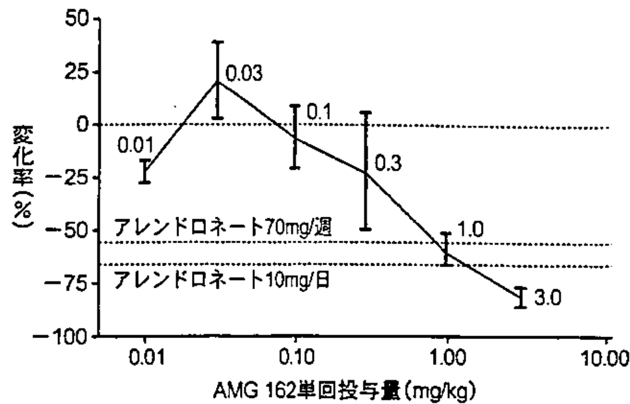

図5 AMG 162単回投与6カ月後の尿中NTX/Creatinine値(フェーズⅠ試験)

49人の閉経後の健常女性に対し，AMG 162 (0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg, 3.0 mg/kg) あるいはプラセボを1回皮下投与6カ月後の尿中NTX/Creatinine値の変化率%で示した(平均値 ± SE)。参考のため，アレンドロネート70 mgを週1回[a]，あるいはアレンドロネート10 mgを毎日投与[a)b)]して得られたデータも示した。

NTX : N-telopeptide/creatinine

[a)]Schnitzer T, et al : Aging (Milano) 2 : 1-12, 2000.

[b)]Tonino RP, et al : J Clin Endocrinol Metab 85 : 3109-3115, 2000.

(文献3，4より改変)

た[5)]。AMG 162を3カ月ごとに6 mg，14 mg，30 mg，および6カ月ごとに14 mg，60 mg，100 mg，210 mgあるいはプラセボを皮下投与した。さらに1群の治験者には，アレンドロネート70 mgを週1回投与した。12カ月間骨量を経時的に測定し，次のことが明らかとなった。

① AMG 162を6カ月に1回投与した群では，最短で72時間で骨代謝マーカーの血清NTX値が減少し，アレンドロネート群よりも有意に低い状態を，14 mg群では2カ月間，それ以外の群では4カ月維持した ($p < 0.0001$)。

② 骨密度は，投与1カ月以内に増加した。AMG 162すべての投与群は投与量の増加に伴って増え，12カ月後において腰椎で4～7％の増加を示した（アレンドロネート投与群では5％の増加）。股関節部で2～4％増加した。

③ すべての群で最も多く見られた副作用は消化障害で，「AMG 162投与群」では5％，「アレンドロネート投与群」では20%，「プラセボ投与群」では4％だった。なお，AMG 162に対する抗体が2人の治験者で出現したものの，その後抗体は消滅し，治療効果に影響はなかったという。

今回の結果から，閉経後の女性の骨量を増加させるためには，60 mgのAMG 162を6カ月に1回投与するのが最も有効であるという[5)]。

AMG 162の特徴

AMG 162に利点として，以下のことが挙げられる。

① マウス抗体やマウス-ヒトキメラ抗体と異なり，AMG 162は，完全なヒト抗体であるために血中に長く留まる。そのため，6カ月に一度の皮下注射で骨吸収抑制と骨量の十分な増加が認められる。

② OPGは，RANKL以外にTRAILと結合することが報告されているが，AMG 162は，RANKL以外の他のTNFファミリーメンバーとは結合しない。

③ AMG 162はヒト抗体であるために，AMG 162に対する抗体ができにくい。また，抗AMG 162抗体ができても，OPG-RANKLの相互作用を阻害することはない。

おわりに

以上のように，完全にヒト型のRANKL抗体AMG 162は，骨粗鬆症に対して有効な治療薬となりえることが報告された。さらに，癌関連の骨病変（骨転移のある乳癌患者）へのAMG 162の投与も行われており，こちらも有効であると報告された[10)]。AMG 162は，RANKLを直接ブロックす

る治療薬で，強い骨量増加作用を有するとともに副作用がないことから，骨粗鬆症を始め他の骨疾患の治療薬として認可される可能性が高いと思われる。RANKLの発見から6年目で，そのRANKLを標的とした効果的な治療薬が出現した。驚くべきスピードで研究と開発が進んでいることを示すものである。

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卷頭連載

目で見るBone Biology

第1回　破骨細胞の分化と機能の調節機構

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松本歯科大学総合歯科医学研究所

キーワード　RANKL, TRAF, NFATc1, OSCAR, DAP12

Summary　骨組織は骨吸収と骨形成をくり返し，常に新しい組織に置きかえられる．骨吸収を担う破骨細胞は，骨芽細胞が発現するM-CSFおよびRANKLにより，マクロファージから分化する．近年，破骨細胞前駆細胞膜上に発現するOSCARやTREM-2などの免疫受容体と，骨芽細胞あるいは破骨細胞前駆細胞の細胞膜上に発現するリガンドとの相互作用も破骨細胞の分化に重要であることが示された．これらの免疫受容体は，ITAMモチーフを有するアダプター分子FcRγやDAP12と共役してはたらき，その下流で破骨細胞分化のキーファクターであるNFATc1を活性化する．これらアダプター分子のダブルノックアウトマウスは，破骨細胞の形成阻害による重篤な大理石骨病を呈する．

略語一覧

M-CSF : macrophage-colony stimulating factor, RANKL : receptor activator of NF-κB ligand, OSCAR : osteoclast-associated receptor, TREM-2 : triggering receptor expressed by myeloid cells-2, ITAM : immunoreceptor tyrosine based activation motif, DAP12 : DNAX-activation protein 12, FcRγ: Fc receptor common γ subunit, NFATc1 : nuclear factor of activated T cell c1, TRAF : TNF receptor-associated factor, RANK : receptor activator of NF-κB, JNK : c-jun N-terminal kinase, p38MAPK : p38 mitogen-activated protein kinase, ERK : extracellular signal regulated kinase, VDR : vitamin D receptor, PTH : parathyroid hormone, PGE_2 : prostaglandin E_2, PKA : protein kinase A, IL-6 : interleukin-6, IL-11 : interleukin-11, IL-1 : interleukin-1, LPS : lipopolysaccharide, PKC : protein kinase C, TLR-4 : Toll-like receptor4, PLCγ: phospholipase Cγ

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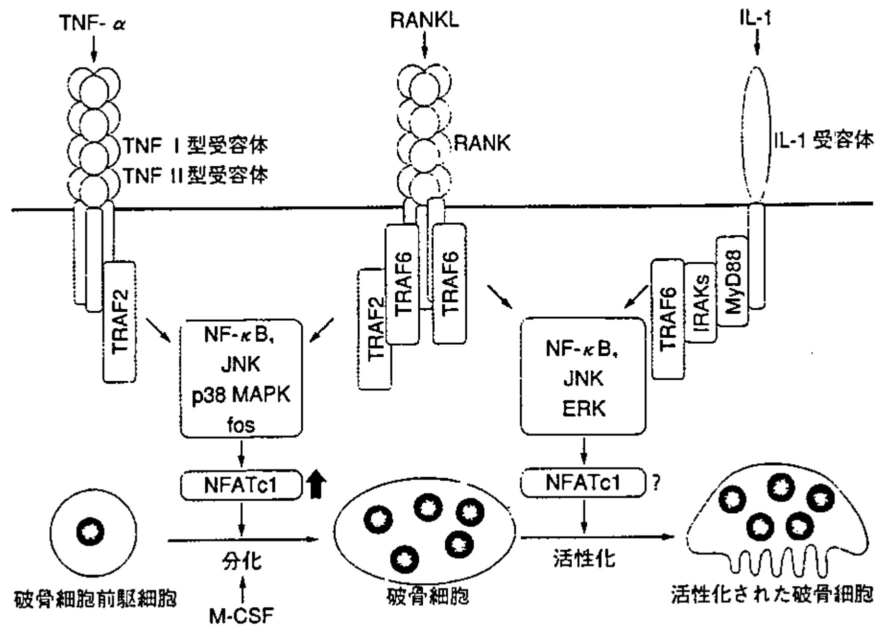

図1　破骨細胞の分化と機能発現を誘導するRANKL，TNF-αおよびIL-1のシグナル系

解説

骨芽細胞が発現するRANKLとM-CSFは破骨細胞の分化に必須なサイトカインである．骨芽細胞が提示するRANKLは，破骨細胞前駆細胞あるいは成熟破骨細胞が発現する受容体RANKを介して，破骨細胞の分化と活性化を誘導する．RANKシグナルは，シグナル伝達因子TRAFを介してNF-κB，JNK，p38MAPK，ERK，fosなどを活性化する．TNF-αは破骨細胞の分化のみを誘導し，IL-1は破骨細胞の機能のみを促進する．このことから，破骨細胞の分化にはTRAF2が，機能にはTRAF6がはたらくように思われたが，破骨細胞分化におけるTRAF6の重要性も指摘されている．破骨細胞の分化誘導には，転写因子NFATc1の活性化が必須である．一方，NFATc1が破骨細胞の機能発現に関与するか否かは不明である．

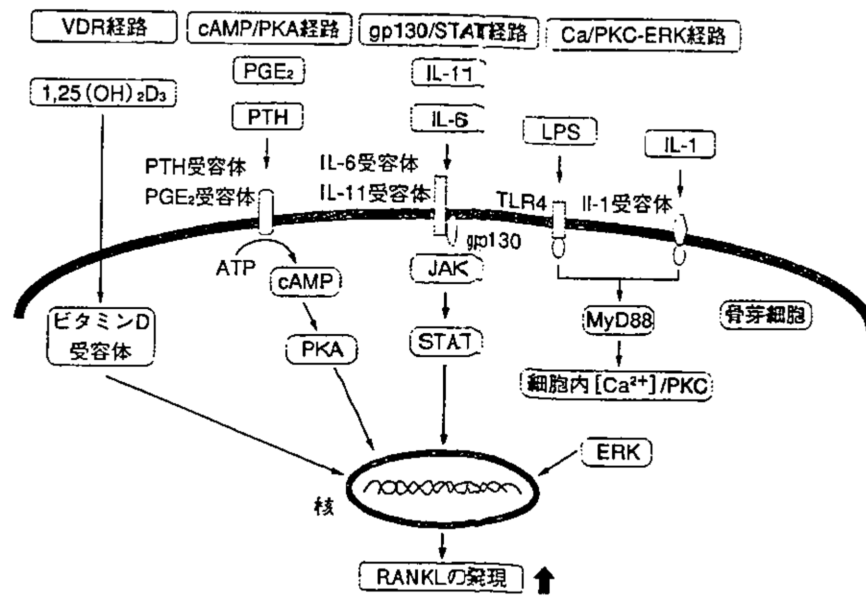

図2　RANKL発現を誘導する4つのシグナル系

解説

骨芽細胞におけるRANKL発現を誘導する経路は，①活性型ビタミンD_3〔1,25$(OH)_2D_3$〕のシグナルを伝達するvitamin D receptor(VDR)経路，②副甲状腺ホルモン(PTH)やPGE_2のシグナルを伝達するcAMP/PKA経路，③IL-6やIL-11のシグナルを伝達するgp130/STAT3経路，④IL-1やLPSのシグナルを伝達するCa/PKC-ERK経路が存在する．LPS受容体(TLR4)とIL-1受容体の下流にはMyD88が存在し，IL-1やLPSによるRANKL発現誘導はMyD88を介することが示されている．

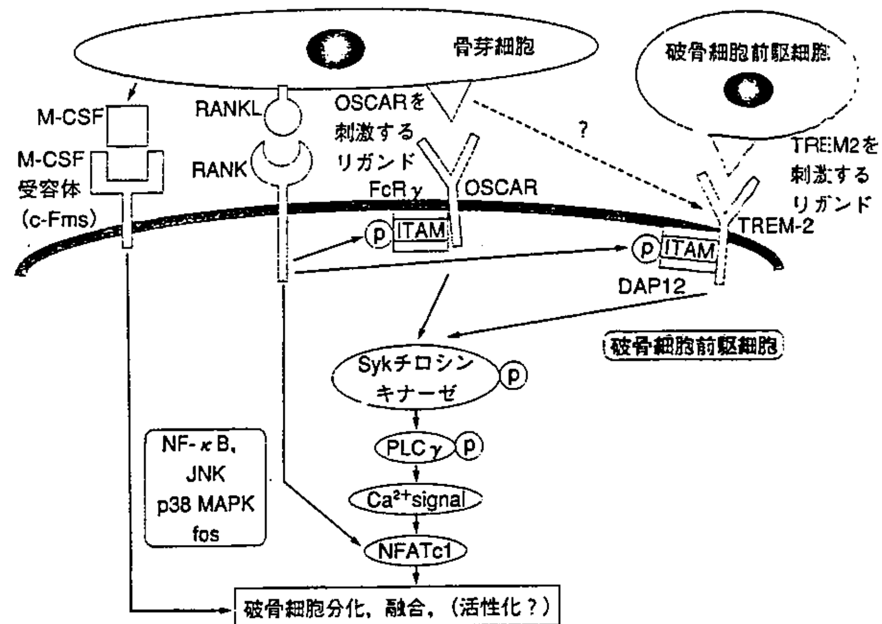

図3　破骨細胞形成におけるITAMを介するシグナル系

解説

近年，破骨細胞前駆細胞の細胞膜上に発現するOSCARやTREM-2などの免疫受容体と，骨芽細胞あるいは破骨細胞前駆細胞の細胞膜上に発現するリガンドとの相互作用も破骨細胞の分化に重要であることが示された．RANKL刺激はFcRγやDAP12のITAMモチーフのチロシンリン酸化を誘導する．そのチロシンリン酸化は非受容体型チロシンキナーゼのSykをリクルートする．SykはPLCγを活性化し，細胞内Caシグナルを誘導する．この細胞内Caシグナルは，転写因子NFATc1を活性化し，RANKLとM-CSFにより誘導される破骨細胞の分化を促進する．FcRγとDAP12のダブルノックアウトマウスは，破骨細胞の形成が著しく抑制された大理石骨病を呈する．骨芽細胞がTREM-2を刺激できるか否かは不明である．

Suppression of Osteoprotegerin Expression by Prostaglandin E_2 Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation[1]

Koji Suda,*† Nobuyuki Udagawa,‡ Nobuaki Sato,‡ Masamichi Takami,† Kanami Itoh,† Je-Tae Woo,¶ Naoyuki Takahashi,[2]§ and Kazuo Nagai¶

LPS is a potent stimulator of bone resorption in inflammatory diseases. The mechanism by which LPS induces osteoclastogenesis was studied in cocultures of mouse osteoblasts and bone marrow cells. LPS stimulated osteoclast formation and PGE_2 production in cocultures of mouse osteoblasts and bone marrow cells, and the stimulation was completely inhibited by NS398, a cyclooxygenase-2 inhibitor. Osteoblasts, but not bone marrow cells, produced PGE_2 in response to LPS. LPS-induced osteoclast formation was also inhibited by osteoprotegerin (OPG), a decoy receptor of receptor activator of NF-κB ligand (RANKL), but not by anti-mouse TNFR1 Ab or IL-1 receptor antagonist. LPS induced both stimulation of RANKL mRNA expression and inhibition of OPG mRNA expression in osteoblasts. NS398 blocked LPS-induced down-regulation of OPG mRNA expression, but not LPS-induced up-regulation of RANKL mRNA expression, suggesting that down-regulation of OPG expression by PGE_2 is involved in LPS-induced osteoclast formation in the cocultures. NS398 failed to inhibit LPS-induced osteoclastogenesis in cocultures containing OPG knockout mouse-derived osteoblasts. IL-1 also stimulated PGE_2 production in osteoblasts and osteoclast formation in the cocultures, and the stimulation was inhibited by NS398. As seen with LPS, NS398 failed to inhibit IL-1-induced osteoclast formation in cocultures with OPG-deficient osteoblasts. These results suggest that IL-1 as well as LPS stimulates osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of OPG expression, which is mediated by PGE_2 production. *The Journal of Immunology*, **2004, 172: 2504–2510.**

Osteoclasts are bone-resorbing multinucleated cells that originate from hemopoietic progenitors of the monocyte/macrophage lineage (1–4). Osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitors (4, 5). Studies of M-CSF-deficient *op/op* mice have shown that M-CSF produced by osteoblasts is an essential factor for osteoclastogenesis (6, 7). Receptor activator of NF-κB ligand (RANKL)[3] (3) was also identified as another factor essential for osteoclastogenesis (8–11). RANKL is a member of the TNF-ligand family that is expressed by osteoblasts/stromal cells as a membrane-associated factor. Osteoclast precursors express RANK, a receptor of RANKL; recognize RANKL through cell-cell interaction; and differentiate into osteoclasts in the presence of M-CSF (12, 13). Osteoblasts/Stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation in vivo and in vitro by interrupting the interaction between RANKL and RANK (14, 15).

In mouse cell cocultures, osteoclasts are formed in response to bone-resorbing factors such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), PGE_2, and IL-11 (4). Almost all of the bone-resorbing factors stimulate expression of RANKL in osteoblasts/stromal cells (4). Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by $1,25(OH)_2D_3$, cAMP/protein kinase A (PKA)-mediated signals induced by PTH or PGE_2, and gp130-mediated signals induced by IL-11 (4). Among these signals, vitamin D receptor- and cAMP/PKA-mediated signals suppress OPG expression in osteoblasts/stromal cells. Recently, we reported that compounds that elevate intracellular calcium, such as ionomycin, A23187, cyclopiazonic acid, and thapsigargin, stimulated osteoclast formation in mouse cocultures (16). Treatment of primary osteoblasts with those compounds stimulated the expression of RANKL. Thus, the signal mediated by calcium and protein kinase C (PKC) is proposed to be another (fourth) signal that induces RANKL expression in osteoblasts/stromal cells.

Severe bone loss due to excessive bone resorption is observed in inflammatory diseases such as periodontitis and osteomyelitis and some types of arthritides (17). LPS, a major constituent of Gram-negative bacteria, is proposed to be a potent stimulator of bone loss in these inflammatory diseases (17–22). Recently, Toll-like receptor 4 (TLR4) was identified as the signal-transducing receptor for LPS (23, 24). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1Rs. Both TLR4 and IL-1Rs use common signaling molecules such as myeloid differentiation factor 88 and TNFR-associated factor 6 (25–29). Macrophages, lymphocytes,

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[3] Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; COX2, cyclooxygenase 2; EIA, enzyme immunoassay; ERK, extracellular signal-regulated kinase; IL-1ra, IL-1 receptor antagonist; OPG, osteoprotegerin; PKA, protein kinase A; PKC, protein kinase C; PTH, parathyroid hormone; RANK, receptor activator of NF-κB; TLR, Toll-like receptor; TRAP, tartrate-resistant acid phosphatase.

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and osteoblasts/stromal cells express TLR4, and produce PGE_2 and proinflammatory cytokines such as TNF-α and IL-1 in response to LPS (30, 31). These inflammatory factors also stimulate osteoclastogenesis directly or indirectly (17–22, 32).

LPS stimulates PGE_2 production in the target cells through the induction of mitogen-inducible cyclooxygenase 2 (COX2) expression (33). NS398, a nonsteroidal anti-inflammatory agent, specifically inhibits COX2 without affecting COX1 activity (34). Therefore, NS398 has been used as a tool to explore the role of PGE_2 in pathological processes involving COX2 activity. PGE_2 exerts its biological actions through binding to four specific membrane receptors (EP1, EP2, EP3, and EP4) (35). Sakuma et al. (20, 21) reported that induction of osteoclast formation by LPS, TNF-α, and IL-1 was barely observed in cell cultures prepared from EP4 knockout ($EP4^{-/-}$) mice, and that urinary excretion of deoxypyridinoline, a sensitive marker for bone resorption, was not increased in $EP4^{-/-}$ mice injected with LPS. These results suggest that PGE_2 is a key factor in the enhancement of osteoclastogenesis by LPS in vivo and in vitro. However, it is still not known how PGE_2 is involved in the induction of osteoclastogenesis by LPS.

In the present study, we examined the mechanism of the induction of osteoclast formation by LPS in cocultures of mouse osteoblasts and bone marrow cells. We showed that LPS promoted osteoclastogenesis through two parallel events: one was direct enhancement of RANKL expression, and the other was suppression of OPG production mediated by PGE_2 in osteoblasts. In addition, IL-1 stimulated osteoclast formation in the cocultures in a manner similar to LPS.

Materials and Methods

Reagents and mice

LPS (*Escherichia coli* O26:B6) and PGE_2 were purchased from Sigma-Aldrich (St. Louis, MO). NS398 was from Calbiochem (San Diego, CA). Human rOPG and mouse rIL-1β were obtained from PeproTech (London, U.K.). Mouse rTNF-α and mouse rIL-1 receptor antagonist (rIL-1ra) were obtained from R&D Systems (Minneapolis, MN). Anti-mouse TNFR1 Ab was obtained from Genzyme Diagnostics (Cambridge, MA). Six- to 9-wk-old male and newborn ddY mice were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan). C57BL/6 (B6) mice and OPG-deficient ($OPG^{-/-}$) mice (C57BL/6 (B6)) were obtained from Clear Japan Clea (Tokyo, Japan). This study was reviewed and approved by the Showa University Animal Care and Use Committee.

Cell preparation and osteoclast formation assay

Primary osteoblasts were obtained from calvariae of newborn ddY mice, C57BL/6 (B6) mice, and $OPG^{-/-}$ mice by the conventional method using collagenase (36). Bone marrow cells were collected from femora and tibiae of 6- to 9-wk-old male mice. Primary osteoblasts (1×10^4 cells) and bone marrow cells (2×10^5 cells) were cocultured for 5 days in α-MEM containing 10% FCS (CSL, Victoria, Australia) in 96-well tissue culture plates (Corning, Corning, NY) (0.2 ml/well). Cocultures were incubated in the presence of LPS (0.001–10 μg/ml), PGE_2 (1 μM), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for the final 3 days. Some cocultures were pretreated with NS398 (1 μM), OPG (100 ng/ml), IL-1ra (10 μg/ml), and TNFR1 Ab (10 μg/ml) for 1 h before adding LPS, PGE_2, IL-1β, or TNF-α. Then cocultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. The results obtained from a typical experiment of three independent experiments are expressed as the mean ± SD of four cultures.

Measurement of PGE_2 production

Primary osteoblasts (3×10^4 cells) and bone marrow cells (6×10^5 cells) were cultured separately or in combination with or without LPS (1 μg/ml) or IL-1β (10 ng/ml) in α-MEM containing 10% FCS in 48-well culture plates (Corning). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture medium was determined using an enzyme immunoassay (EIA; Cayman Chemicals, Ann Arbor, MI). The Ab showed the following cross-reactivity determined by comparing the bond/free ratios with several eicosanoids: PGE_2, 100%; PGE_2 ethanolamide, 100%; PGE_3, 43%; PGE_1, 18.7%; 6-keto $PGF_1\alpha$, 1%; and 8-*iso* $PGF_2\alpha$, 0.25%.

Northern blot analysis

Primary osteoblasts (1×10^6 cells) were seeded in cell culture dishes (60 mm in diameter; Corning) and cultured in α-MEM containing 10% FCS for 3 days. After incubation in α-MEM containing 0.1% FCS for 3 h, the cells were incubated with LPS (1 μg/ml) or IL-1β (10 ng/ml). In some experiments, osteoblasts were cocultured with bone marrow cells (2×10^7 cells) in the presence of LPS (1 μg/ml) for 3 or 48 h. Some cultures were also treated with NS398 (1 μM) for 1 h before the addition of LPS. Total RNA was isolated from cultures using TRIzol (Life Technologies, Grand Island, NY). Northern blot analysis was performed using denaturing formaldehyde/agarose gels, as described (16). Double-stranded cDNA fragments encoding mouse RANKL OPG and COX2 were kindly provided by H. Yasuda (Snow Brand Milk Products, Tochigi, Japan). cDNA probes (RANKL, OPG, COX2, and β-tubulin) labeled with ^{32}P were synthesized using a cDNA labeling kit (Takara, Tokyo, Japan). The RANKL, OPG, COX2, and β-tubulin probes were hybridized with membranes to which total RNA isolated from osteoblasts had been transferred. The membranes were exposed to Kodak BioMax MS film (Rochester, NY) for 3–48 h. Signals of RANKL, OPG, COX2, and β-tubulin mRNA were quantified using a radioactive image analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). Signals of RANKL, OPG, and COX2 mRNAs were normalized with the respective β-tubulin mRNA expression levels to calculate the relative intensity.

Results

PGE_2 is required for osteoclast formation induced by LPS

LPS stimulated TRAP-positive osteoclast formation in cocultures of primary osteoblasts and bone marrow cells in a dose-dependent manner (Fig. 1*A*). The maximal number of osteoclasts was observed at 1 μg/ml of LPS. We then examined whether PGE_2 is involved in the induction of osteoclast formation by LPS. LPS (1 μg/ml) as well as PGE_2 (1 μM) induced TRAP-positive osteoclast formation in the cocultures (Fig. 1, *B* and *C*). NS398 (1 μM), a specific inhibitor of COX2, suppressed the induction of osteoclast formation by LPS, but not by PGE_2 in the cocultures (Fig. 1, *B* and *C*). Both LPS- and PGE_2-induced osteoclast formation in the cocultures was strongly inhibited by simultaneous addition of OPG (100 ng/ml) (Fig. 1, *B* and *C*). These results suggest that both PGE_2 production and RANKL-RANK interaction are required for LPS-induced osteoclast formation in the cocultures. We next examined whether LPS induces osteoclastogenesis through IL-1 and TNF-α. Recombinant IL-1β (10 ng/ml) and TNF-α (10 ng/ml) induced osteoclastogenesis in the cocultures. IL-1ra (10 μg/ml) and anti-mouse TNFR1 Ab (10 μg/ml) strongly inhibited the osteoclast formation induced by IL-1β and TNF-α, respectively. However, neither IL-1ra nor TNFR1 Ab affected osteoclastogenesis induced by LPS (Fig. 1*D*). These results suggest that PGE_2 is a critical factor in LPS-induced osteoclastogenesis.

Osteoblasts mainly produce PGE_2 in response to LPS

We then measured the PGE_2 concentration in the conditioned medium of cocultures incubated with or without LPS (1 μg/ml) for 6 h (Fig. 2*A*). LPS significantly increased the PGE_2 concentration in the culture medium (Fig. 2*A*). The addition of NS398 (1 μM) to the cocultures completely blocked the induction of PGE_2 production by LPS in the cocultures (Fig. 2*A*). To determine the type of cells that respond to LPS in the cocultures, osteoblasts and bone marrow cells were cultured separately in the presence or absence of LPS for 6 h (Fig. 2*B*). LPS stimulated PGE_2 production in the cultures of osteoblasts, but not bone marrow cells. LPS-induced PGE_2 production was strongly inhibited by the addition of NS398 (Fig. 2*B*). Northern blot analysis showed that treatment of osteoblasts with LPS for 3 h stimulated the expression of COX2 mRNA (Fig. 2*C*). These results suggest that osteoblasts in the cocultures

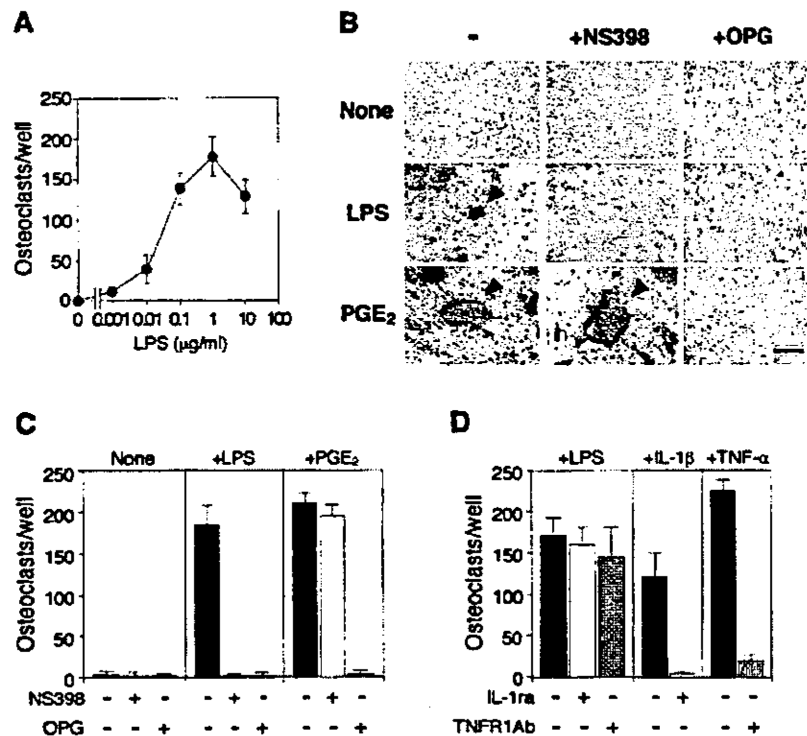

FIGURE 1. Effects of NS398, OPG, IL-1ra, and TNFR1 Ab on osteoclast formation in cocultures treated with LPS. *A*, Mouse primary osteoblasts and bone marrow cells were cocultured for 5 days. LPS (0.001–10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, LPS (1 μg/ml) or PGE_2 (1 μM) together with or without NS398 (1 μM) or OPG (100 ng/ml) was added to the cocultures for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate TRAP-positive multinucleated cells. Bar, 200 μm. *C*, TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *D*, LPS (1 μg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) together with or without IL-1ra (10 μg/ml) and TNFR1 Ab (10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

produce PGE_2 in response to LPS via up-regulation of COX2 mRNA expression.

LPS regulates RANKL and OPG gene expression in osteoblasts

We next analyzed the effects of LPS on RANKL and OPG mRNA expression levels in primary osteoblasts by Northern blot analysis (Fig. 3). Treatment of the osteoblasts with LPS increased RANKL mRNA expression with two peaks at 3 and 48 h. The expression of RANKL mRNA after treatment with LPS was increased within 1 h, and was still higher than that of the control cultures even after 72 h (Fig. 3*A*). The expression of OPG mRNA in osteoblasts was also enhanced by the treatment with LPS for 3 h (Fig. 3*A*). However, the expression of OPG mRNA in osteoblasts treated with LPS for 48 or 72 h was decreased to a level lower than that of the control culture (Fig. 3*A*). NS398 (1 μM) had no effect on the level of RANKL mRNA induced by LPS at 3 h (Fig. 3*B*). The LPS-induced up-regulation of RANKL mRNA expression at 48 h was slightly inhibited by the COX2 inhibitor, but the level of the mRNA was much higher than that in the control cultures. In contrast, the LPS-induced down-regulation of OPG mRNA expression in osteoblasts at 48 h after treatment with LPS was completely blocked by the addition of NS398, although the OPG mRNA expression at 3 h was not affected by the COX2 inhibitor (Fig. 3*B*). The expression levels of RANKL and OPG mRNAs in bone marrow cells were lower than those in primary osteoblasts, and were unchanged even after treatment with NS398 for 3 or 48 h (data not shown). These results suggest that PGE_2 produced by osteoblasts plays an important role in the down-regulation of OPG expression, but not the up-regulation of RANKL expression in osteoblasts treated with LPS.

Suppression of OPG expression is involved in induction of osteoclast formation by LPS

We next examined how PGE_2 production is involved in LPS-induced osteoclast formation using osteoblasts from OPG-deficient ($OPG^{-/-}$) mice. Primary osteoblasts prepared from $OPG^{-/-}$ mice were cocultured with bone marrow cells from wild-type mice in the presence or absence of NS398, OPG, and/or LPS (Fig. 4). In agreement with previously reported findings (37), TRAP-positive osteoclasts were formed in cocultures containing $OPG^{-/-}$ osteoblasts even in the absence of any stimulus (Fig. 4). The number of osteoclasts was further increased in the LPS-treated cocultures containing $OPG^{-/-}$ osteoblasts. NS398 strongly suppressed the spontaneous osteoclast formation in the control cocultures containing $OPG^{-/-}$ osteoblasts. This suggests that endogenous production of PGE_2 plays an important role in the osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts. NS398 slightly, but not completely, inhibited LPS-induced osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 4). OPG completely suppressed osteoclast formation in the cocultures treated or not treated with LPS (Fig. 4). These results suggest that the down-regulation of OPG expression by PGE_2 is crucially involved in the osteoclast formation induced by LPS in the cocultures.

IL-1 stimulates osteoclast formation in a manner similar to LPS

Because the signaling pathway of IL-1Rs is quite similar to that of TLR4 (26–30), we finally examined whether IL-1 stimulates osteoclastogenesis in the cocultures in a manner similar to LPS. IL-1β (10 ng/ml) induced osteoclast formation in the wild-type cocultures, and the induction was inhibited by NS398 (1 μM) and OPG (100 ng/ml) (Fig. 5*A*). IL-1β (10 ng/ml) also stimulated PGE_2 production in osteoblasts, but not in bone marrow cells after treatment for 6 h (Fig. 5*B*). Northern blot analysis showed that IL-1β up-regulated COX2 mRNA expression in osteoblasts at 3 h (Fig. 5*C*). IL-1β also stimulated RANKL mRNA expression at 3 h (data not shown). IL-1β stimulated osteoclast formation in the cocultures of $OPG^{-/-}$ osteoblasts and wild-type bone marrow cells (the control: 53 ± 11, the mean ± SD of four cultures) (Fig. 5*D*). NS398 (1 μM) did not completely suppress LPS-induced osteoclast formation in the cocultures with $OPG^{-/-}$ osteoblasts, but

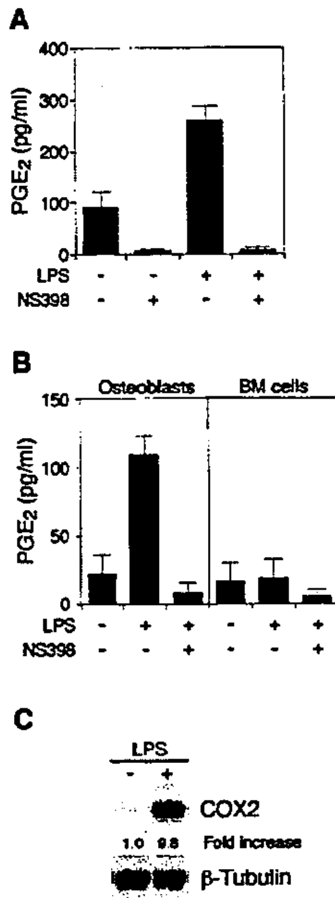

FIGURE 2. LPS induces PGE_2 production and COX2 expression in osteoblasts. *A*, Primary osteoblasts and bone marrow (BM) cells were cocultured with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE_2 concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 h. Total RNA was isolated from the osteoblasts, and COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals.

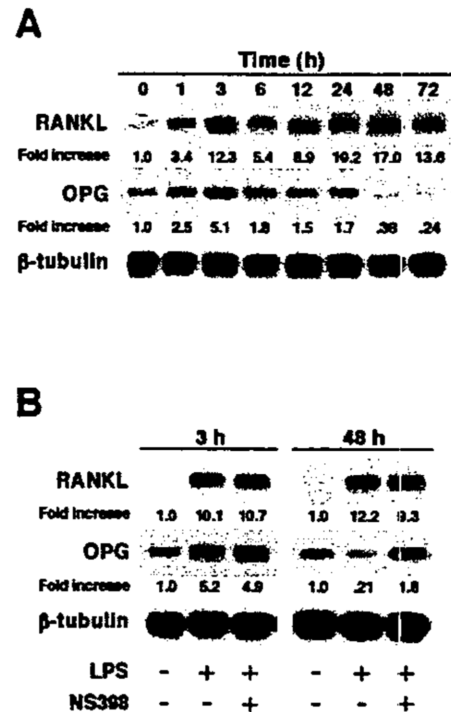

FIGURE 3. LPS regulates the expression of RANKL and OPG mRNAs in osteoblasts. *A*, Primary osteoblasts were treated with LPS (1 μg/ml) for 0–72 h. Total RNA was isolated from the osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals. *B*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 or 48 h in the presence of bone marrow cells. NS398 (1 μM) was also added to some cultures. After incubation for the indicated periods, bone marrow cells were removed by pipetting. Total RNA was isolated from osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals.

OPG did (Fig. 5*D*). These results suggest that IL-1 and LPS stimulate osteoclast formation in the same manner in the cocultures.

Discussion

In vivo and in vitro experiments have shown that PGE_2 is crucially involved in the induction of osteoclastic bone resorption by IL-1, TNF-α, and LPS (20). EP4 subtype-mediated signaling has been shown to be particularly important for the induction of bone resorption by such inflammation-related factors as well as PGE_2 (20). The present study showed that LPS stimulated COX2 expression and PGE_2 production in osteoblasts, and NS398, a specific inhibitor of COX2, strongly blocked the LPS-induced osteoclast formation in cocultures containing wild-type osteoblasts (Figs. 1 and 2). These results suggest that PGE_2 is somehow involved in LPS-induced osteoclast formation in the cocultures through PGE_2 receptors of EP4 subtype.

It was reported that LPS stimulated the expression of RANKL mRNA in osteoblasts obtained from $EP4^{-/-}$ mice, and that COX inhibitors did not block this stimulation (21). In agreement with this finding, NS398 failed to inhibit the induction of RANKL expression by LPS in osteoblasts (Fig. 3). These results suggest that LPS induced RANKL expression in a manner that was independent of PGE_2 production in osteoblasts. In contrast, the treatment of osteoblasts in the cocultures with LPS together with NS398 blocked the down-regulation of OPG mRNA expression at 48 h (Fig. 3). This suggests that suppression of OPG by PGE_2 is an important event in osteoclast formation in the cocultures treated with LPS. This notion was further supported by the finding that LPS stimulated osteoclast formation even in the presence of NS398 in cocultures containing osteoblasts derived from $OPG^{-/-}$ mice (Fig. 4). Thus, PGE_2 appears to play an important role as a suppressor of OPG expression rather than an activator of RANKL expression in LPS-induced osteoclast formation (Fig. 6). Recently, Fu et al. (38) reported that the activation of CREB by PTH is required for PTH-induced down-regulation of OPG expression. This suggests that the cAMP-PKA signals play a role in PGE_2-induced suppression of OPG mRNA expression. Further study will elucidate the detail mechanism of the down-regulation of OPG expression by PGE_2.

PGE_2 has been shown to induce RANKL mRNA expression in osteoblasts (10). Suzawa et al. (39) reported that PGE_2-induced RANKL expression is mediated through the cAMP signaling pathway. In our experiments, NS398 failed to inhibit RANKL expression in osteoblasts treated with LPS for as long as 48 h (Fig. 3).

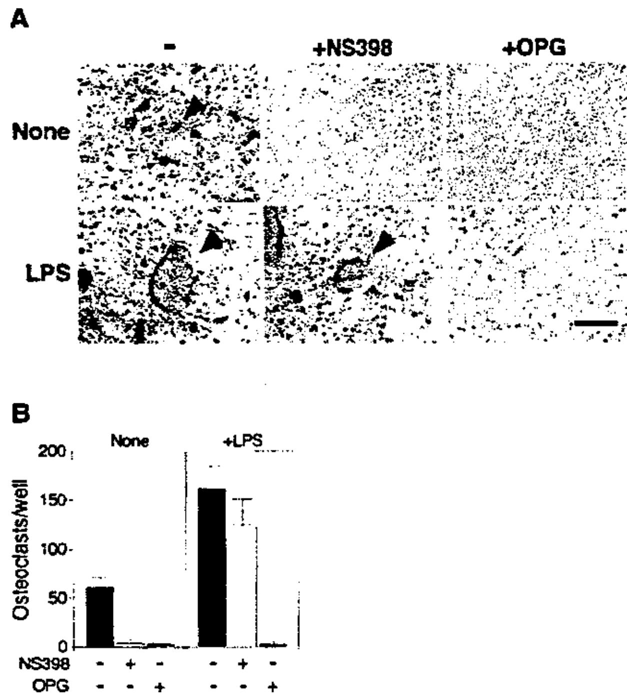

FIGURE 4. Effects of NS398 on LPS-induced osteoclast formation in cocultures containing $OPG^{-/-}$ mouse-derived osteoblasts. *A*, Primary osteoblasts prepared from $OPG^{-/-}$ mice were cocultured with wild-type bone marrow cells for 5 days. LPS (1 μg/ml) was added to the cocultures with or without NS398 (1 μM) or OPG (100 ng/ml) for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate the TRAP-positive osteoclasts. Bar, 200 μm. *B*, TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

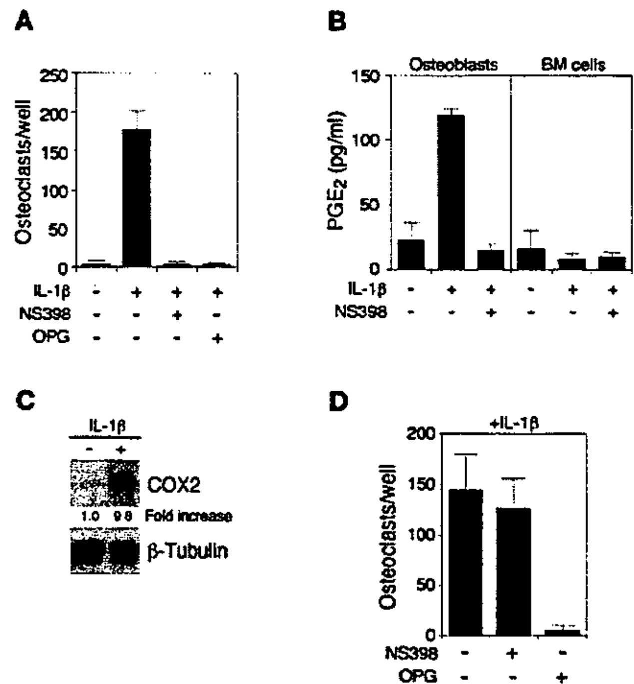

FIGURE 5. IL-1 induces osteoclast formation in the cocultures in a manner similar to LPS. *A*, Mouse primary osteoblasts and bone marrow (BM) cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE_2 concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with IL-1β (10 ng/ml) for 3 h, and then COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals. *D*, Primary osteoblasts prepared from $OPG^{-/-}$ mice and wild-type bone marrow cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

This suggests that LPS induces RANKL expression by the mechanism independent of PGE_2 production. Kikuchi et al. (40) reported that LPS induces RANKL through extracellular signal-regulated kinase (ERK) and PKC. We also confirmed that calcium/PKC inhibitors, such as BAPTA-AM (an intracellular calcium chelator) and Ro-32-0432 (a PKC inhibitor), and ERK inhibitor PD98059 inhibited LPS-induced RANKL mRNA expression in osteoblasts (K.S., unpublished observation). PD98059 failed to inhibit the induction of RANKL mRNA expression by PGE_2 and the induction of osteoclast formation in cocultures treated with PGE_2 (data not shown). These results suggest that LPS directly stimulates RANKL expression through calcium/PKC signals, followed by ERK signals in osteoblasts. It is unlikely that PGE_2-induced signals directly cross talk with LPS-induced signals in the induction of RANKL expression in osteoblasts.

The intracellular signaling pathway of TLR4 is quite similar to that of IL-1Rs (25–29). Like LPS, IL-1 stimulated COX2 mRNA expression at 3 h and PGE_2 production at 6 h in osteoblast cultures (Fig. 5). IL-1β also induced RANKL mRNA expression in osteoblasts, as previously reported (data not shown) (32). NS398 inhibited IL-1-induced osteoclast formation strongly in cocultures containing wild-type osteoblasts, but only partially in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 5). These results suggest that PGE_2 produced by osteoblasts in response to IL-1 plays a similar role to LPS in osteoclast formation through the suppression of OPG expression (Fig. 6).

In cocultures containing $OPG^{-/-}$ osteoblasts, osteoclasts were formed even in the absence of any stimulus (Fig. 4). The spontaneous osteoclast formation was strongly inhibited by the addition of either OPG or NS398 (Fig. 4). These results suggest that RANKL is involved in the spontaneous osteoclast formation, and that PGE_2 constitutively produced in the cocultures stimulates RANKL expression in osteoblasts. LPS and IL-1β further enhanced osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 4), suggesting that the up-regulation of RANKL expression by LPS and IL-1 enhances the osteoclast formation. The induction of osteoclast formation by LPS and IL-1 in cocultures containing $OPG^{-/-}$ osteoblasts was partially inhibited by the addition of NS398. Therefore, PGE_2 induced by LPS and IL-1 appears to be involved in RANKL expression in osteoblasts. Our results indicate that the full inhibition of LPS- and IL-1-induced osteoclast formation by NS398 requires PGE_2-dependent suppression of OPG production (Fig. 6).

The previous studies have shown that OPG production by osteoblasts is down-regulated by bone-resorbing factors such as $1,25(OH)_2D_3$, PTH, and PGE_2 (38, 41–44). Our results confirmed the previous finding that osteoclasts spontaneously form in the control cocultures containing $OPG^{-/-}$ osteoblasts. The decrease in OPG production by osteoblasts was a key event for the induction

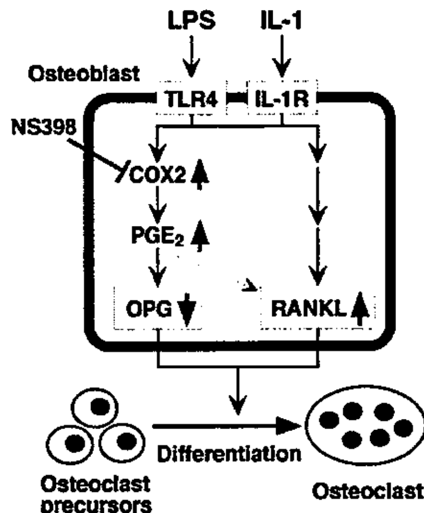

FIGURE 6. A possible mechanism of the induction of osteoclastogenesis by LPS and IL-1. LPS and IL-1 promote the differentiation of osteoclast precursors into osteoclasts through two parallel events in osteoblasts: direct enhancement of RANKL expression, and suppression of OPG production mediated by PGE_2. PGE_2 induced by LPS and IL-1 also stimulates RANKL expression, but the suppression of OPG production in osteoblasts appears to be more important than the induction of RANKL expression in osteoblasts for the stimulation of osteoclastogenesis. See text for details.

of osteoclastogenesis by LPS and IL-1. $OPG^{-/-}$ mice exhibited severe osteoporosis caused by enhanced osteoclast formation and function (14, 15, 45, 46). These results suggest that OPG is a physiological regulator of bone resorption, and that the balance between RANKL and OPG expressions at bone is particularly important for the regulation of bone resorption in vivo and in vitro.

We previously reported that LPS and IL-1 directly stimulated the survival, fusion, and pit-forming activity of osteoclasts (47). Those results together with the results shown in this study suggest that LPS and IL-1 are involved in the stimulation of osteoclastic bone resorption in several ways: LPS and IL-1 directly stimulate osteoclast function, induce RANKL expression in osteoblasts, and suppress OPG expression through enhancement of PGE_2 production. Further studies will be necessary to elucidate the precise mechanism of the regulation of osteoclastic bone resorption induced by these inflammatory factors.

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MyD88 But Not TRIF Is Essential for Osteoclastogenesis Induced by Lipopolysaccharide, Diacyl Lipopeptide, and IL-1α

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Abstract

Myeloid differentiation factor 88 (MyD88) plays essential roles in the signaling of the Toll/interleukin (IL)-1 receptor family. Toll–IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF)-mediated signals are involved in lipopolysaccharide (LPS)-induced MyD88-independent pathways. Using MyD88-deficient (MyD88$^{-/-}$) mice and TRIF-deficient (TRIF$^{-/-}$) mice, we examined roles of MyD88 and TRIF in osteoclast differentiation and function. LPS, diacyl lipopeptide, and IL-1α stimulated osteoclastogenesis in cocultures of osteoblasts and hemopoietic cells obtained from TRIF$^{-/-}$ mice, but not MyD88$^{-/-}$ mice. These factors stimulated receptor activator of nuclear factor-κB ligand mRNA expression in TRIF$^{-/-}$ osteoblasts, but not MyD88$^{-/-}$ osteoblasts. LPS stimulated IL-6 production in TRIF$^{-/-}$ osteoblasts, but not TRIF$^{-/-}$ macrophages. LPS and IL-1α enhanced the survival of TRIF$^{-/-}$ osteoclasts, but not MyD88$^{-/-}$ osteoclasts. Diacyl lipopeptide did not support the survival of osteoclasts because of the lack of Toll-like receptor (TLR)6 in osteoclasts. Macrophages expressed both TRIF and TRIF-related adaptor molecule (TRAM) mRNA, whereas osteoblasts and osteoclasts expressed only TRIF mRNA. Bone histomorphometry showed that MyD88$^{-/-}$ mice exhibited osteopenia with reduced bone resorption and formation. These results suggest that the MyD88-mediated signal is essential for the osteoclastogenesis and function induced by IL-1 and TLR ligands, and that MyD88 is physiologically involved in bone turnover.

Key words: Toll-like receptor • osteoprotegerin • RANKL • bone resorption • osteoporosis

Introduction

Osteoclasts, the multinucleated cells that resorb bone, originate from monocyte-macrophage lineage cells. Osteoblasts (or bone marrow stromal cells) are involved in osteoclastogenesis (1, 2). Macrophage CSF (M-CSF) produced by osteoblasts is an essential factor for osteoclast formation. Receptor activator of NF-κB ligand (RANKL) is another cytokine essential for osteoclastogenesis expressed by osteoblasts as a membrane-associated cytokine (1–5). Osteoclast precursors

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Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage CSF; MEK, MAPK/ERK kinase; MyD88, myeloid differentiation factor 88; MyD88$^{-/-}$, MyD88-deficient; OPG, osteoprotegerin; PKC, protein kinase C; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule; TRAM$^{-/-}$, TRAM-deficient; TRAP, tartrate-resistant acid phosphatase; TRIF, TIR domain-containing adaptor-inducing IFN-β; TRIF$^{-/-}$, TRIF-deficient.

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express RANK (a receptor of RANKL), recognize RANKL expressed by osteoblasts through cell–cell interaction, and differentiate into osteoclasts in the presence of M-CSF. Osteoprotegerin (OPG) produced mainly by osteoblasts is a soluble decoy receptor for RANKL (6). OPG blocks osteoclastogenesis by inhibiting the RANKL–RANK interaction. Bone resorption-stimulating hormones and cytokines enhance the expression of RANKL in osteoblasts. Mature osteoclasts also express RANK, and RANKL supports the survival and stimulates the bone-resorbing activity of osteoclasts (1–5).

LPS, a major constituent of gram-negative bacteria, is proposed to be a potent stimulator of bone resorption in inflammatory diseases (7). CD14 is a membrane-anchored glycoprotein that functions as a member of the LPS receptor system. Toll-like receptor (TLR)4 is a critical receptor and signal transducer for LPS (8, 9). Bacterial lipoprotein/lipopeptides have pathogen-specific molecular patterns. The complex of TLR6 and TLR2 recognizes diacyl lipopeptide (9, 10). We found that lipoproteins derived from *Mycoplasma salivarium*, a member of the human oral microbial flora, and a synthetic diacyl lipopeptide (FSL-1) activate human gingival fibroblasts to induce inflammatory cytokine production via p38 mitogen-activated protein kinase (MAPK)-mediated signals (11).

TLR family members have an intracytoplasmic region, called the Toll/IL-1 receptor (TIR) homology domain. Through the homophilic interaction of TIR domains, myeloid differentiation factor 88 (MyD88) is associated not only with cytokine receptors for IL-1 and IL-18 but also with various TLRs (9, 12). MyD88-deficient ($MyD88^{-/-}$) mice showed resistance to LPS-induced responses including cytokine production by macrophages, B cell proliferation, and endotoxin shock (12, 13). $MyD88^{-/-}$ mice did not respond to IL-1, IL-18, or other microbial cell wall components such as peptidoglycan and lipopeptides (14). However, $MyD88^{-/-}$ macrophages showed a delayed activation of NF-κB and MAPK cascades in response to LPS (13). In addition, LPS induced the functional maturation of $MyD88^{-/-}$ dendritic cells, including the up-regulation of costimulatory molecules (15). These results indicate the existence of a MyD88-independent pathway through TLR4.

Recently, TIR domain-containing adaptor-inducing IFN-β (TRIF) was identified as an adopter involved in MyD88-independent signaling pathways (16). TRIF plays essential roles in TLR4- and TLR3-mediated pathways (17, 18). TRIF-related adaptor molecule (TRAM) was identified as an adopter specifically involved in the TLR4-mediated MyD88-independent signaling pathway (19, 20). Using TRIF-deficient ($TRIF^{-/-}$) mice and TRAM-deficient ($TRAM^{-/-}$) mice, it was shown that both MyD88-dependent and TRAM–TRIF-dependent pathways were required for LPS-induced proinflammatory cytokine production in macrophages and for LPS-induced activation of B cells (19). In addition, p38 MAPK- and MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK-mediated signals are shown to be involved in LPS-induced proinflammatory cytokine production in human osteoblastic cells (21).

Using $MyD88^{-/-}$ mice and $TRIF^{-/-}$ mice, we explored the roles of MyD88 and TRIF in osteoclast differentiation and function induced by LPS, IL-1α, and diacyl lipopeptide. We also examined whether both MyD88 and TRIF signals are involved in cytokine production in osteoblasts as well as bone marrow macrophages. We have shown that MyD88-mediated signals, but not TRIF-mediated signals, induced RANKL expression in osteoblasts. LPS stimulated IL-6 production in $TRIF^{-/-}$ osteoblasts, but not $TRIF^{-/-}$ macrophages. MyD88- but not TRIF-mediated signals supported the survival of osteoclasts induced by LPS. Bone histomorphometry revealed that $MyD88^{-/-}$ mice exhibited typical osteopenia with reduced bone resorption and formation.

Materials and Methods

Animals and Drugs. $MyD88^{-/-}$, TLR4-deficient ($TLR4^{-/-}$), and $TRIF^{-/-}$ mice with the genetic background of C57BL/6J were generated and maintained as described previously (12, 17, 22). After heterozygous (+/−) mating, heterozygous (+/−), homozygous (−/−), and WT (+/+) mice were identified by PCR analysis of DNA obtained from the tail of each mouse. WT (C57BL/6J) mice were obtained from Japan Clea Co. All procedures for animal care were approved by the Animal Management Committee of Matsumoto Dental University. LPS (*Escherichia coli* O55:B5) and H-89 were purchased from Sigma-Aldrich. A synthetic diacyl lipopeptide (FSL-1) was prepared as described previously (23). PD98059, BAPTA-AM, Ro-32-0432, A23187, and phorbol-12-myristate-13-acetate (PMA) were obtained from Calbiochem Co. Recombinant human soluble RANKL and human OPG were purchased from PeproTech. Recombinant mouse IL-1α was obtained from Genzyme. Recombinant human M-CSF (Leukoprol) was obtained from Kyowa Hakko Kogyo Co. 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and prostaglandin E_2 (PGE_2) were purchased from Wako Pure Chemical Industries Ltd. Rabbit anti–mouse phospho-ERK1/2 (Thr202/Tyr204) antibody and rabbit anti–mouse ERK1/2 antibody were purchased from Cell Signaling Technology Inc. An ELISA kit for mouse IL-6 was obtained from R&D Systems. Specific PCR primers for mouse TLR2, TLR4, TLR6, IL-1R, CD14, RANKL, TRIF, and TRAM and GAPDH were synthesized by Invitrogen. Other chemicals and reagents were of analytical grade.

Osteoclast Differentiation Assay. To isolate primary osteoblasts from either $MyD88^{-/-}$, $TLR4^{-/-}$, $TRIF^{-/-}$, or WT mice, calvaria from 2-d-old mice (male and female) were cut into small pieces and cultured for 5 d in type I collagen gel (cell matrix type-IA; Nitta Gelatin, Inc.) prepared in an α-MEM (Sigma-Aldrich) containing 10% FBS (JRH Biosciences; reference 6). Osteoblasts grown from the calvarium were collected by treating the collagen gel cultures with collagenase and stored at −80°C before use. Bone marrow cells obtained from tibiae of 5–8-wk-old male mice were suspended in an α-MEM supplemented with 10% FBS in 60-mm-diameter dishes for 16 h in the presence of 50 ng/ml M-CSF. Next, nonadherent cells were harvested as hemopoietic cells. The hemopoietic cells (1.5×10^5 cells/well) were cocultured with osteoblasts (1.5×10^4 cells/well) prepared from each mouse for 7 d in a 48-well plate with 0.3 ml of α-MEM containing 10% FBS in the presence of test chemicals. In some experiments, the hemopoietic cells prepared from male $MyD88^{-/-}$

and WT mice were cultured in the presence of 100 ng/ml RANKL and 50 ng/ml M-CSF for 5 d. All cultures were incubated in quadruplicate, and cells were replenished on day 3 with fresh medium. Adherent cells were fixed with 10% formaldehyde in PBS, treated with ethanol-acetone (50:50), and stained for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) as described previously (24). TRAP positive multinucleated cells containing more than three nuclei were counted as osteoclasts. The results obtained from one experiment typical of at least three independent experiments were expressed as the mean ± SEM of three cultures. The significance of the differences was determined using Student's *t* test.

Survival Assay of Mature Osteoclasts. Osteoblasts and freshly prepared bone marrow cells were cocultured in α-MEM containing 10% FBS and 10^{-8} M $1,25(OH)_2D_3$ and 10^{-6} M PGE_2 in 100-mm-diameter dishes precoated with type I collagen gel as described previously (24). Osteoclasts were formed within 6 d in the cocultures. All the cells in the cocultures were recovered from the dishes by treatment with α-MEM containing 0.3% collagenase (Wako Pure Chemical Industries Ltd.). The cocultures at day 6 contained ~5% osteoclasts. To purify osteoclasts, the crude osteoclast preparation was plated in 48-well culture dishes. After cells were incubated for 6 h, osteoblasts were removed by treatment with trypsin (0.05%) and EDTA (0.53 mM; Invitrogen) for 5 min on the dishes. Some cultures were fixed and stained for TRAP. These cultures contained ~95% osteoclasts. Purified osteoclasts were further incubated for 24 h in the presence of test chemicals and stained for TRAP. TRAP positive multinucleated cells containing more than three nuclei were counted as osteoclasts. The results were expressed as the mean ± SEM of three cultures.

Preparation of Bone Marrow Macrophages. Bone marrow macrophages were prepared from $MyD88^{-/-}$, $TRIF^{-/-}$, and WT mice to examine LPS-induced IL-6 production. Bone marrow cells obtained from tibiae of $MyD88^{-/-}$, $TRIF^{-/-}$, and WT mice (5–8-wk-old adults) were cultured for 16 h in α-MEM supplemented with 10% FBS in the presence of 50 ng/ml M-CSF. Nonadherent cells were harvested as hemopoietic cells and further cultured with 50 ng/ml M-CSF for 2 d. Adherent cells were used as bone marrow macrophages. Bone marrow macrophages were incubated for 24 h with 100 ng/ml LPS in the presence of 50 ng/ml M-CSF, and the conditioned medium was collected for the determination of IL-6.

PCR Amplification of Reverse-transcribed mRNA. For semiquantitative RT-PCR analysis, osteoblasts prepared from the $MyD88^{-/-}$, $TRIF^{-/-}$, or WT mice were cultured in α-MEM containing 10% FBS in the presence of test chemicals on 60-mm-diameter dishes. After cells were cultured, total cellular RNA was extracted from osteoblasts using TRIzol solution (Life Technologies). First-strand cDNA was synthesized from total RNA with random primers and subjected to PCR amplification with EX *Taq* polymerase (Takara Biochemicals) using specific PCR primers: mouse, TLR2, forward, 5′-AAACAACTTACCGAAACCTCAGAC-3′ (nucleotides 273–296) and reverse, 5′-TGTAAATTTGTGAGATTGGGAAAA-3′ (nucleotides 748–771); mouse, TLR4, forward, 5′-AGTGGGTCAAGGAACAGAAGCA-3′ (nucleotides 1766–1787) and reverse, 5′-CTTTACCAGCTCATTTCTCACC-3′ (nucleotides 2055–2076); mouse TLR6, forward, 5′-GCCTGACTCTTACAGGTGTGACTA-3′ (nucleotides 1698–1721) and reverse, 5′-TTATGATGGGACAAATAGAGTTCA-3′ (nucleotides 2175–2198); mouse CD14, forward, 5′-ACATCTTGAACCTCCGCAAC-3′ (nucleotides 454–473) and reverse, 5′-AGGGTTCCTATCCAGCCTGT-3′ (nucleotides 934–953); mouse IL-1R, forward, 5′-TAATGAGTTACCCGAGGTCCA-3′ (nucleotides 570–590) and reverse, 5′-AGGCATCGTATGTCTTTCCA-3′ (nucleotides 1257–1276); mouse RANKL, forward, 5′-CGCTCTGTTCCTGTACTTTCGAGCG-3′ (nucleotides 195–219) and reverse, 5′-TCGTGCTCCCTCCTTTCATCAGGTT-3′ (nucleotides 757–781); mouse TRIF, forward, 5′-ATGGATAACCCAGGGCCTT-3′ (nucleotides 187–205) and reverse, 5′-TTCTGGTCACTGCAGGGGAT-3′ (nucleotides 696–715); mouse TRAM, forward, 5′-ATGGCCAGTCCTGGACTTC-3′ (nucleotides 126–144) and reverse, 5′-CAAGCAGGCTTCCTCAGAATT-3′ (nucleotides 576–596); and mouse GAPDH, forward, 5′-ACCACAGTCCATGCCATCAC-3′ (nucleotides 566–585) and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ (nucleotides 998–1017). The PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining with UV light illumination. The sizes of the PCR products for mice TLR2, TLR4, TLR6, CD14, IL-1R, RANKL, TRIF, TRAM, and GAPDH are 499, 311, 501, 500, 707, 587, 535, 476, and 452 bp, respectively.

Northern Blot Analysis. WT mouse-derived osteoblasts (10^6 cells) were seeded in cell culture dishes (60 mm in diameter) and cultured in α-MEM containing 10% FBS for 3 d. After incubation in α-MEM containing 0.1% FBS for 3 h, cells were treated with LPS for 3 h. Some cultures were also treated with several kinds of signal inhibitors for 1 h before the addition of LPS. Total RNA was isolated from cultures using TRIzol. Northern blot analysis was performed using denaturing formaldehyde/agarose gels as described previously (25). Double stranded complementary DNA (cDNA) fragments encoding mouse RANKL were provided by H. Yasuda (Snow Brand Milk Products, Tokyo, Japan). cDNA probes (RANKL and β-tubulin) labeled with ^{32}P were synthesized using a cDNA labeling kit (Takara). The RANKL and β-tubulin probes were hybridized with membranes to which total RNA isolated from osteoblasts had been transferred. The membranes were exposed to Kodak BioMax MS film. Signals for RANKL and β-tubulin mRNA were quantified using a radioactive image analyzer (BAS2000; Fuji Photo Film Co., Ltd.). Signals for RANKL were normalized with the respective β-tubulin mRNA expression levels to calculate the relative intensity.

Western Blot Analysis. Confluent $MyD88^{-/-}$- and WT mouse-derived osteoblasts were further incubated with test chemicals for 30 min, washed twice with PBS, and lysed in cell lysate buffer. Whole cell extracts were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Millipore). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), the antiphospho-ERK1/2 antibody or anti-ERK antibody (1:1,000) was added to TBS-T containing 5% skim milk and the bound antibodies were visualized using the enhanced chemiluminescence assay with reagents from Amersham Biosciences followed by exposure to X-ray film.

Bone Histomorphometry. Seven male $MyD88^{-/-}$ and WT (14-wk-old) mice were killed for bone histomorphometric analysis. For in vivo fluorescent labeling, intraperitoneal injections of tetracycline hydrochloride (Sigma-Aldrich) (30 mg/kg of body weight) and calcein (Sigma-Aldrich) (6 mg/kg of body weight) were administered at days 0 and 2. Mice were killed on day 4. Their vertebrae were removed, fixed in 70% ethanol, and embedded in glycol-methacrylate without decalcification. Sections were prepared and stained with Villanueva Goldner to discriminate between mineralized and unmineralized bone and to identify cellular components. Quantitative histomorphometric analy-

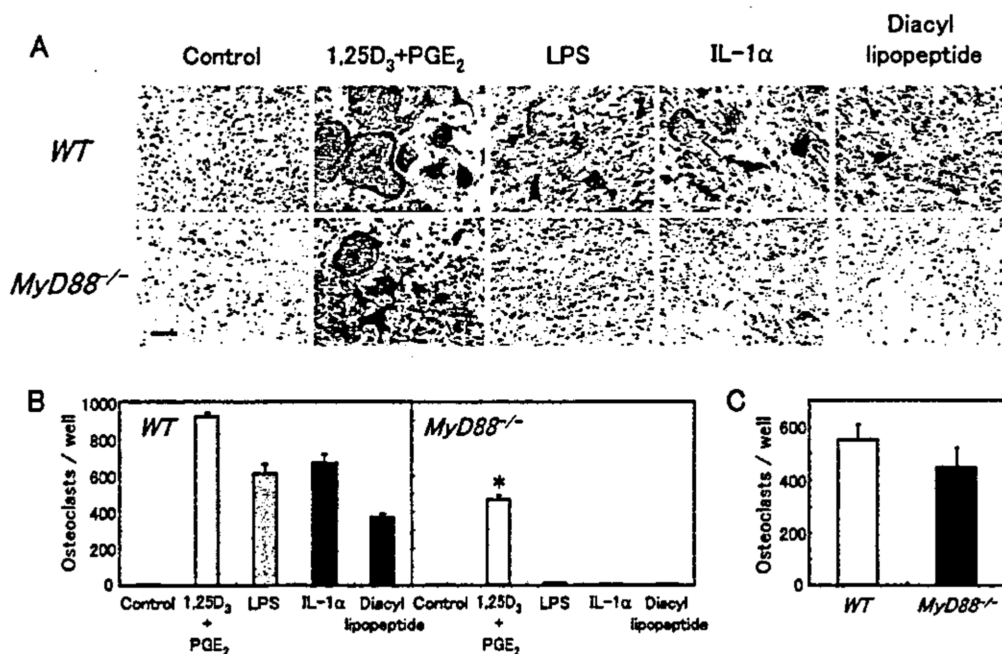

Figure 1. MyD88 is essential for osteoclastogenesis induced by LPS, IL-1α, and diacyl lipopeptide. (A and B) Effects of $1,25(OH)_2D_3$ plus PGE_2, LPS, IL-1α, and diacyl lipopeptide on osteoclast formation in cocultures of osteoblasts and hemopoietic cells prepared from male WT and $MyD88^{-/-}$ mice. Calvarial osteoblasts (1.5×10^4 cells/well) and bone marrow–derived hemopoietic cells (1.5×10^5 cells/well) prepared from WT and $MyD88^{-/-}$ mice were cocultured for 7 d in a 48-well plate in the presence or absence of 1 μg/ml LPS, 10 ng/ml IL-1α, 10^{-8} M diacyl lipopeptide, and 10^{-8} M $1,25(OH)_2D_3$ plus 10^{-6} M PGE_2. Cells were fixed and stained for TRAP. TRAP positive osteoclasts appeared dark red (A). Bar, 100 μm (A). TRAP positive multinucleated cells containing three or more nuclei were counted as osteoclasts (B). Values were expressed as the mean ± SD of three cultures. Significant difference between WT and $MyD88^{-/-}$ cultures (*, $P < 0.005$). (C) Effect of M-CSF plus RANKL on osteoclast formation in hemopoietic cells prepared from WT and $MyD88^{-/-}$ mice. Bone marrow–derived hemopoietic cells (1.5×10^5 cells/well) prepared from WT and $MyD88^{-/-}$ mice were cultured for 5 d in the presence of 50 ng/ml M-CSF plus 100 ng/ml RANKL. Cells were fixed and stained for TRAP. TRAP positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Values were expressed as the mean ± SD of three cultures. Experiments were repeated five times with similar results.

sis was conducted in a blind fashion. Images were also visualized by fluorescent microscopy. Nomenclature and units were used according to the recommendation of the Nomenclature Committee of the American Society for Bone and Mineral Research (26). Statistical analysis was done using Student's *t* test.

Tissue Preparation for the Histological Analysis of Bone. 12-wk-old $MyD88^{-/-}$ and WT mice (two males of each type) were anesthetized with sodium pentobarbital (Nembutal; Dainippon Pharmaceutical Co., Ltd.), and perfused for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, through the left ventricle. Tibiae were removed and immersed into the same fixative for 20 h at 4°C. Specimens were washed with the phosphate buffer, and decalcified in 10% EDTA, pH 7.3, for 2 wk at 4°C. Decalcified specimens were dehydrated in a graded series of ethanol solutions, embedded in paraffin, and cut into 4-μm-thick sections. TRAP staining was performed on the specimens as described previously (27, 28) and TRAP positive osteoclasts were detected under a light microscope.

Results

MyD88 Is an Essential Molecule for Osteoclastogenesis Induced by LPS, IL-1α, and Diacyl Lipopeptide First, we examined the effects of LPS, IL-1α, and a synthetic diacyl lipopeptide (FSL-1) on osteoclast formation in the murine coculture system. LPS, IL-1α, and diacyl lipopeptide as well as $1,25(OH)_2D_3$ plus PGE_2 stimulated the formation of TRAP positive osteoclasts (cells stained red) in cocultures of primary osteoblasts and bone marrow–derived hemopoietic cells obtained from WT mice (Fig. 1, A and B). In contrast, LPS, IL-1α, and diacyl lipopeptide did not in-

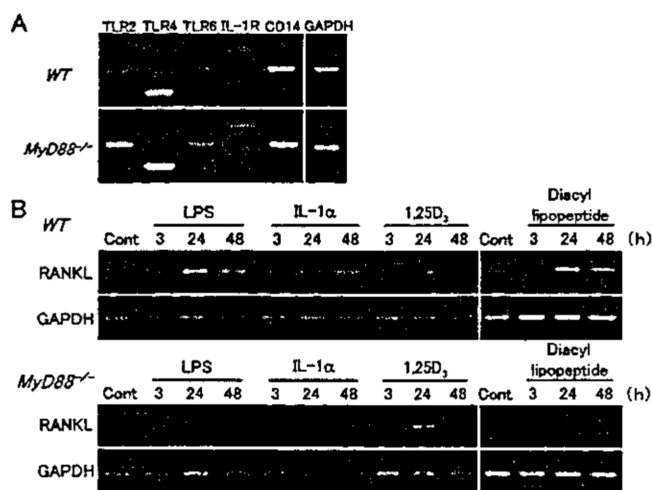

Figure 2. MyD88-mediated signals are involved in RANKL expression in osteoblasts treated with LPS, diacyl lipopeptide, and IL-1α. (A) Expression of TLR2, TLR4, TLR6, IL-1R, and CD14 in osteoblasts prepared from WT and $MyD88^{-/-}$ mice. Osteoblasts prepared from WT and $MyD88^{-/-}$ mice were cultured in 60-mm-diameter dishes. Total cellular RNA was extracted from osteoblasts, reverse transcribed, and amplified by PCR for mouse TLR2 (32 cycles), TLR4 (32 cycles), TLR6 (32 cycles), IL-1R (32 cycles), CD14 (32 cycles), or GAPDH (20 cycles) using the specific primers described in Materials and Methods. (B) Effects of LPS, IL-1α, $1,25(OH)_2D_3$, and diacyl lipopeptide on RANKL mRNA expression in osteoblasts prepared from WT and $MyD88^{-/-}$ mice. WT and $MyD88^{-/-}$ osteoblasts were treated with or without 1 μg/ml LPS, 10 ng/ml IL-1α, 10^{-8} M $1,25(OH)_2D_3$ ($1,25D_3$), and 10^{-8} M diacyl lipopeptide for the periods indicated. Total cellular RNA was extracted from osteoblasts, reverse transcribed, and amplified by PCR for mouse RANKL (28 cycles) or GAPDH (20 cycles) using the specific primers described in Materials and Methods.

duce osteoclast formation in the coculture of MyD88$^{-/-}$-derived osteoblasts and hemopoietic cells (Fig. 1, A and B). The number of osteoclasts that formed in response to $1,25(OH)_2D_3$ plus PGE_2 in cocultures prepared from MyD88$^{-/-}$ mice was always significantly smaller than that from WT mice (Fig. 1, A and B). In contrast, bone marrow–derived hemopoietic cells obtained from MyD88$^{-/-}$ mice and those from WT mice similarly differentiated into osteoclasts in response to RANKL plus M-CSF (Fig. 1 C). 100 ng/ml OPG completely inhibited the osteoclast formation induced by LPS, IL-1α, diacyl lipopeptide, and $1,25(OH)_2D_3$ plus PGE_2 in WT cocultures (unpublished data). These results suggest that MyD88-mediated signals are important to osteoblasts but not osteoclast precursors in the osteoclast formation induced by LPS, IL-1α, and diacyl lipopeptide in the coculture system.

RT-PCR analysis showed that primary osteoblasts obtained from WT and MyD88$^{-/-}$ mice similarly expressed TLR2, TLR4, TLR6, IL-1R, and CD14 mRNAs (Fig. 2 A). These results suggest that osteoblasts express LPS receptors (TLR4 and CD14), diacyl lipopeptide receptors (TLR2 and TLR6), and IL-1R. Treatment of WT osteoblasts with LPS, IL-1α, and diacyl lipopeptide stimulated the expression of RANKL mRNA within 24 h (Fig. 2 B). However, these bacterial components and IL-1α failed to enhance RANKL mRNA expression in MyD88$^{-/-}$ osteoblasts. $1,25(OH)_2D_3$ stimulated the expression of RANKL mRNA in WT and MyD88$^{-/-}$ osteoblasts (Fig. 2 B). These results suggest that the MyD88-mediated pathway is essentially involved in osteoclast formation induced by LPS, diacyl lipopeptide, and IL-1α through the expression of RANKL in osteoblasts.

LPS Stimulates RANKL Expression in Osteoblasts through MyD88 followed by Protein Kinase C (PKC) and MEK/ERK Signals. We have shown that LPS stimulates osteoclast formation in the coculture through two parallel events: direct enhancement of RANKL expression and indirect suppression of OPG expression, which is mediated by PGE_2 production (29). Northern blot analysis confirmed that LPS stimulated the expression of RANKL mRNA in osteoblasts (Fig. 3 A). Kikuchi et al. (30) reported previously that LPS-induced RANKL expression was mediated by PKC- and ERK-mediated signals. We also showed that PMA (a potent PKC activator), high concentrations of extracellular Ca^{2+}, and compounds such as A23187 (an intracellular calcium-elevating compound) stimulated RANKL expression in osteoblasts (25). Next, we examined how MyD88 is involved in the RANKL expression induced by PKC-, ERK-, and intracellular calcium-mediated signals in osteoblasts. Pretreatment of osteoblasts with BAPTA-AM (an intracellular calcium chelator), Ro-32-0432 [a PKC inhibitor], and PD98059 [a MEK/ERK inhibitor] strongly inhibited RANKL mRNA expression induced by LPS (Fig. 3 A). In contrast, H-89 (a protein kinase A inhibitor) failed to inhibit LPS-induced RANKL mRNA expression in osteoblasts (Fig. 3 A). A23187 and PMA stimulated the expression of

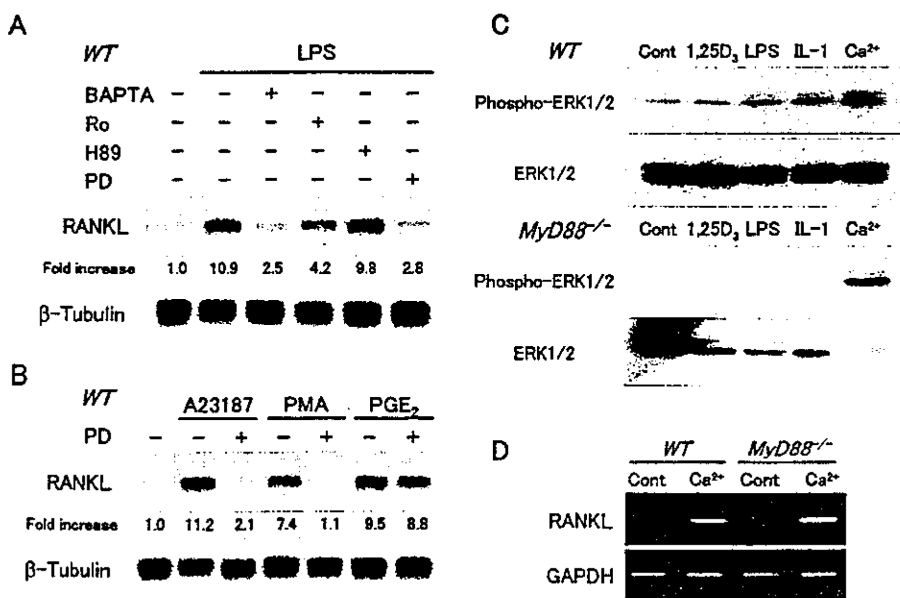

Figure 3. LPS stimulates RANKL expression in osteoblasts through MyD88 followed by PKC and MEK/ERK signals. (A) Effects of several inhibitors on LPS-induced RANKL mRNA expression in WT osteoblasts. Osteoblasts were pretreated for 1 h with 10^{-5} M BAPTA-AM (BAPTA), 10^{-6} M Ro-32-0432 (Ro), 10^{-6} M H-89, or 10^{-5} M PD98059 (PD) and further treated with 1 μg/ml LPS for 3 h. Total RNA was isolated from osteoblasts, and the expression of RANKL and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of RANKL mRNA expression relative to β-tubulin mRNA expression. (B) Effects of PD98059 on RANKL mRNA expression induced by A23187, PMA, or PGE_2 in WT osteoblasts. Osteoblasts were pretreated for 1 h with 10^{-5} M PD98059 (PD), and further treated with or without 10^{-6} M A23187, 10^{-6} M PMA, or 10^{-6} M PGE_2 for 3 h. Total RNA was isolated from osteoblasts, and the expression of RANKL and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of RANKL mRNA expression relative to β-tubulin mRNA expression. (C) Effects of $1,25(OH)_2D_3$, LPS, IL-1α, and extracellular Ca^{2+} on phosphorylation of ERK1/2 in osteoblasts prepared from WT and MyD88$^{-/-}$ mice. WT and MyD88$^{-/-}$ osteoblasts were treated with or without 10^{-8} M $1,25(OH)_2D_3$ ($1,25D_3$), 1 μg/ml LPS, 10 ng/ml IL-1α, and 5 mM high calcium medium (final concentration). After culture for 30 min, cells were washed twice with PBS and lysed in cell lysate buffer. Whole cell extracts were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking, the antiphospho-ERK1/2 antibody or anti-ERK antibody (1:1,000) was added and the bound antibodies were visualized using the enhanced chemiluminescence assay followed by exposure to X-ray film. (D) Effects of extracellular Ca^{2+} on RANKL mRNA expression in osteoblasts prepared from WT and MyD88$^{-/-}$ mice. WT and MyD88$^{-/-}$ osteoblasts were treated with or without high calcium medium (5 mM, final concentration) for 24 h. Total cellular RNA was extracted from osteoblasts, reverse transcribed, and amplified by PCR for mouse RANKL (28 cycles) or GAPDH (20 cycles) using specific primers.

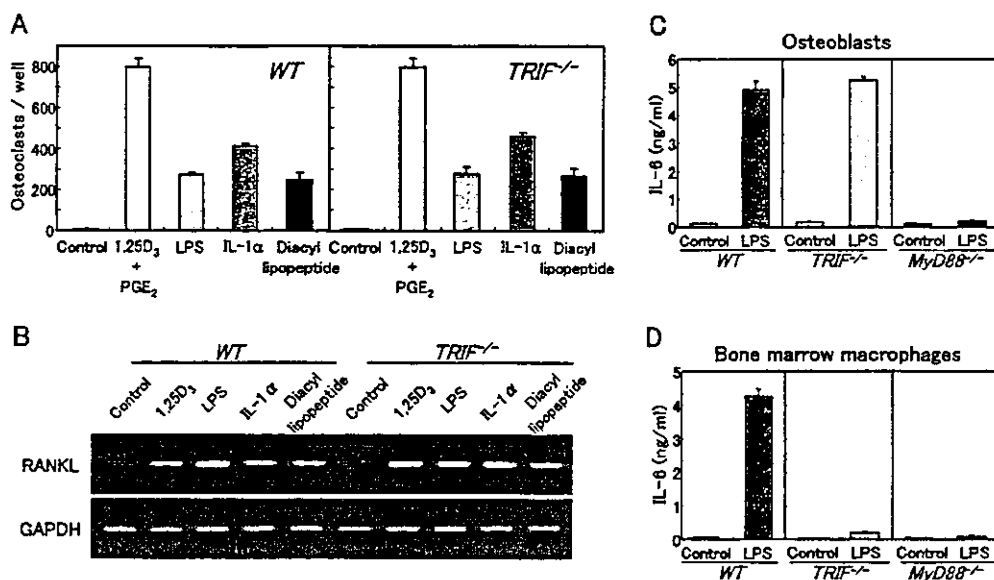

Figure 4. TRIF is not involved in osteoclast differentiation induced by TLR4 ligand. (A) Effects of $1,25(OH)_2D_3$ plus PGE_2, LPS, IL-1α, and diacyl lipopeptide on osteoclast formation in cocultures of osteoblasts and hemopoietic cells prepared from WT and $TRIF^{-/-}$ mice. Calvarial osteoblasts (1.5×10^4 cells/well) and bone marrow–derived hemopoietic cells (1.5×10^5 cells/well) prepared from WT and $TRIF^{-/-}$ mice were cocultured for 7 d in a 48-well plate in the presence or absence of 1 μg/ml LPS, 10 ng/ml IL-1α, 10^{-8} M diacyl lipopeptide, and 10^{-8} M $1,25(OH)_2D_3$ plus 10^{-6} M PGE_2. Cells were fixed and stained for TRAP. TRAP positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Values were expressed as the mean ± SD of three cultures. (B) Effects of LPS, IL-1α, $1,25(OH)_2D_3$, and diacyl lipopeptide on RANKL mRNA expression in osteoblasts prepared from WT and $TRIF^{-/-}$ mice. WT and $TRIF^{-/-}$ osteoblasts were treated with or without 1 μg/ml LPS, 10 ng/ml IL-1α, 10^{-8} M $1,25(OH)_2D_3$ ($1,25D_3$), and 10^{-8} M diacyl lipopeptide for 24 h. Total cellular RNA was extracted from osteoblasts, reverse transcribed, and amplified by PCR for mouse RANKL (28 cycles) or GAPDH (20 cycles) using specific primers. (C) Effects of LPS on the production of IL-6 in osteoblasts prepared from WT, $TRIF^{-/-}$, and $MyD88^{-/-}$ mice. Osteoblasts were incubated for 24 h in the presence or absence of 100 ng/ml LPS. The conditioned medium was collected, and the concentration of IL-6 in the medium was measured using an ELISA kit. Values are expressed as the mean ± SD of quadruplicate cultures. (D) Effects of LPS on the production of IL-6 in M-CSF–treated bone marrow macrophages prepared from WT, $TRIF^{-/-}$, and $MyD88^{-/-}$ mice. Bone marrow–derived macrophages were incubated for 24 h in the presence or absence of 100 ng/ml LPS. The conditioned medium was collected, and the concentration of IL-6 in the medium was measured using an ELISA kit. Values are expressed as the mean ± SD of quadruplicate cultures.

RANKL mRNA in osteoblasts (Fig. 3 B). Pretreatment of osteoblasts with PD98059 suppressed RANKL mRNA expression induced by A23187 and PMA as well as LPS (Fig. 3 B). In contrast, PD98059 showed no inhibitory effect on the PGE_2-induced expression of RANKL mRNA in osteoblasts (Fig. 3 B). These results suggested that MEK/ERK is a down-stream target of PKC-mediated signals in LPS-induced RANKL expression in osteoblasts.

Next, we examined the effects of $1,25(OH)_2D_3$, LPS, IL-1α, and high concentrations of extracellular Ca^{2+} on the phosphorylation of ERK1/2 in osteoblasts prepared from $MyD88^{-/-}$ and WT mice. LPS and IL-1α stimulated phosphorylation of ERK1/2 within 30 min in WT osteoblasts, but not in $MyD88^{-/-}$ osteoblasts (Fig. 3 C). This indicates that the MyD88 signal is essential for LPS-induced phosphorylation of ERK1/2 in osteoblasts. In contrast, high calcium concentrations in the culture medium (5 mM, final concentration) stimulated the phosphorylation of ERK1/2 and the expression of RANKL mRNA in both $MyD88^{-/-}$ and WT osteoblasts (Fig. 3, C and D). This suggests that the MEK/ERK signals in osteoblasts are active even in the absence of MyD88. $1,25(OH)_2D_3$ did not induce the phosphorylation of ERK1/2 in either type of osteoblast (Fig. 3 C). These results suggest that MyD88 is located upstream of PKC/ERK signals in the pathway leading to RANKL expression induced by LPS and IL-1α in osteoblasts.

TRIF Is Not Involved in Osteoclast Formation in the Cocultures. Both MyD88-dependent and TRIF-dependent pathways are essential for proinflammatory cytokine production induced by LPS in peritoneal macrophages (17, 18). Using $TRIF^{-/-}$ mice, we examined the importance of TRIF-mediated signals in LPS-induced osteoclast formation. LPS stimulated osteoclast formation in cocultures prepared from $TRIF^{-/-}$ mice as well as WT mice (Fig. 4 A). Similarly, IL-1α and diacyl lipopeptide stimulated osteoclast formation in cocultures prepared from $TRIF^{-/-}$ mice (Fig. 4 A). Consistent with these results, treatment of $TRIF^{-/-}$ osteoblasts with LPS, IL-1α, diacyl lipopeptide or $1,25(OH)_2D_3$ for 24 h stimulated the expression of RANKL mRNA (Fig. 4 B). These results suggest that the TRIF-mediated pathway is not involved in osteoclast formation induced by IL-1 and TLR ligands.

Next, we examined proinflammatory cytokine production in osteoblasts and macrophages prepared from $TRIF^{-/-}$ and $MyD88^{-/-}$ mice. Treatment with LPS for 24 h stimulated IL-6 production in $TRIF^{-/-}$ and WT osteoblasts, but not in $MyD88^{-/-}$ osteoblasts (Fig. 4 C). LPS stimulated IL-6 production in WT bone marrow macrophages, but not in $TRIF^{-/-}$ or $MyD88^{-/-}$ bone marrow macrophages (Fig. 4 D). These results suggest that the TRIF-dependent pathway is involved in LPS-induced IL-6 production in macrophages but not in osteoblasts.

MyD88 Is Involved in the Survival of Osteoclasts Supported by LPS and IL-1α. We reported previously that purified osteoclasts spontaneously died due to apoptosis within 36 h, and LPS and IL-1α promoted the survival of osteoclasts (31, 32). Next, we examined whether the survival of osteoclasts supported by LPS, IL-1α, and diacyl lipopeptide is mediated by MyD88, TRIF, or both. Purified osteoclasts were pre-

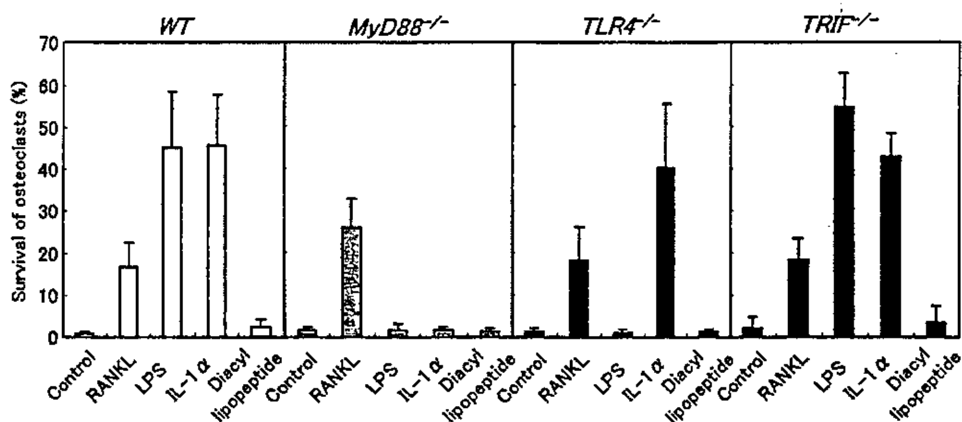

Figure 5. Effects of RANKL, LPS, IL-1α, and diacyl lipopeptide on the survival of osteoclasts prepared from WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ mice. Purified osteoclasts were prepared in cocultures of osteoblasts and bone marrow cells obtained from WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ mice. WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ osteoclasts were treated with or without 100 ng/ml RANKL, 1 μg/ml LPS, 10 ng/ml IL-1α, and 10^{-8} M diacyl lipopeptide. After culture for 24 h, cells were fixed and stained for TRAP. TRAP positive multinucleated cells containing three or more nuclei were counted as viable osteoclasts. Values were expressed as the mean ± SD of three cultures. Experiments were repeated five times with similar results.

pared from cocultures of osteoblasts and bone marrow cells obtained from WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ mice. Most of the osteoclasts died spontaneously and disappeared within 24 h. RANKL promoted the survival of osteoclasts derived from MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ mice. LPS and IL-1α supported the survival of WT and TRIF$^{-/-}$ osteoclasts, but not MyD88$^{-/-}$ osteoclasts (Fig. 5). IL-1α and RANKL, but not LPS, promoted the survival of osteoclasts derived from TLR4$^{-/-}$ mice. Diacyl lipopeptide (a ligand for the TLR2 plus TLR6 complex) did not support the survival of osteoclasts derived from any of the mice. Takami et al. (33) reported that mature osteoclasts expressed the mRNA of TLR2 and TLR4, but not TLR6. We have confirmed that TLR6 mRNA is not expressed in mature osteoclasts (unpublished data). These results suggest that diacyl lipopeptide did not support the survival of osteoclasts because of the lack of TLR6 in osteoclasts. Thus, MyD88-mediated signals, but not TRIF-mediated ones, were essential for the survival of osteoclasts supported by LPS and IL-1α.

TRAM Is Not Expressed in Osteoblasts and Osteoclasts. TRAM was shown to be involved in the LPS-induced, TRIF-mediated signaling pathway (19, 20). We examined the expression of TRIF and TRAM mRNAs in osteoblasts, bone marrow macrophages, and osteoclasts prepared from MyD88$^{-/-}$, TRIF$^{-/-}$, and WT mice. TRIF mRNA was expressed in osteoblasts, macrophages, and osteoclasts derived from WT and MyD88$^{-/-}$ mice (Fig. 6). Interestingly, TRAM was expressed in macrophages, but not in osteoblasts or mature osteoclasts (Fig. 6). The fact that TRIF-mediated signals are not required for LPS-induced RANKL expression in osteoblasts and osteoclast survival may be related to the lack of TRAM expression in osteoblasts and osteoclasts.

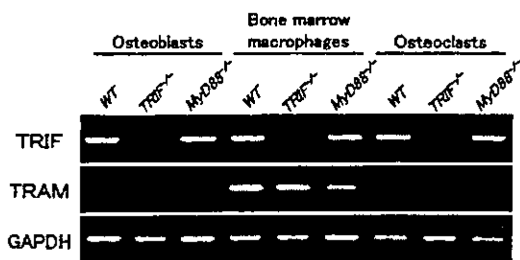

Figure 6. Expression of TRIF and TRAM in osteoblasts, macrophages and mature osteoclasts. Expression of TRIF and TRAM in osteoblasts, macrophages and osteoclasts prepared from WT, TRIF$^{-/-}$, and MyD88$^{-/-}$ mice. Total cellular RNA was extracted from osteoblasts, M-CSF-induced bone marrow macrophages, and osteoclasts; reverse transcribed; and amplified by PCR for mouse TRIF (30 cycles), TRAM (30 cycles), or GAPDH (18 cycles) using the specific primers described in Materials and Methods.

MyD88$^{-/-}$ Mice Exhibited Profound Osteopenia with Reduced Bone Resorption and Formation. Histomorphometric measurements of vertebrae showed that MyD88$^{-/-}$ mice exhibited osteopenia with reduced bone resorption and formation. Bone resorption–related parameters such as osteoclast surface/bone surface and osteoclast number/bone surface were 37.4 and 46.8% lower in MyD88$^{-/-}$ mice than WT mice, respectively (Fig. 7 A). Bone formation–related parameters such as osteoid volume/tissue volume and osteoblast surface/bone surface were also significantly reduced in MyD88$^{-/-}$ mice (Fig. 7 A). Both trabecular bone volume (bone volume per tissue volume) and trabecular number were significantly decreased in 14-wk-old MyD88$^{-/-}$ mice in comparison with the WT mice. No significant differences in body size and shape were observed between MyD88$^{-/-}$ and WT mice (unpublished data). Histological analysis showed that a loss of trabecular bone in the tibiae was evident in MyD88$^{-/-}$ mice. The number of TRAP positive osteoclasts (cells stained red) was reduced in MyD88$^{-/-}$ mice compared with WT mice (Fig. 7 B). These results suggest that MyD88 is involved in the physiological regulation of bone resorption and formation.

Discussion

Using MyD88$^{-/-}$ and TRIF$^{-/-}$ mice, we examined the possible involvement of MyD88 and TRIF in osteoclast differentiation and function. LPS, diacyl lipopeptide, and IL-1α