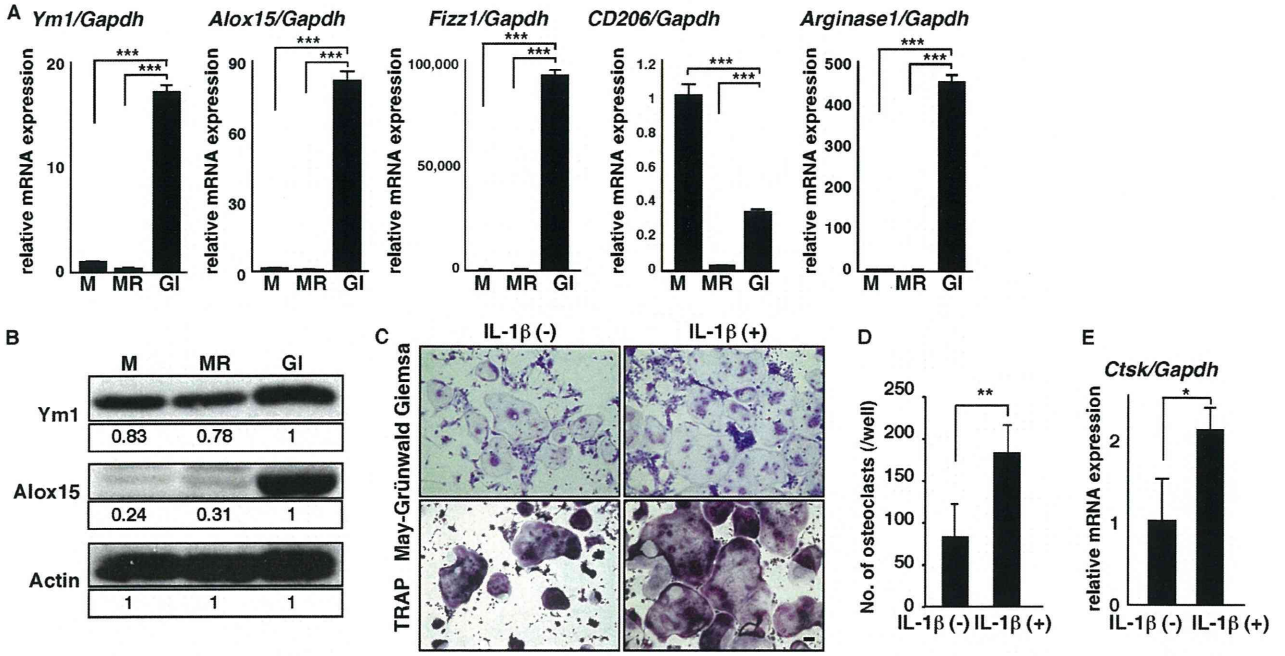


FIGURE 3. **FBGCs express wound-healing molecules, and IL-1β stimulates osteoclastogenesis.** *A* and *B*, osteoclast and FBGC common progenitor cells were cultured in the presence of M-CSF alone (*M*) to induce macrophages, M-CSF plus RANKL (*MR*) to induce osteoclasts, or GM-CSF plus IL-4 (*GI*) to induce FBGCs. Expression of *Ym1*, *Alox15*, *Fizz1*, *CD206*, or ariginase1 transcripts relative to *Gapdh* and expression of Ym1 and Alox15 protein were analyzed by quantitative real time PCR (*A*) and Western blotting (*B*), respectively. Data represent means ± S.D. of *Ym1/Gapdh*, *Alox15/Gapdh*, *Fizz1/Gapdh*, *CD206/Gapdh*, or arginase1/*Gapdh* levels (***, $p < 0.001$; $n = 3$). Actin protein expression served as an internal control. Relative Ym1 or Alox15 protein levels determined by immunoblot were quantified by densitometry and are shown as values relative to levels in FBGCs (*GI*). Representative data of at least three independent experiments are shown. *C* and *D*, osteoclast and FBGC common progenitor cells were cultured in the presence of M-CSF plus RANKL with or without IL-1β (10 ng/ml) for 5 days and then subjected to May-Grünwald Giemsa and TRAP staining (*bar*, 100 μm) (*C*). The number of multinuclear osteoclasts containing more than three nuclei was then scored (**, $p < 0.01$, $n = 3$) (*D*). *E*, total RNAs were prepared from osteoclasts treated with or without IL-1β (10 ng/ml) for 3 days, and *Ctsk* expression relative to *Gapdh* was analyzed by quantitative real time PCR. Data represent means ± S.D, of *Ctsk/Gapdh* levels (*, $p < 0.05$; $n = 3$). Representative data from at least three independent experiments are shown.

size of Ym1 and Alox15 proteins (Fig. 3*B* and data not shown). By contrast, osteoclastogenesis shown by multinuclear TRAP-positive cell formation and cathepsin K expression was significantly stimulated by IL-1β (Fig. 3, *C–E*). Overall, these results suggest that FBGCs function in wound healing and FBR termination and that FBGCs and osteoclasts are reciprocally regulated.

*IRAK4 Is a Reciprocal Switch for FBGC and Osteoclast Differentiation*—We next asked what factor(s) might stimulate FBGCs while inhibiting excessive osteoclastogenesis. Although inflammatory cytokine or LPS stimulation activates various downstream factors (44, 45), we focused on IRAK4, because it is reportedly critical for both IL-1 and toll-like receptor signaling (28). To assess IRAK4 function, we isolated FBGCs and osteoclast common progenitor cells from IRAK4-deficient or wild-type mice and cultured them in the presence of M-CSF plus RANKL (Fig. 4). Multinuclear TRAP-positive osteoclast formation and cathepsin K (*Ctsk*) expression did not differ between IRAK4-deficient and wild-type cells *in vitro* (Fig. 4, *A–C*). Bone-resorbing activity, as determined by a pit formation assay, was comparable in IRAK4-deficient and wild-type osteoclasts *in vitro* (Fig. 4*D*). BMD, as analyzed by a dual energy x-ray absorptiometric scan, was also equivalent between IRAK4-deficient and wild-type mice *in vivo* (Fig. 4*E*). Furthermore, bone morphometric analysis and toluidine blue O staining of tibial bones in knock-out and wild-type mice indicated that IRAK4 loss did not alter osteoclastic and osteoblastic parameters such as eroded surface/bone surface (BS), number of osteoclasts per bone perimeter, Oc surface/BS, osteoblast surface/BS, mineral apposition rate, or bone formation rate/BS *in vivo* (Fig. 4*F*). Thus, we conclude that IRAK4 does not regulate physiological osteoclast differentiation or bone mass.

By contrast, we found that increased osteoclastogenesis and osteolysis induced by IL-1β in wild-type cells was significantly inhibited in IRAK4-deficient cells *in vitro* (Fig. 5, *A–D*). IL-1β promoted osteoclast survival in wild-type but not in IRAK4-deficient osteoclasts *in vitro* (Fig. 5*E*). Osteoclastogenesis activated by IL-1β is considered a phenotypic indicator of M1 polarization. Accordingly, we observed significantly up-regulated expression of M1 markers such as *TNFα*, *IL-12*, and nitric oxide synthase 2 (*Nos2*) in wild-type osteoclasts (Fig. 5*F*), an activity blocked in IRAK4-deficient osteoclasts (Fig. 5*F*), suggesting that M1 polarization in osteoclasts promoted by IL-1β treatment is IRAK4-dependent. MAPK activation is known to promote osteoclast formation and survival. We found that, although p38 and ERK activation was unchanged (data not shown), JNK was activated by IL-1β in wild-type cells but not in IRAK4-deficient cells *in vitro* (Fig. 5*G*). These results suggest that IRAK4 transduces activating signals underlying osteoclast formation and survival through JNK activation.

*In vivo*, we found that calvarial osteolysis induced by LPS administration in wild-type mice was abrogated in IRAK4-deficient mice (Fig. 6*A*). Increased TRAP- or cathepsin K-positive osteoclast formation and cellular migration into calvarial bones

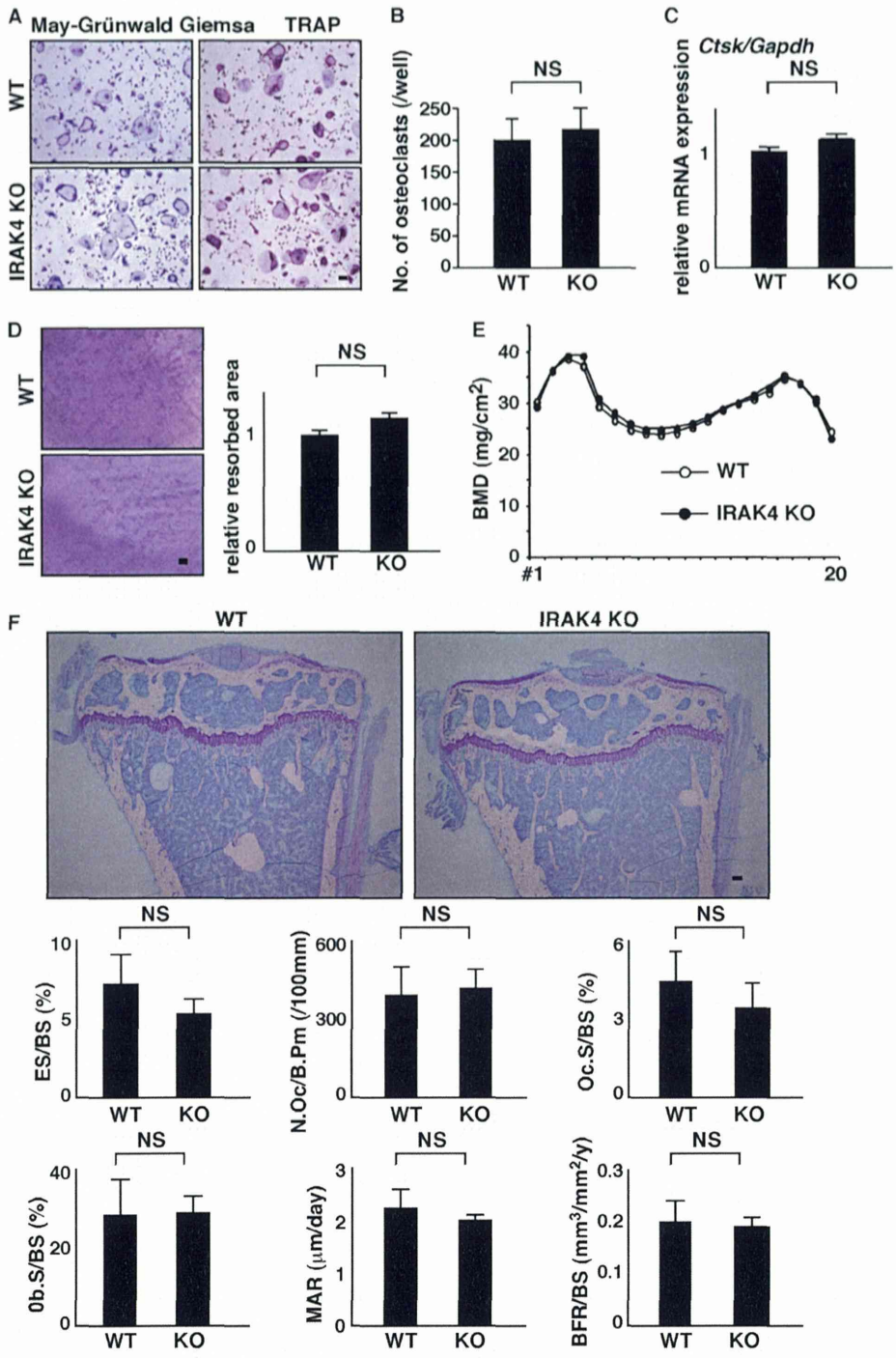


FIGURE 4. **Normal osteoclastogenesis and bone mass in IRAK4-deficient mice.** *A–C,* osteoclast and FBGC common progenitor cells were isolated from wild-type or IRAK4-deficient mice and cultured in the presence of M-CSF plus RANKL for 4 days. Cells were then stained with TRAP (*bar,* 100 μm) (*A*), scored for the number of multinuclear osteoclasts containing more than three nuclei (*NS,* not significant; $n = 3$) (*B*), and analyzed for *Ctsk* expression relative to *Gapdh* by quantitative real time PCR (*C*). Resorption pits appearing on dentine slices were visualized by toluidine blue staining (*D, left panel*), and the relative resorbed area was quantified (*NS,* not significant; $n = 3$) (*D, right panel*) (*bar,* 200 μm). Representative data from at least three independent experiments are shown. *E,* BMD of femurs divided equally longitudinally from wild-type and IRAK4-deficient mice. Representative data of two independent experiments are shown ($n = 5$). *F,* representative toluidine blue O staining images and bone morphometric analysis of 8-week-old female wild-type or IRAK4-deficient mice. Shown are toluidine blue O staining, eroded surface per bone surface (*ES/BS*), the number of osteoclasts per bone perimeter (*N.Oc/B.Pm*), osteoclast surface per bone surface (*OcS/BS*), osteoblast surface per bone surface (*ObS/BS*), mineral apposition rate (*MAR*), and bone formation rate per bone surface (*BFR/BS*). Data are quantified as means ± S.D. *NS,* not significant, $n = 4$ (WT) and 5 (KO).

induced by LPS in wild-type mice were absent in IRAK4-deficient mice *in vivo* (Fig. 6, *B–D*). Because cathepsin K expression is significantly higher in osteoclasts than in osteoblasts (data not shown), we conclude that LPS-stimulated cathepsin K expression in wild-type calvarial bones is due to activated osteoclast formation. Receptor activator of nuclear factor κB ligand

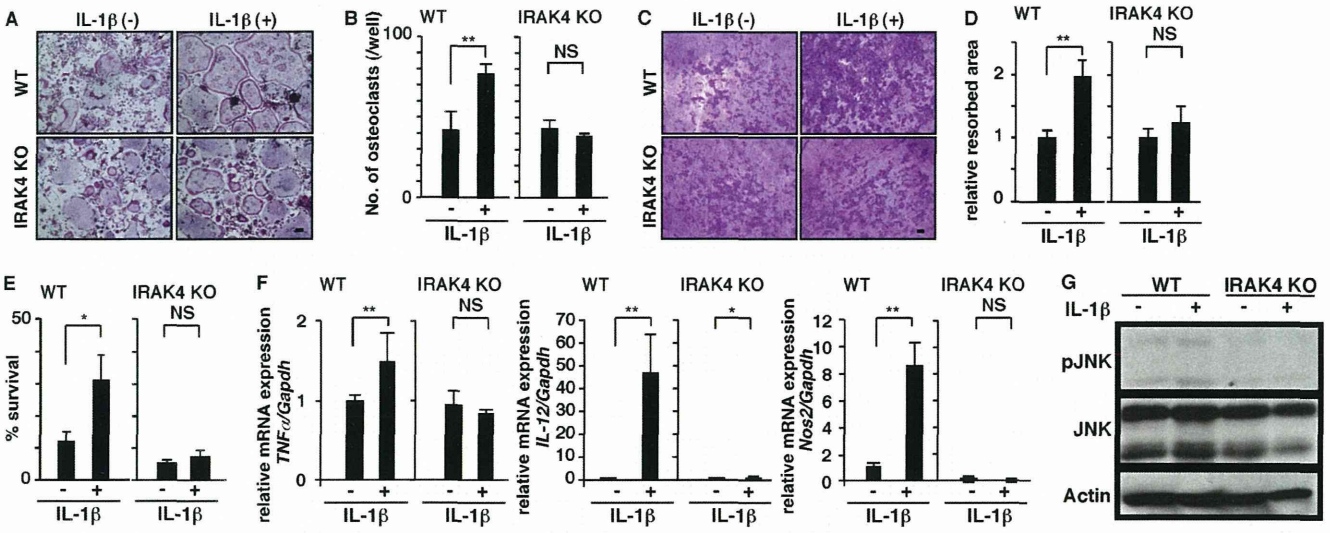


FIGURE 5. **IRAK4 transduces an osteoclast-activating signal by IL-1β.** *A–D*, osteoclast and FBGC common progenitor cells were isolated from wild-type or IRAK4-deficient mice and cultured in the presence of M-CSF plus RANKL with or without IL-1β (10 ng/ml) for 4 days. Cells were then stained with TRAP (*bar*, 100 μm) (*A*), and the number of multinuclear osteoclasts containing more than 10 nuclei was scored (**, $p < 0.01$; *NS*, not significant; $n = 3$) (*B*). A bone resorption assay on dentine slices was visualized by toluidine blue staining (*C*) (*bar*, 100 μm), and the relative resorbed area was quantified (**, $p < 0.01$; *NS*, not significant, $n = 3$) (*D*). *E*, osteoclasts formed in wild-type and IRAK4-deficient cells in the presence of M-CSF plus RANKL with or without IL-1β (10 ng/ml). Cells were then stained with TRAP at time 0, or cultured without cytokines for 3 h and then stained with TRAP. The number of surviving cells was scored at time 0 and 3 h. Osteoclast survival rate is represented as the percentage of living osteoclasts present after 3 h of incubation relative to the number at time zero. Data represent mean number of surviving cells ± S.D. (*, $p < 0.05$; *NS*, not significant; $n = 3$). *F*, total RNAs were prepared from wild-type or IRAK4-deficient osteoclasts treated with or without IL-1β (10 ng/ml) for 2 days, and *TNFα*, *IL-12* or *Nos2* expression relative to *Gapdh* was analyzed by quantitative real time PCR. Data represent means ± S.D. of *TNFα*, *IL-12*, or *Nos2* relative to *Gapdh* (*, $p < 0.05$; **, $p < 0.01$, $n = 3$). *G*, osteoclast and FBGC common progenitor cells were isolated from wild-type or IRAK4-deficient mice, starved in serum-free media for 2 h, and stimulated with or without IL-1β (10 ng/ml) for 10 min. JNK activation was then analyzed by Western blot. Representative data of at least three independent experiments are shown.

(Rankl), a cytokine essential for osteoclastogenesis, is reportedly induced by LPS (46). In our model, *Rankl* expression was induced to similar levels by LPS in both wild-type and IRAK4-deficient mice *in vivo* (Fig. 6*D*).

FBGC formation in IRAK4-deficient cells not treated with exogenous factors was significantly elevated compared with wild-type cells, and inhibition of FBGC formation by IL-1β, LPS, or zymosan was significantly rescued in IRAK4-deficient cells *in vitro* (Fig. 7, *A* and *B*). Although *DC-STAMP* expression was significantly inhibited in IRAK4-deficient cells by IL-1β treatment, that inhibition was less significant in IRAK4-deficient compared with wild-type cells (Fig. 7*C*).

Finally, we asked whether blocking IRAK4 regulates the FBR *in vivo*. To do so, we implanted PVA sponges either with or without LPS into the peritoneal cavity of IRAK4-deficient or wild-type mice to represent foreign bodies, and we then analyzed FBGC formation in sponges and expression of wound-healing factors in cells infiltrating those bodies by real time PCR (Fig. 8). FBGCs formed in sponges lacking LPS in both knockout and wild-type mice, but FBGC formation was more robust in IRAK4-deficient mice (Fig. 8*A*). Also, in the absence of LPS, expression of *TNFα*, an inflammatory cytokine and an M1 macrophage marker, was significantly lower IRAK4-deficient relative to wild-type mice, whereas expression of *Ym1* and *Fizz1*, both M2 markers, was significantly higher (Fig. 8*B*), suggesting that cells infiltrating sponges in IRAK4-deficient mice were significantly M2-polarized compared with those in wild-type mice. Furthermore, in wild-type mice FBGC formation was inhibited by inclusion of LPS in the sponge, but that activity was blocked in IRAK4-deficient mice (Fig. 8*A*). Also in the presence of LPS, *TNFα* expression was significantly lower in IRAK4-deficient compared with wild-type mice (Fig. 8*B*). In contrast, *Ym1*, *Alox15*, *Fizz1*, *CD206*, and arginase1 expression was significantly higher in cells from sponges implanted in IRAK4-deficient compared with wild-type mice in the presence of LPS *in vivo* (Fig. 8*B*). Differences in expression of mRNAs encoding M2 markers, such as *Fizz1*, *CD206*, and arginase1, seen following LPS treatment of wild-type mice, were less apparent in IRAK4-deficient mice (Fig. 8*B*). Taken together, these results suggest that IRAK4 functions as a differentiation switch in reciprocal regulation of FBGCs and osteoclasts (Fig. 8*C*).

## DISCUSSION

Failure of biomedical implants severely limits activities of daily living and increases health care expenses. Biomaterial implant into tissues promotes FBR development, a condition associated with implant failure (2, 3, 37). The FBR develops in response to implantation of almost all biomaterials; this can occur throughout the body and is detrimental to device function (47, 48). Thus, controlling FBR is crucial to protect implants from failures and for human lives. We show that FBGCs do not resorb bones, but rather they express wound healing and inflammation-terminating molecules. Our study also demonstrates that targeting IRAK4 could inhibit elevation of pathologically activated osteoclasts and enable normal FBGC formation, thereby preventing osteolysis (Fig. 8*C*).

Some investigators have concluded that repressing FBGCs may prevent implant failure in part because FBGCs express enzymes such as MMP9, which can degrade biomaterials or

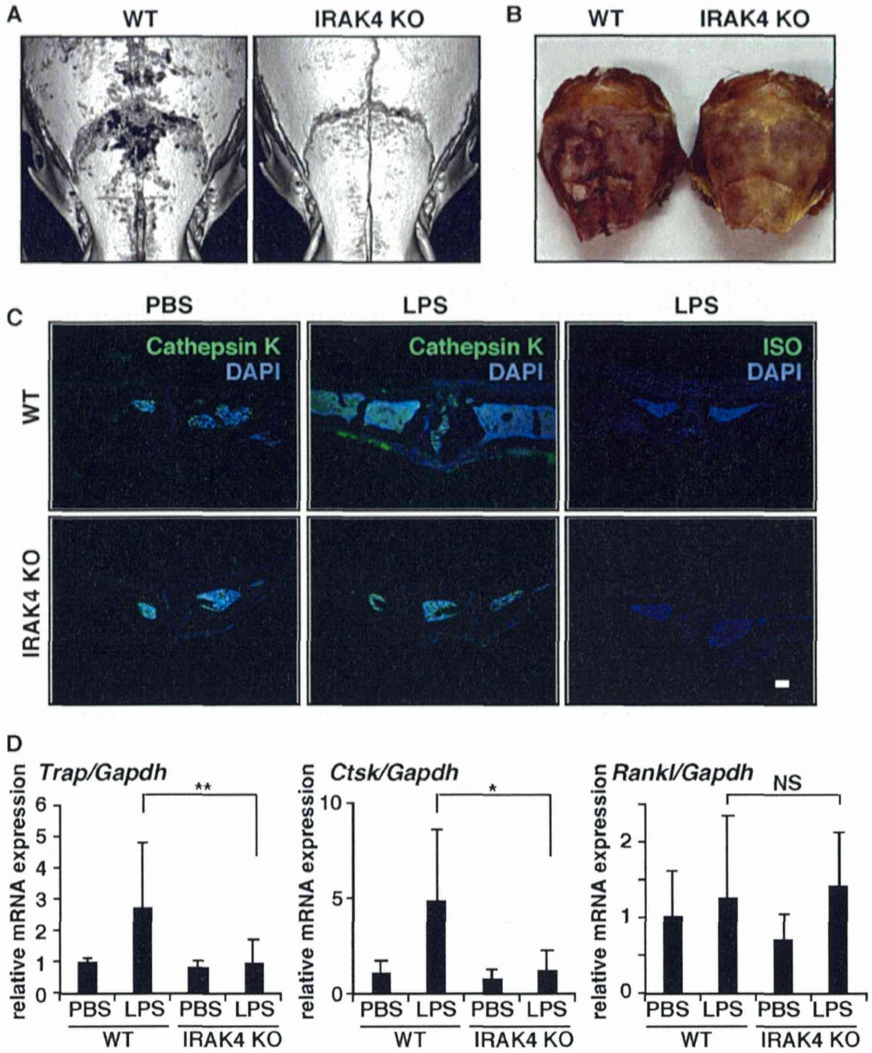


FIGURE 6. **IRAK4 is a specific target for pathological osteolysis.** *A–D*, PBS or LPS (50 mg/kg) was administered subcutaneously to the skull of wild-type or IRAK4-deficient mice. Five days later, osteolysis in calvariae was evaluated by micro-computed tomography (*A*), and osteoclast formation in calvariae was examined by TRAP staining (*B*) and immunohistochemical staining for cathepsin K or ISO-type control antibody (*C*) (*bar*, 100 $\mu$m; $n = 3–5$). Expression of *Trap*, *Ctsk*, and *Rankl* was also analyzed by real time PCR (*D*). Data represent means ± S.D. of *Trap/Gapdh*, *Ctsk/Gapdh*, or *Rankl/Gapdh* levels (*, $p < 0.05$; **, $p < 0.01$; *NS*, not significant; $n = 4–11$).

tissues (7). Interestingly, however, MMP9 also reportedly functions in tissue or extracellular matrix protein remodeling (49, 50). Khan *et al.* (36) reported a lower expression of *MMP9* and *Trap* in FBGCs than in osteoclasts and that FBGCs did not degrade gelatin. We also found that FBGCs expressed significantly lower *Trap* levels than did osteoclasts and failed to resorb bone. Instead, FBGCs expressed factors characteristic of M2 macrophages such as *Ym1* and *Alox15* (27).

A caveat of our study is that we did not assess implanted bones or evaluate the relationship of osteoclast and FBGC formation in implanted materials in bones. Thus, further studies are needed to examine IRAK4 function in the FBR in those contexts. Furthermore, M1/M2 polarization is considered microenvironment-dependent, and although FBGCs express wound-healing factors, it remains to be tested whether FBGCs themselves would have beneficial effects on implant device longevity.

A balance in M1 and M2 macrophage polarization likewise determines the balance between inflammation and anti-inflammatory/wound healing status. Recently, studies of tissues harvested from revised joint replacements reported that pro-inflammatory M1 factors were predominant over M2 anti-inflammatory molecules (51–54). Moreover, Rao *et al.* (55) reported that IL-4, an M2 macrophage activator, mitigated polyethylene particle-induced osteolysis through macrophage polarization. Our data indicate that osteoclasts excessively activated by pro-inflammatory cytokines or TLR ligands such as IL-1$\beta$ and LPS are phenotypically M1 macrophages and are required for osteolysis. By contrast, activity of FBGCs, which are considered M2 macrophages and are induced by IL-4, could terminate the FBR and prevent osteolysis.

A major finding of this study is that FBGC and osteoclast differentiation is reciprocally regulated. IRAK4 is a key molecule required for M1/M2 polarization. Antagonizing

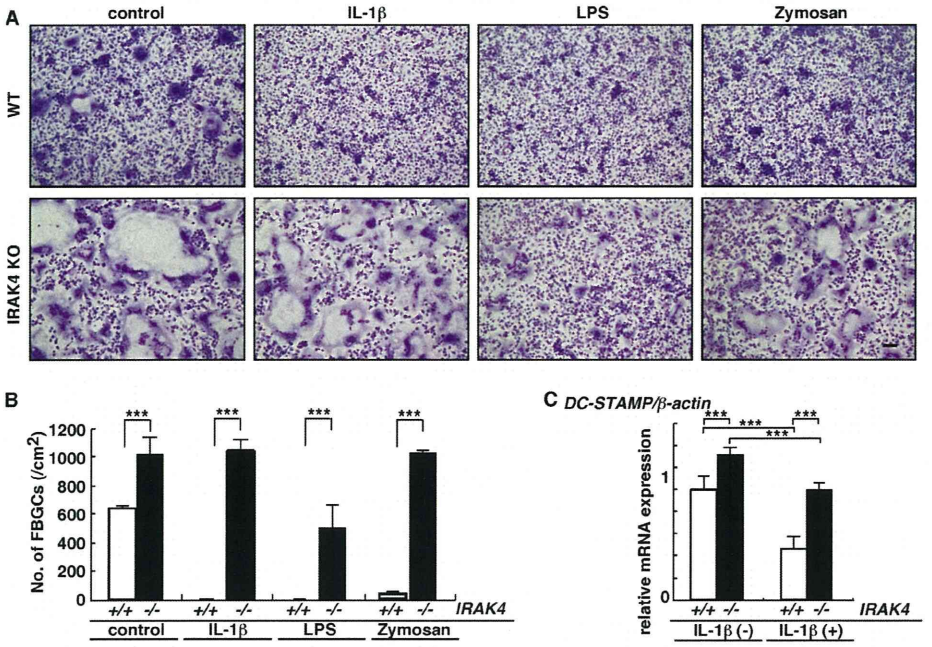


FIGURE 7. **Loss of IRAK4 rescues FBGC formation inhibited by IL-1β, LPS, or zymosan.** *A* and *B*, osteoclast and FBGC common progenitor cells were isolated from wild-type or IRAK4-deficient mice and cultured as FBGCs in the presence of GM-CSF plus IL-4 with or without IL-1β (2 ng/ml), LPS (0.2 ng/ml), or zymosan (100 ng/ml). Cells were stained with May-Grünwald Giemsa (*bar*, 100 μm) (*A*), and the number of multinuclear FBGCs containing more than three nuclei was scored (*B*). (***, $p < 0.001$; $n = 3$.) *C*, total RNAs were prepared from FBGCs treated with (+) or without (−) IL-1β, and *DC-STAMP* expression relative to β-actin was analyzed by quantitative real time PCR. Data represent means ± S.D. of *DC-STAMP*/β-actin levels (***, $p < 0.001$, $n = 3$). Representative data of at least three independent experiments are shown.

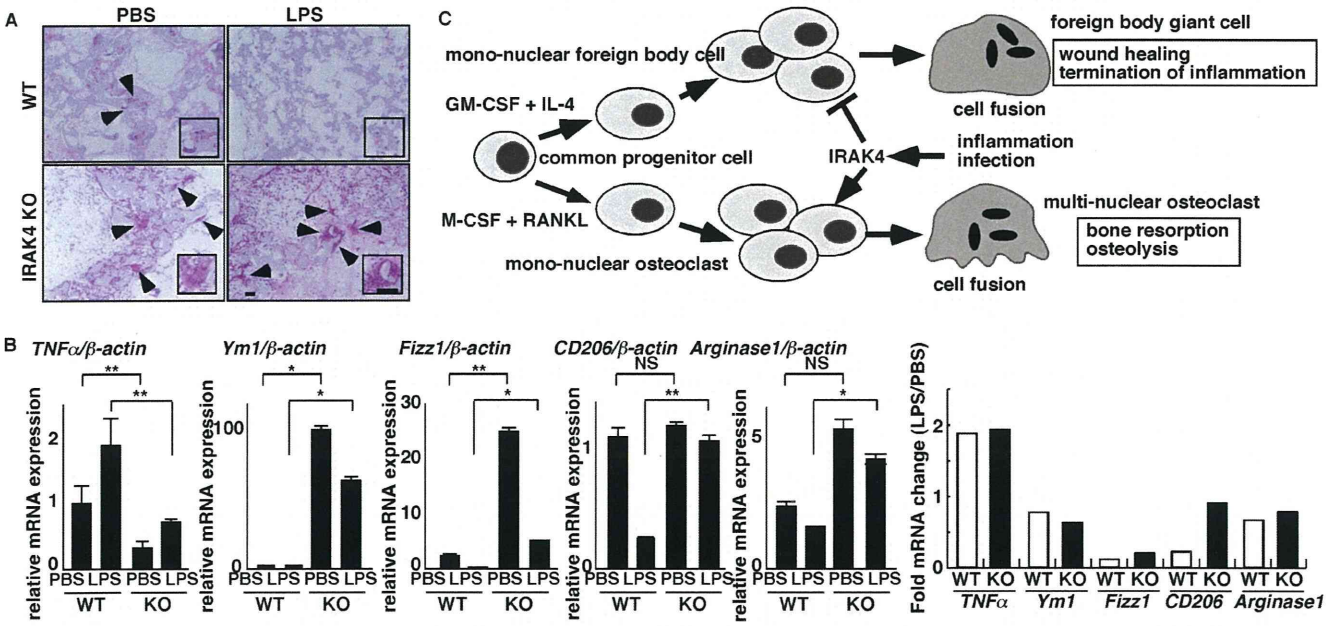


FIGURE 8. **IRAK4 is a potential therapeutic target regulating the FBR *in vivo*.** *A* and *B*, foreign bodies (PVA sponges soaked with PBS or LPS (25 mg/kg)) were implanted into peritoneal spaces in wild-type or IRAK4-deficient mice. After 6 days, foreign bodies were dissected, and tissue sections were stained with H&E (*A*) (*bar*, 100 μm). Alternatively, total RNAs were prepared from cells contained in foreign bodies and *TNFα*, *Ym1*, *Fizz1*, *CD206*; and arginase1 expression relative to β-actin was analyzed by quantitative real time PCR (*B, left panels*). Data represent means ± S.D. of *TNFα*/β-actin, *Ym1*/β-actin, *Fizz1*/β-actin, *CD206*/β-actin, or arginase1/β-actin levels (*, $p < 0.05$; **, $p < 0.01$; *NS*, not significant; $n = 5$–12) (*B, right panel*). Fold changes in mRNA expression between LPS-induced *versus* PBS-treated samples, shown as LPS/PBS. Representative data of at least three independent experiments are shown. *C*, schematic model of FBGC and osteoclast formation regulated by IRAK4 under inflammatory or infectious conditions. FBGC or osteoclast formation is induced in the presence of GM-CSF plus IL-4 or M-CSF plus RANKL, respectively, from common progenitor cells. Both FBGCs and osteoclasts form multinuclear cells by fusion of mononuclear cells. Inflammation or infection activates IRAK4 and inhibits FBGC formation while stimulating osteoclastogenesis, an event underlying implant failure. Multinucleation of FBGCs likely elevates wound healing efficiency, although osteoclasts mediate bone-resorbing activity.

IRAK4 activity could potentially stimulate FBGC formation and inhibit osteoclastogenesis under inflammatory conditions. A recent report shows that LPS induces multinuclear cell formation by Raw264.7 pre-osteoclastic macrophage cells *in vitro* (46). Although M2 marker expression was not addressed in that study, TNFα expression was induced in

those multinuclear cells, suggesting that LPS likely promotes their M1 polarization.

Severe inhibition of osteoclast activity beyond physiological levels by osteoclast-inhibiting agents such as bisphosphonate frequently promotes osteonecrosis of the jaws and severely suppressed bone turnover (21–23, 56, 57). The inability of bone to heal microcracks due to low bone turnover is considered a cause of atypical fracture (58). However, our findings demonstrate that IRAK4 loss did not alter physiological osteoclast differentiation/function required for bone turnover but rather inhibited pathologically activated osteoclasts. Taken together, IRAK4 could serve as a therapeutic target to antagonize inflammatory osteolysis and implant failure without adversely affecting physiological bone metabolism.

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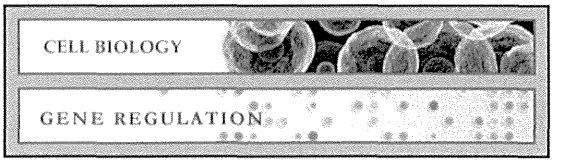
Cell Biology:

# Interleukin-1 Receptor-associated Kinase-4 (IRAK4) Promotes Inflammatory Osteolysis by Activating Osteoclasts and Inhibiting Formation of Foreign Body Giant Cells




Eri Katsuyama, Hiroya Miyamoto, Tami Kobayashi, Yuiko Sato, Wu Hao, Hiroya Kanagawa, Atsuhiro Fujie, Toshimi Tando, Ryuichi Watanabe, Mayu Morita, Kana Miyamoto, Yasuo Niki, Hideo Morioka, Morio Matsumoto, Yoshiaki Toyama and Takeshi Miyamoto

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PLOS ONE

# The Vitamin D Analogue ED71 but Not $1,25(OH)_2D_3$ Targets HIF1α Protein in Osteoclasts

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## Abstract

Although both an active form of the vitamin D metabolite, $1,25(OH)_2D_3$, and the vitamin D analogue, ED71 have been used to treat osteoporosis, anti-bone resorbing activity is reportedly seen only in ED71- but not in $1,25(OH)_2D_3$ -treated patients. In addition, how ED71 inhibits osteoclast activity in patients has not been fully characterized. Recently, HIF1α expression in osteoclasts was demonstrated to be required for development of post-menopausal osteoporosis. Here we show that ED71 but not $1,25(OH)_2D_3$, suppress HIF1α protein expression in osteoclasts *in vitro*. We found that $1,25(OH)_2D_3$ or ED71 function in osteoclasts requires the vitamin D receptor (VDR). ED71 was significantly less effective in inhibiting M-CSF and RANKL-stimulated osteoclastogenesis than was $1,25(OH)_2D_3$ *in vitro*. Downregulation of c-Fos protein and induction of *Ifnβ* mRNA in osteoclasts, both of which reportedly block osteoclastogenesis induced by $1,25(OH)_2D_3$ *in vitro*, were both significantly higher following treatment with $1,25(OH)_2D_3$ than with ED71. Thus, suppression of HIF1α protein activity in osteoclasts *in vitro*, which is more efficiently achieved by ED71 rather than by $1,25(OH)_2D_3$, could be a reliable read-out in either developing or screening reagents targeting osteoporosis.

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## Introduction

A cause for concern in developed countries is the increasing number of osteoporosis patients and individuals suffering fragility fractures due to osteoporosis [1]. Estrogen-deficiency due to menopause is a risk factor for both [2]. Vitamin D insufficiency is also reportedly observed in osteoporosis patients with fragility fractures and considered a cause of osteoporotic fractures [3]. Indeed, vitamin D is known to play a crucial role in skeletal development, and lack of the vitamin D receptor (VDR) or low vitamin D intake results in Rickets [4] [5].

Currently, active vitamin D analogues are used in several countries to treat patients with bone and mineral disorders associated with chronic renal disease or osteoporosis [6]. Interestingly, $1,25(OH)_2D_3$ has been demonstrated to promote osteoclastogenesis in co-cultures of osteoclast progenitor cells and osteoblastic cells [7]; in addition, $1,25(OH)_2D_3$ elevated receptor activator of nuclear factor kappa B ligand (RANKL), an essential cytokine for osteoclastogenesis, but inhibited expression of OPG, a decoy receptor of RANKL, in osteoblastic cells to promote osteoclast differentiation [8] [9]. In contrast, $1,25(OH)_2D_3$ was shown to inhibit osteoclast differentiation in osteoblastic cell-free culture systems: osteoclast formation induced by macrophage colony stimulating factor (M-CSF) and RANKL was inhibited in the presence of $1,25(OH)_2D_3$ [10] [11]. c-Fos protein, an essential transcription factor for osteoclast differentiation, or interferon beta (Ifnβ), an inhibitor of osteoclastogenesis, was downregulated or elevated by $1,25(OH)_2D_3$, respectively, in osteoclast progenitor cells [10] [11]. However, patients treated with a $1,25(OH)_2D_3$ pro-drug, alfacalcidol, did not show inhibition of osteoclastic activity or increased bone mass, while patients treated with the vitamin D analogue ED71 exhibited significantly reduced osteoclast activities and increased bone mass [12].

Since postmenopausal osteoporosis is caused in part by estrogen-deficiency, treating of patients with estrogen is one option. However, continuous estrogen administration is associated with adverse effects such as uterine or mammary gland tumors or cardio-vascular disease [13]. Recently, we reported that hypoxia inducible factor 1 alpha (HIF1α) is required for osteoclast activation following estrogen-deficiency and for development of postmenopausal osteoporosis in animal models [14]. We found that in pre-menopausal mice, HIF1α activity in osteoclasts is continuously suppressed by estrogen but then HIF1α accumulate in osteoclasts following estrogen deficiency due to menopause, which in turn activates osteoclastic activity and promotes bone

loss. Osteoclast specific HIF1α knockout or administration of a HIF1α inhibitor completely abrogated ovariectomy (OVX)-induced osteoclast activation and bone loss [14]. This study suggests that HIF1α could be a therapeutic target for postmenopausal osteoporosis.

Here, we show that HIF1α is a target of ED71 *in vitro*. HIF1α in osteoclasts was suppressed by ED71 but not by $1,25(OH)_2D_3$. Since inhibition of osteoclast activity was seen in the patients treated with ED71 but not with $1,25(OH)_2D_3$, this work confirms that HIF1α could be a target to treat postmenopausal osteoporosis patients.

## Materials and Methods

### Mice

C57BL/6 background wild-type mice were purchased from Sankyo Labo Service (Tokyo, Japan). VDR-deficient mice were established previously [4]. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University School of Medicine animal care committee. All animal procedures were approved by the Keio University School of Medicine animal care committee.

### Cell culture

To assess *in vitro* osteoclast formation, bone marrow cells isolated from $Hif^{flox/flox}$ or Ctsk Cre/$Hif^{flox/flox}$ mouse femurs and tibias were cultured for 72 hours in αMEM (Sigma-Aldrich Co., St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, JRH Biosciences Lenexa, KS, USA) and GlutaMax (Invitrogen Corp., Carlsbad, CA, USA) supplemented with M-CSF (50 ng/ml, Kyowa Hakko Kirin Co. Tokyo, Japan). Subsequently, adherent cells were collected and cultured under indicated conditions containing M-CSF (50 ng/ml), recombinant soluble RANKL (25 ng/ml, PeproTech Ltd., Rocky Hill, NJ, USA) using $1\times10^5$ cells per well in 96-well plates. Osteoclastogenesis was evaluated by TRAP staining [15] [16]. Raw264.7 cells were maintained in DMEM (Sigma-Aldrich Co.) containing 10% heat-inactivated FBS (JRH Biosciences) and GlutaMax (Invitrogen

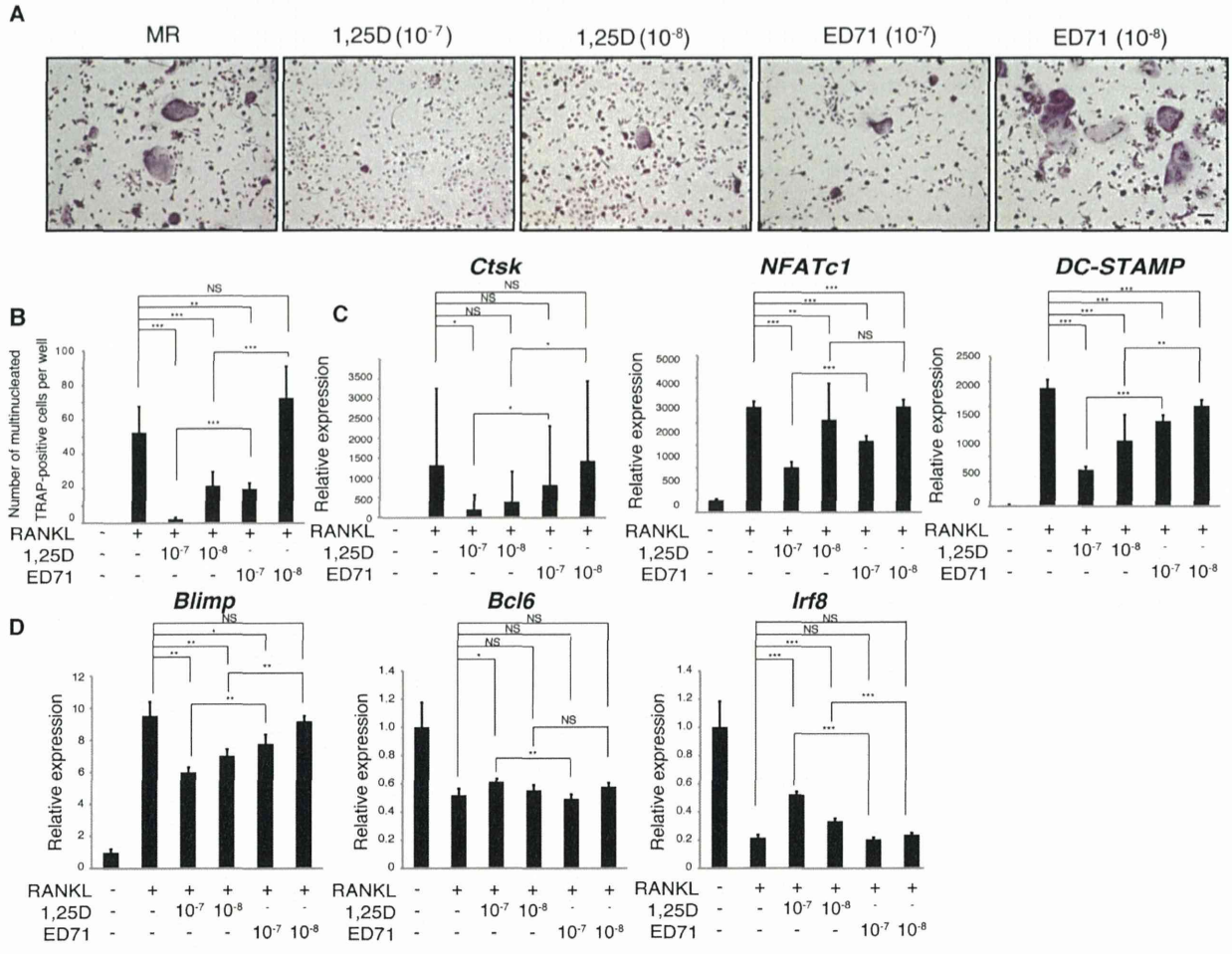


**Figure 1. $1,25(OH)_2D_3$ is a more potent inhibitor of osteoclastogenesis *in vitro* than is ED71.** (**A, B** and **C**) M-CSF-dependent osteoclast progenitor cells were isolated from wild-type mice and cultured in the presence of M-CSF (M, 50 ng/ml) + RANKL (R, 25 ng/ml) with or without indicated concentrations of ED71 or $1,25(OH)_2D_3$ (1,25D) for 5 days. Cells were then stained with TRAP (**A**) and the number of multi-nuclear TRAP-positive cells was counted (**B**). Expression of *Ctsk*, *NFATc1* and *DC-STAMP*, all of which are osteoclastic genes, was analyzed by realtime PCR (**C**). Expression of *Blimp1*, *Bcl6* and *Irf8* was analyzed by realtime PCR (**D**). Data represent mean expression of each relative to *Actb* ± SD ($n=5$). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant.

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