded in methyl methacrylate (Wako) without decalcification (3). The metaphyseal region of each tibia was subjected to histologic analysis using a light microscope equipped with a micrometer.

ELISA for GTP-Bound Rac. Osteoclasts were serum-starved for 15 min before exposure to 100 nM LTB4 for 2 min. After cell lysis, a total of 12.5 μg protein was subjected to ELISA for GTP-Rac with a G-LISA Rac Activation Assay Biochem kit (Cytoskeleton).

Statistical Analysis. All values are expressed as means ± SD. The statistical significance of differences was determined by two-tailed unpaired t test. Values of P < 0.05 were considered to indicate statistical significance.

Fig. S1. Radiographic and morphometric analyses of hindlimb bones from mice treated with a BLT1 antagonist. (A) Areal bone mineral density (BMD) of the metaphyseal region of the femur measured by DXA. Female mice treated with the BLT1 antagonist CP105696 (n = 8) or vehicle (n = 12) were ovariectomized. *, P < 0.05 vs. vehicle, as determined by two-tailed unpaired t test. (B) Trabecular bone mineral content per tissue volume (BMC/TV) of the metaphyseal region of the femur measured by microCT. Data are shown as described for A. *, P < 0.0001 vs. vehicle, as determined by two-tailed unpaired t test (n = 8 animals in CP105696-treated group and n = 12 animals in vehicle-treated group). (C) Representative microCT photographs of the metaphyseal regions of femurs. Note the highly porous inside of the bone (transparent regions) in the vehicle-treated ovariectomized mice compared with CP105696-treated ovariectomized mice. (Scale bar, 1 mm.) (D) Trabecular bone volume per tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) of the metaphyseal region of the femur from ovariectomized mice. These bone mass indices were quantified based on analyses of three-dimensional microCT images of the metaphyseal region of the femur. *, P < 0.001 vs. vehicle, as determined by two-tailed unpaired t test (n = 8 animals in CP105696-treated group and n = 12 animals in vehicle-treated group).
**Fig. S2.** Histology of tibias from LPS-injected mice. (A) Images of Villanueva bone staining of the metaphyseal region of the tibia. (Scale bar, 100 μm.) (B) High magnification images of osteoclasts. White arrows, osteoclasts. (Scale bar, 50 μm.)
Fig. S3. Rac activation in LTB₄-treated osteoclasts. ELISA for Rac in an active GTP-bound state was performed. The absorbance ratio (LTB₄-treated cells per control cells) of WT mice was compared with that of BLT1-KO mice. *, $P < 0.05$ vs. WT osteoclasts, as determined by two-tailed unpaired t test ($n = 6$ in WT group and $n = 5$ in BLT1-KO group).
Fig. S4. Role of Rac1 in preosteoclast number. Number of preosteoclasts defined as TRAP-positive cells with less than three nuclei was measured. *, $P < 0.001$ vs. WT osteoclasts without drug treatment, as determined by ANOVA with Tukey’s multiple comparison test ($n = 6$ each).