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5-Lipoxygenase Mediates RANKL-Induced Osteoclast Formation via the Cysteinyl Leukotriene Receptor 1

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5-Lipoxygenase (5-LO) catalyzes the formation of two major groups of leukotrienes, leukotriene B4 and cysteinyl leukotrienes (CysLTs), and it has been implicated as a promising drug target to treat various inflammatory diseases. However, its role in osteoclastogenesis has not been investigated. In this study, we used mouse bone marrow–derived macrophages (BMMs) to show that 5-LO inhibitor suppresses RANKL-induced osteoclast formation. Inhibition of 5-LO was associated with impaired activation of multiple signaling events downstream of RANK, including ERK and p38 phosphorylation, and IκB degradation, followed by a decrease in NFATc1 expression. Ectopic overexpression of a constitutively active form of NFATc1 partly rescued the antosteoclastogenic effect of 5-LO inhibitor. The knockdown of 5-LO in BMMs also resulted in a significant reduction in RANKL-induced osteoclast formation, accompanied by decreased expression of NFATc1. Similar effects were shown with CysLT receptor (CysLTR)1/2 antagonist and small RNA for CysLTR1 in BMMs, indicating the involvement of CysLT and CysLTR1 in 5-LO–mediated osteoclastogenesis. Finally, 5-LO inhibitor suppressed LPS-induced osteoclast formation and bone loss in the in vivo mouse experiments, suggesting a potential therapeutic strategy for treating diseases involving bone destruction. Taken together, the results of this study demonstrate that 5-LO is a key mediator of RANKL-induced osteoclast formation and possibly a novel therapeutic target for bone-resorption diseases. The Journal of Immunology, 2012, 189: 5284–5292.

Osteoclasts are unique bone-resorbing, multinucleated cells derived from the monocyte–macrophage lineage. Two molecules, M-CSF and RANKL, which are mainly produced by osteoblasts and stromal cells, are essential for osteoclast formation from osteoclast precursors (1, 2). RANKL can promote osteoclast development from osteoclast precursors in vitro in the presence of M-CSF (1–4). Binding of RANKL to its receptor RANK can activate downstream signaling pathways, including those involving the MAPKs ERK1/2, p38, and JNK (5). This triggers activation of the transcription factors NF-κB and c-Fos, which, in turn, induces expression of the key transcription factor NFATc1. Accelerated osteoclastic bone resorption plays an important role in the pathogenesis of osteoporosis and in the focal bone loss that accompanies diseases such as rheumatoid arthritis and bone metastases. Thus, defining the molecular mechanisms underlying osteoclastogenesis is essential to gain a better understanding of the molecular basis for the pathogenesis of bone diseases with altered osteoclastic activity. This knowledge will be important for the prevention and treatment of these diseases.

Leukotrienes, biologically active lipid mediators that play important roles in inflammation, are involved in pathological states with an inflammatory component, such as asthma, cardiovascular disease, or cancer (6). They are derived from arachidonic acid (AA) through the action of 5-lipoxygenase (5-LO) (7), an enzyme expressed in a limited number of cells, including neutrophils, eosinophils, monocytes, macrophages, mast cells, and basophils (8). AA is released from phospholipids by cytosolic phospholipase A2, and 5-LO oxidizes free AA to 5-hydroperoxyeicosatetraenoic acid. This intermediate can be reduced by peroxidases to 5-hydroxyeicosatetraenoic acid or dehydrated in a second 5-LO–catalyzed reaction to leukotriene A4 (LTA4). LTA4 must find its way either to leukotriene C4 (LTC4), or LTA4 oxidizes free AA to 5-hydroperoxyeicosatetraenoic acid. This intermediate can be reduced by peroxidases to 5-hydroxyeicosatetraenoic acid or dehydrated in a second 5-LO–catalyzed reaction to leukotriene A4 (LTA4). LTA4 must find its way either to leukotriene C4 (LTC4), which conjugates LTC4 with glutathione to form LTC4, or to LTA4 hydrolase, which forms leukotriene B4 (LTB4). LTC4 can be metabolized by sequential proteolytic hydrolysis to leukotriene D4 (LTD4) and leukotriene E4 (LTE4); these three compounds are collectively...
termed cysteiny1 leukotrienes (CysLTs). LB4 exerts its effect through two G protein-coupled receptors (BLT1 and BLT2) (9, 10) and CysLTs through two G protein-coupled receptors (CysLT receptor [CysLTR1] and CysLTR2) (11–13).

Previous studies suggested that mice lacking the functional gene for 5-LO have increased cortical bone thickness and show less bone loss following ovariectomy than do wild-type controls (14). These observations suggest that increased bone formation or decreased bone resorption may occur in the absence of the 5-LO enzyme and suggest that 5-LO and its metabolites may act as regulators of bone metabolism. Although some studies indicated that LB4 functions by inhibiting bone formation and stimulating osteoclastic bone resorption (15–17), little is known about the roles of 5-LO and its metabolites in osteoclast differentiation. In the current study, we clarified their critical roles and intracellular signaling pathways in osteoclast differentiation. Furthermore, the findings of our in vivo study suggest that 5-LO and its metabolites are potential therapeutic targets for bone-resorption diseases.

Materials and Methods
Reagents

MK886, REV5901, montelukast, U75302, and LY255283 were purchased from Cayman Chemical (Ann Arbor, MI). Abs against ERK, p-ERK, IkB, β-actin, p-p38, p-p38, and NFATc1 were purchased from Cell Signaling Technology (Beverly, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cells and culture system

Bone marrow cells were obtained from the long bones of 4–6-week-old male ICR mice. Bone marrow cells were cultured in the presence of M-CSF (30 ng/ml; R&D Systems) for 3 d to generate the bone marrow–derived macrophages (BM-Ms). To examine osteoclast formation, BM-Ms were treated with reagents in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml; PeproTech) in 96-well culture plates (Corning, Corning, NY). Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts.

RT-PCR analysis

Total RNA was extracted from BM-Ms by EasyBlue (iNtRON Biotechnology). cDNA was synthesized from total RNA using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD) and amplified using PCR. The following primers for osteoclastogenic genes were used in this study: calcitonin receptor (CTR), 5′-TTTCAAGGATTTCTGCTGCCAGAG-3′ (forward), 5′-CAAGGCAGAGATTTCC-3′ (reverse); cathepsin K, 5′-CTGCCAAATGAGTTCAGAGTGATG-3′ (forward), 5′-ACCGCACAATTTCTGGAC3′ (reverse); β-actin, 5′-TTTATGTC-CACCAGCACCTTTC-3′ (forward), 5′-TTGATGTTGGGATGTCGAC3′ (reverse); ATP6v0d2, 5′-TCAGATCTCTTCAAGGCTGTGCTG-3′ (forward), 5′-GTGCCAAATGAGTTCAGAGTGATG-3′ (reverse); DC- STAMP, 5′-TTGGAATTCTTCAAGGCTGTGCTG-3′ (forward), 5′-CTCCGTTTCCCCTGAGCCCTCTC-3′ (reverse); αv-Integrin, 5′- CACCAGAGGGATTTCTGTCAC-3′ (forward), 5′-AATGGCC AAGATGATCCACCAC3′ (reverse); and β3-Integrin, 5′-GATGACATCGAGAGGATTTCTGTCAC-3′ (forward), 5′-CCGGTCTACGTA GTTGTAGAGT3′ (reverse). The PCR program was as follows: 32 cycles (β3-Integrin), 30 cycles (ATP6v0d2 and DC- STAMP), 28 cycles (CTR and αv-Integrin), or 22 cycles (cathepsin K and β-actin), after an initial denaturation step at 94°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at 60°C (αv-Integrin), 59°C (ATP6v0d2), or 58°C (CTR, cathepsin K, β-actin, and DC- STAMP) for 45 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min.

Immunoblot analysis

Total-cell lysates were isolated, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in PBS-T and then immunostained with anti-p-ERK (1:1000), anti-p-p38 (1:1000), anti-ERK (1:1000), anti-p-p38 (1:1000), anti-β-actin (1:200), or anti-β-actin (1:4000), followed by secondary HRP-conjugated Ab (1:5000). The membranes were developed using an advanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, U.K.).

Small interfering RNA transfection

5-LO–specific small interfering RNA (siRNA) (sense 5′-AAUUUGCA-GAGCACGCAGAACCUC-3′ and antisense 5′-GAGUUUGGGUCGUGUCACGCUU-3′) or CysLTR1-specific siRNA (sense 5′-AAGAUUAGUG- UAAAUCUUU-3′ and antisense 5′-UCCGAUAGAAGUAUUACUUU-3′) was prepared from corresponding oligonucleotides provided by QIAGEN (Germany). BM-Ms were plated on a 48-well plate (3.5 × 10^5 cells/well) for osteoclast formation or on a 60-mm dish (1.5 × 10^5 cells/dish) for RT-PCR and immunoblot analysis with 30 ng/ml M-CSF. After 24 h, cells were transfected with 10 nM 5-LO or CysLTR1 siRNA using Lipofectamine 2000 (Invitrogen, Grand Island, NY), according to the manufacturer’s instructions. The control contained 10 nM AllStars Negative Control siRNA (QIAGEN).

Retroviral gene transduction

The retroviral vectors PMX-puro GFP and PMSCV-GFP CA-NFATc1 were kindly provided by Prof. Soo Young Lee (Ewha University). Plat-E retroviral packaging cells were seeded in a culture dish. The following day, PMX-puro GFP or PMSCV-GFP CA-NFATc1 were transfected into Plat-E cells using Lipofectamine 2000 CD (Invitrogen). Two days later, culture supernatants of the retrovirus-producing cells were collected. BM-Ms were seeded with culture supernatants of PMX-puro GFP or PMSCV-GFP CA-NFATc1 virus-producing Plat-E cells together with Polybrene (10 µg/ml) and M-CSF (30 ng/ml) overnight. Infected cells were selected with puromycin (2 µg/ml) for 2 d and then further cultured or not with MK886 or REV5901 in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 d.

Bone-resorption assay

BM-Ms were differentiated on dentin slices with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of MK886 or REV5901. The cells were removed from the dentin slice by wiping the surface, and slices were stained with toluidine blue (1 µg/ml; J.T. Baker, Center Valley, PA). The number of pits formed by bone resorption on the dentin slices were counted.

In vivo experiment

To study the effects of MK886 on LPS-induced osteoclast formation in vivo, 6-wk-old male ICR mice, divided into three groups of six mice, were administered MK886 (5 mg/kg) or vehicle (EtOH) i.p. daily. After a day, mice were injected s.c. with vehicle (PBS) or LPS (0.5 mg) over calvarial bone. The mice were sacrificed 6 d after LPS or vehicle injection, and whole calvariae were fixed in 4% paraformaldehyde and stained for TRAP. For histological analysis, whole calvariae were decalcified in 12% EDTA and embedded in paraffin. Histological sections (5 µm) were prepared, stained for TRAP, and counterstained using methyl green (0.5% [pH 4]). To study the effects of MK886 on LPS-induced bone loss in vivo, bone loss was induced by LPS administration using previously described methods (18), with a slight modification. Mice received i.p. injections of LPS (5 mg/kg) or vehicle (PBS) on days 0 and 4 and also received i.p. injections of MK886 (5 mg/kg) or vehicle (EtOH) daily beginning on day −1. The left femurs of mice were collected on day 8 after the first injection and were scanned using dual-energy x-ray absorptiometry (DEXA) or high-resolution microcomputed tomography (µCT). All animal experiments were reviewed and approved by the Sookmyung Women’s University Animal Care Committee.

Statistical analysis

Data are presented as the means ± SD from at least three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by the Student t test. A p value < 0.05 was considered statistically significant.

Results

5-LO inhibitor and CysLTR1/2 antagonist inhibit RANKL-induced osteoclast formation

RANKL is essential and sufficient for the differentiation of osteoclast precursors into mature osteoclasts in the presence of M-CSF (2–5). Therefore, we examined the effects of 5-LO inhibitor and antagonists for CysLTR1/2, BLT1, or BLT2 on RANKL-induced osteoclast formation from BM-Ms. When BM-Ms were incubated with M-CSF and RANKL for 4 d, numerous TRAP^ multilamellated
osteoclasts were generated. Treatment of the same cultures with 5-LO inhibitor (MK886) or CysLTR1/2 antagonist (REV5901) completely suppressed osteoclast formation in a dose-dependent manner (Fig. 1A–D). In contrast, antagonists for BLT1 (U75302) or BLT2 (LY255228) showed weak (<50%) inhibition (Fig. 1A, 1B). The results of the MTT assay showed that the anti-osteoclastogenic effects of MK886 or REV5901 were not due to cellular toxicity (Supplemental Fig. 1). We next examined at which stage 5-LO inhibitor (MK886) or CysLTR1/2 antagonist (REV5901) impaired osteoclast development. Because complete inhibition of osteoclast formation was achieved with 10 μM MK886 or 10 μM REV5901, these concentrations were subsequently used, unless otherwise noted. We added MK886 or REV5901 to BMM cultures treated with RANKL and M-CSF at early (days 0–2) and late (days 2–4) stages of differentiation, and TRAP staining was performed on day 4 (Fig. 1E). Although the maximal inhibitory effect was seen with exposure for the entire culture period, similar effects on osteoclast formation were observed when MK886 or REV5901 was added on days 2–4. The 50% inhibitory effect was also observed with treatment on days 0–2. These findings suggested that 5-LO inhibitor and CysLTR1/2 antagonist affect both early and late stages of osteoclastogenesis, with preference for the latter.

To examine whether the effect of 5-LO inhibitor or CysLTR1/2 antagonist on osteoclastogenesis could be reflected in osteoclastic activity, we performed an in vitro resorption pit assay using dentin slices. Many resorption pits were generated in wells with RANKL-treated cells (Fig. 1F, 1G). In contrast, treatment with MK886 or REV5901 strongly inhibited the formation of resorption pits by RANKL-treated cells.

We next analyzed the gene-expression profiles of various candidate genes involved in RANKL-mediated osteoclastogenesis by 5-LO inhibitor or CysLTR1/2 antagonist (Fig. 2). When BMM precursors were cultured with M-CSF and RANKL for 4 d, various genes related to osteoclast function, including CTR, cathepsin K, and integrin αv/β3, showed upregulation of expression, which was markedly suppressed by the presence of MK886 or REV5901. Furthermore, they also decreased the RANKL-induced expression of v-ATPase subunit d2 (ATP6v0d2) and DC-STAMP, both of which are known to be involved in fusion. Finally, we observed that pretreatment with LTC4 significantly rescued the blockade of osteoclast formation in the presence of MK886 or REV5901 (Fig. 2G, 2H). Taken together, these results suggest that 5-LO exerts an important role in osteoclast differentiation via CysLTR1/2.

5-LO inhibitor and CysLTR1/2 antagonist inhibit the activation of MAPKs, NF-κB, and NFATc1 induced by RANKL

Engagement of RANK by RANKL stimulation on osteoclast precursors permits multiple intracellular signaling cascades that lead to osteoclast differentiation (5). To define the molecular mechanism of the inhibitory effects of 5-LO inhibitor or CysLTR1/2 antagonist on osteoclastogenesis, we next examined the effects of MK886 or REV5901 on the signaling pathways induced by RANKL in BMMs. As shown in Fig. 3A and 3B, ERK and p38 phosphorylation were observed 15 min after RANKL treatment and were suppressed by pretreatment with MK886 or REV5901. We also examined their effects on the NF-κB signaling pathway. RANKL stimulation led to the degradation of IκB within 30 min, which was completely blocked by MK886 or REV5901 (Fig. 3C).

NFATc1 plays a critical and fundamental role in osteoclast development, and the lack of NFATc1 results in the arrest of osteoclastogenesis (19). Therefore, we investigated the effects of 5-LO inhibitor or CysLTR1/2 antagonist on the level of NFATc1 expression. As reported previously, RANKL stimulation increased the expression of NFATc1 in BMMs, which was completely abrogated by MK886 or REV5901 (Fig. 3D). To investigate whether downregulation of the NFATc1 protein level was involved in mediating the antiosteoclastogenic effect of MK886 or REV5901, we overexpressed a constitutively active form of NFATc1 in BMMs using a retroviral infection system. The overexpression of NFATc1 was confirmed by Western blotting analysis (Fig. 3E). These NFATc1-transduced BMMs were cultured with M-CSF and RANKL in the absence or presence of MK886 or REV5901 for 4 d. As shown in Fig. 3F and 3G, overexpression of NFATc1 partly rescued the blockade of osteoclast formation in the presence of MK886 or REV5901. These data indicate that 5-LO and CysLTR1/2 mediate osteoclast differentiation in part via NFATc1 expression.

Knockdown of 5-LO or CysLTR1 blocks RANKL-induced osteoclast formation

To further investigate the role of 5-LO or CysLTR1 in osteoclastogenesis, we performed loss-of-function experiments using siRNAs. Because CysLTR1 antagonist (montelukast) showed ~70% inhibition of osteoclast formation compared with vehicle (Fig. 4A), we used siRNA specific for 5-LO or CysLTR1. Compared with nontargeting control siRNA, 5-LO- or CysLTR1-specific siRNA showed significantly reduced gene expression in BMM cells (Fig. 4B, 4C). These BMMs were then differentiated into osteoclasts using RANKL, and the effects of 5-LO or CysLTR1 on osteoclastogenesis were examined. As shown in Fig. 4D–G, introduction of siRNA specific for 5-LO or CysLTR1 decreased the proportion of TRAP⁺ multinuclear cells (MNCs) compared with controls. We further confirmed the effects of these siRNAs on NFATc1 expression by RANKL. As shown in Fig. 4H and 4I, we found that induction of NFATc1, which is normally upregulated by RANKL stimulation, was attenuated in cells expressing 5-LO or CysLTR1 siRNAs. These results strongly suggested that 5-LO plays an essential role in osteoclast differentiation of BMMs, at least via CysLTR1.

5-LO inhibitor prevents LPS-induced osteoclast formation and bone loss in vivo

Because 5-LO inhibitor suppressed RANKL-induced osteoclast formation in vitro, we examined the in vivo efficacy of MK886 for the treatment of osteoclastic bone loss using a mouse model of bone destruction (18). First, we injected LPS into the calvarial bones of mice with or without MK886. TRAP staining of whole calvariae and histological sections showed that LPS markedly increased osteoclast number (Fig. 5A, 5B). In parallel with the effects observed in vitro, MK886 markedly reduced LPS-induced osteoclast formation when administered systemically (Fig. 5A, 5B).

To further investigate the effects of 5-LO inhibitor in vivo, mice were injected i.p. with LPS with or without MK886. Femurs were collected on day 8 after the first LPS injection, and the areal bone mineral density (BMD; bone mineral content divided by the coronal area of the bone tissue measured) was determined using DEXA (Fig. 5C). The BMD decreased significantly in LPS-challenged mice compared with controls, and treatment with MK886 restored the femoral BMD of LPS-challenged mice. Consistent results were obtained by μCT analysis (Fig. 5D–I). The trabecular bone volume per tissue volume (BV/TV) in the metaphyseal region of the femur decreased in LPS-challenged mice, which was significantly elevated in MK886-treated mice (Fig. 5D). Three other indices related to BV/TV—trabecular thickness, trabecular number (linear density of trabecular bone), and trabecular separation (distance between the edges of trabecular bone)—also indicated amelioration of femoral bone volume in LPS-challenged mice by treatment with MK886 (Fig. 5E–G). In addition, the
FIGURE 1. Decreased osteoclast formation by 5-LO inhibitor or CysLTR1/2 antagonists. (A) BMMs were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the presence of 10 μM MK886 (5-LO inhibitor), 10 μM REV5901 (CysLTR1/2 antagonist), 20 μM U75302 (BLT1 antagonist), or 20 μM LY255283 (BLT2 antagonist) for 4 d. Cells were then fixed and stained for TRAP. (B) TRAP+ MNCs shown in (A) were counted. (C) BMMs were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the presence of various concentration of MK886 or REV5901 for 4 d. (D) TRAP+ MNCs having more than 3 (n > 3) or more than 5 (n > 5) nuclei in (C) were counted as osteoclasts. (E) MK886 (10 μM; left panel) or REV5901 (10 μM; right panel) was added during the indicated culture days in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml). The TRAP+ MNCs were counted. (F) BMMs were placed on dentin slices and cultured in the presence of MK886 (10 μM) or REV5901 (10 μM) with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 6 d. The remaining cells were removed and stained with toluidine blue. Original magnification ×100. (G) The resorbed pit numbers were counted. Data are means ± SD from triplicate samples. Similar results were obtained in two other experiments. Scale bar, 200 μm. *p < 0.05, **p < 0.005, ***p < 0.001, versus vehicle. N.D., Not detectable; Veh, vehicle.
higher structure model index number, as an indicator of increased fragility, in LPS-challenged mice was reduced by MK886 treatment (Fig. 5H). Consistent with these results, three-dimensional visualization of the femoral area clearly showed that the massive loss of trabecular bone following LPS treatment was much lower in MK886-treated mice (Fig. 5I). Taken together, these results suggest that LPS-induced bone destruction was prevented by 5-LO inhibitor treatment.

Discussion
In the current study, we showed that the 5-LO pathway plays a key role in osteoclast differentiation. Specific pharmacological inhibition of 5-LO and loss of function using siRNA prevented RANKL-induced osteoclastogenesis. Further experiments suggested that the molecular mechanisms underlying the anti-osteoclastogenic effects of 5-LO inhibitor involved the ERK-, p38 MAPK-, and NF-κB–signaling pathways. RANKL-induced expression of the transcription factor NFATc1, which is known to play a critical role in osteoclast development, was downregulated by blockade of 5-LO. Because the forced expression of CA-NFATc1 partially rescued the suppression of osteoclastogenesis by 5-LO inhibitor, we speculated that 5-LO partially mediates NFATc1 activation. Additionally, alternative molecules may exist through which 5-LO mediates osteoclastogenesis. Accumulating evidence indicates that NFATc1 acts in a cooperative manner with other transcription factors, such as MITF and PU.1, to regulate the expression of osteoclast genes, including TRAP, cathepsin K, CTR, and DC-STAMP (20–22). In addition, several negative regulators of osteoclastogenesis, such as Id, Mafb, Irf8, and Bcl6, have been identified (23–26). 5-LO may also target these molecules to regulate osteoclast differentiation. Further investigations are needed to clarify the alternative molecular mechanisms involved in the regulation of osteoclastogenesis by 5-LO.

Consistent with its in vitro effects, pharmacological inhibition of 5-LO markedly suppressed LPS-induced bone loss in vivo. These in vivo effects were most likely the result of suppression of RANKL-induced osteoclast formation, as shown in the calvarial model.
5-LO−/− mice show increased cortical bone thickness and reduced bone loss as observed with ovariectomy compared with wild-type controls (14). Our studies provide additional mechanistic insight into these observations by suggesting that 5-LO−/− mice are protected because RANKL-induced osteoclastogenesis mediates 5-LO pathways. Given that impaired fracture healing can be linked to increased osteoclastic activity (27), our in vivo results are consistent with previous studies indicating that pharmacological blockade of 5-LO significantly accelerated and enhanced fracture healing (28). These findings highlight the role of 5-LO as a direct regulator of bone metabolism.

5-LO catalyzes the conversion of AA first to a series of intermediary metabolites, resulting in the formation of two major groups of leukotrienes: LTB4 and CysLTs (LTC4, LTD4, and LTE4).
CysLTs are known to act via two G protein-coupled receptors (CysLTR1 and CysLTR2) (11–13). CysLTR1 is expressed mainly in peripheral blood leukocytes, including eosinophils, monocytes, and basophils; mast cells; and bronchial smooth muscle cells. CysLTR1 binds LTD4 with high affinity, and it binds LTC4 and LTE4 with progressively lower affinities. CysLTR1 accounts for

**FIGURE 4.** Decreased osteoclast differentiation by silencing of 5-LO or CysLTR1. (A) BMMs were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the presence of 10 μM montelukast (CysLTR1 inhibitor) for 4 d. Cells were then fixed and stained for TRAP. (B and C) BMMs were transfected with siRNAs. Total RNA was then isolated from the cells, and cDNA templates were prepared. mRNA expression was determined by RT-PCR using specific primers. (D and E) BMMs were transfected with siRNAs and then cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 4 d. Cells were then fixed and stained for TRAP. (F and G) TRAP+ MNCs were counted. (H and I) BMMs were transfected with siRNAs and then incubated or not with 200 ng/ml of RANKL for 24 h. Western blotting was performed to determine NFATc1 expression levels. Data are expressed as means ± SD from at least three independent experiments. Scale bar, 200 μm. *p < 0.05, ***p < 0.001. si CysLTR1, siRNA targeting CysLTR1 gene; si 5-LO, siRNA targeting 5-LO gene; si NT, nontargeting siRNA.
most of the reported biological activities of CysLTs (29). CysLTR2 was recently cloned (12, 13) and is expressed at high levels in the heart, coronary vessels, and different regions of the brain, with lower levels of expression present in peripheral blood cells; however, its functional role remains largely unknown. CysLTR2 binds both LTC4 and LTD4 with equal affinity. In the current study, the blockade of CysLTR1 showed a comparable effect to 5-LO inhibition on NFATc1 activation and osteoclast formation, suggesting that the mechanism behind the latter effect is primarily a function of the CysLTs, at least via CysLTR1. We observed that BMM cells produce a basal level of CysLTs, whereas no dramatic increase in CysLTs production by RANKL was detected (J.-H. Kang and M. Yim, unpublished observations). Given that BMM cells express 5-LO mRNA, a basal level of 5-LO activity might be necessary for RANKL-induced osteoclast formation.

FIGURE 5. In vivo effects of MK886 on LPS-induced bone loss. (A) Calvariae of mice that received vehicle, LPS, or LPS plus MK886 (5 mg/kg) were subjected to TRAP staining. Original magnification ×40. (B) Decalcified sections of the calvariae were stained for TRAP to detect osteoclasts. Representative images are shown. Original magnification ×40. (C) The femurs of mice that received vehicle, LPS, or LPS plus MK886 (5 mg/kg) were subjected to DEXA analysis. (D–H) The left femurs of the animals were scanned using high-resolution μCT. (I) Two-dimensional images of μCT analysis. Scale bar, 100 μm; original magnification ×10. *p < 0.05. SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness.
One of the hallmarks of CysLTR1 receptor signaling is the induction of an intracellular Ca\(^{2+}\) signal that originates from intracellular mobilization and influx through the plasma membrane. The increase in intracellular Ca\(^{2+}\) concentration by stimulation of CysLTR1 has been demonstrated in many cell types, including a monocytic leukemia cell line (30), mast cells (31), and monocyte-derived macrophages (32). Recently, Ca\(^{2+}\) signaling was reported to have a key role in the regulation of osteoclast formation (33). At the beginning of the osteoclastogenic process, RANKL increases to have a key role in the regulation of osteoclast formation (33). At monocytic leukemia cell line (30), mast cells (31), and monocyte-excessive bone destruction.

Recent evidence has expanded the potential effects of the leukotrienes in a variety of pathophysiological processes. Leukotriene synthesis inhibitors and leukotriene receptor antagonists have been developed for inflammatory diseases, in particular asthma and allergic rhinitis (34). One leukotriene synthesis inhibitor has been developed for use in patients with asthma: the 5-LO inhibitor zileuton (Zyflo) (34, 35). The CysLTR1 antagonist montelukast developed for use in patients with asthma: the 5-LO inhibitor zileuton (Zyflo) (34, 35). The CysLTR1 antagonist montelukast (Singulair) has also been approved by the U.S. Food and Drug Administration to control the symptoms of asthma and allergies (36). In the current study, we identified a novel role for 5-LO and CysLTR1 in regulating osteoclast formation. In view of this property, 5-LO and CysLTR1 may have therapeutic value for treating or preventing several bone diseases characterized by excessive bone destruction.

Disclosures
The authors have no financial conflicts of interest.

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Figure 1. No cytotoxicity was confirmed by MK886 (5-LO inhibitor) or REV5901 (CysLTR1/2 antagonist). BMMs were cultured with or without of MK886 (10 or 20 μM) or REV5901 (10 or 20 μM) in the presence of M-CSF (30 ng/ml) for 48 h. And cell viability was assessed using MTT assay. veh, vehicle; MK, MK886; REV, REV5901.