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

Nobuaki Sato,[1,4] Naoyuki Takahashi,[2] Koji Suda,[5] Midori Nakamura,[3] Mariko Yamaki,[2] Tadashi Ninomiya,[2] Yasuhiro Kobayashi,[2] Haruhiko Takada,[6] Kenichiro Shibata,[7] Masahiro Yamamoto,[8] Kiyoshi Takeda,[9] Shizuo Akira,[8] Toshihide Noguchi,[4] and Nobuyuki Udagawa[1]

[1]Department of Biochemistry, [2]Institute for Oral Science, and [3]Department of Pediatric Dentistry, Matsumoto Dental University, Nagano 399-0781, Japan
[4]Department of Periodontology, School of Dentistry, Aichi Gakuin University, Nagoya 464-8651, Japan
[5]Department of Biochemistry, School of Dentistry, Showa University, Tokyo 142-8555, Japan
[6]Department of Microbiology and Immunology, Tohoku University School of Dentistry, Sendai, 980-8575, Japan
[7]Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Sapporo 060-8586, Japan
[8]Research Institute for Microbial Diseases, Osaka University, Suita 565-0871, Japan
[9]Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

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

Address correspondence to Nobuyuki Udagawa, Dept. of Biochemistry, Matsumoto Dental University, 1780 Gobara, Hiro-oka, Shiojiri, Nagano 399-0781, Japan. Phone: 81-263-51-2072; Fax: 81-263-51-2223; email: udagawa@po.mdu.ac.jp

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–inducing IFN-β; TRIF$^{-/-}$, TRIF-deficient.

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 adapter 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 adapter 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)$_2$D$_3$] 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 $\pm$ 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 $\pm$ 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′-AAACAACTTACCGAAAC-CTCAGAC-3′ (nucleotides 273–296) and reverse, 5′-TGTA-AATTTGTGAGATTGGGAAAA-3′ (nucleotides 748–771); mouse, TLR4, forward, 5′-AGTGGGTCAAGGAACAGAA-GCA-3′ (nucleotides 1766–1787) and reverse, 5′-CTTTAC-CAGCTCATTTCTCACC-3′ (nucleotides 2055–2076); mouse TLR6, forward, 5′-GCCTGACTCTTACAGGTGTGACTA-3′ (nucleotides 1698–1721) and reverse, 5′-TTATGATGGG-ACAAATAGAGTTCA-3′ (nucleotides 2175–2198); mouse CD14, forward, 5′-ACATCTTGAACCTCCGCAAC-3′ (nucleotides 454–473) and reverse, 5′-AGGGTTCCTATCCAGC-

CTGT-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′-CGCTCTGTTCCT-GTACTTTCGAGCG-3′ (nucleotides 195–219) and reverse, 5′-TCGTGCTCCCTCCTTTCATCAGGTT-3′ (nucleotides 757–781); mouse TRIF, forward, 5′-ATGGATAACCCAGGGC-CTT-3′ (nucleotides 187–205) and reverse, 5′-TTCTGGTCA-CTGCAGGGGAT-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′-ACC-ACAGTCCATGCCATCAC-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-
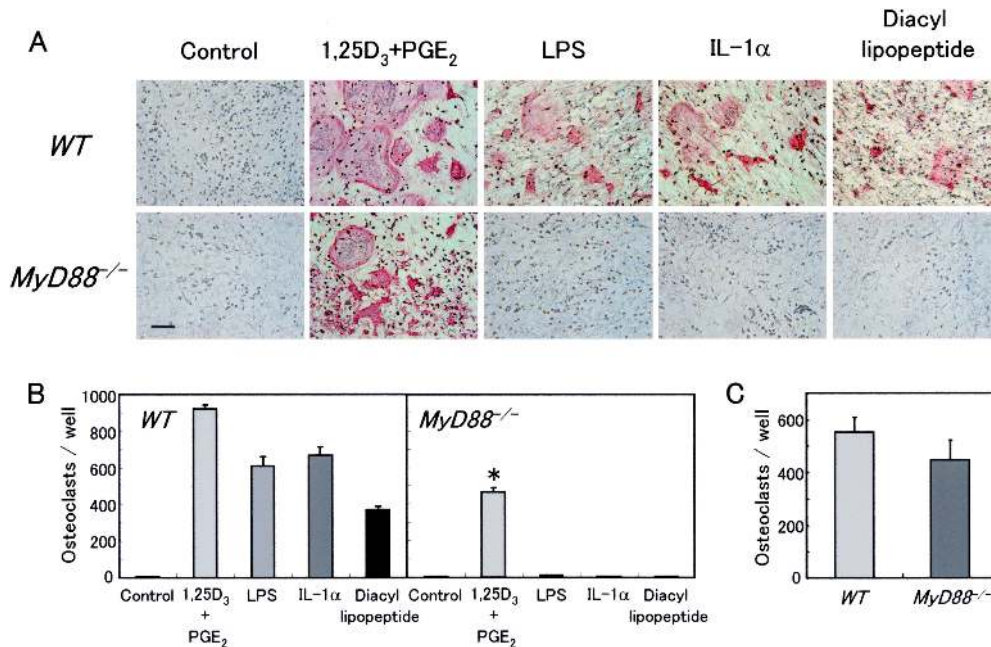
Figure 1. MyD88 is essential for osteoclastogenesis induced by LPS, IL-1α, and diacyl lipopeptide. (A and B) Effects of 1,25(OH)$_2$D$_3$ plus PGE$_2$, LPS, IL-1α, and diacyl lipopeptide on osteoclast formation in cocultures of osteoblasts and hemopoietic cells prepared from male WT and MyD88$^{-/-}$ 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)$_2$D$_3$ 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)$_2$D$_3$ 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-
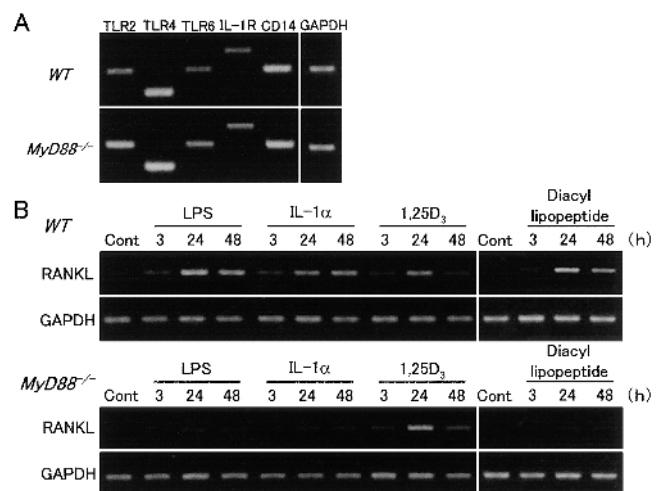

Figure 2. MyD88-mediated signals are involved in RANKL expression in osteoblasts treated with LPS, diacyl lipopeptide, and IL-1α. (A) Expression of TLR2, TLR4, TLR6, IL-1R, and CD14 in osteoblasts prepared from WT and MyD88$^{-/-}$ mice. Osteoblasts prepared from WT and MyD88$^{-/-}$ mice were cultured 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)$_2$D$_3$, 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)$_2$D$_3$ (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)$_2$D$_3$ 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)$_2$D$_3$ 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)$_2$D$_3$ 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
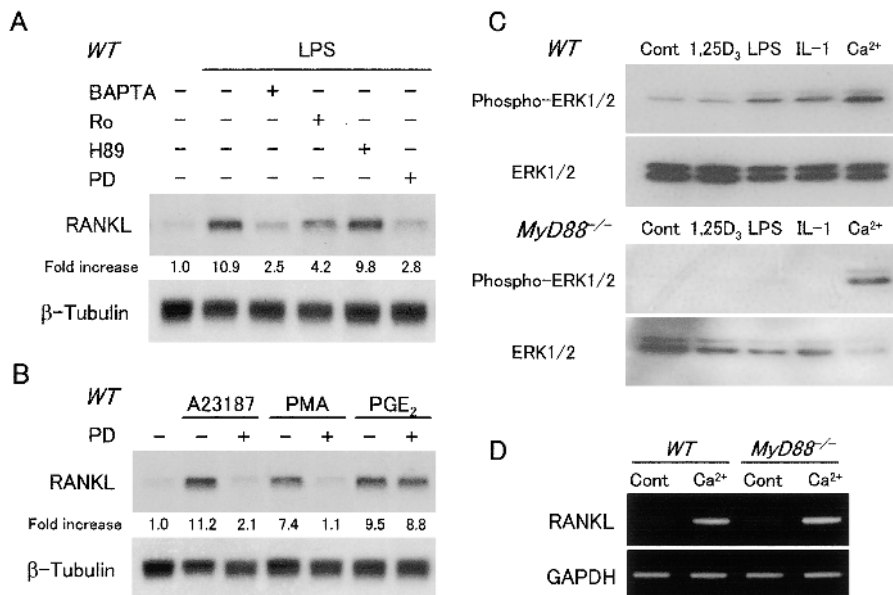

Figure 3. LPS stimulates RANKL expression in osteoblasts through MyD88 followed by PKC and MEK/ERK signals. (A) Effects of several inhibitors on LPS-induced RANKL mRNA expression in WT osteoblasts. Osteoblasts were pretreated for 1 h with 10^{-5} M BAPTA-AM (BAPTA), 10^{-6} M Ro-32-0432 (Ro), 10^{-6} 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)$_2$D$_3$, 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)$_2$D$_3$ (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.
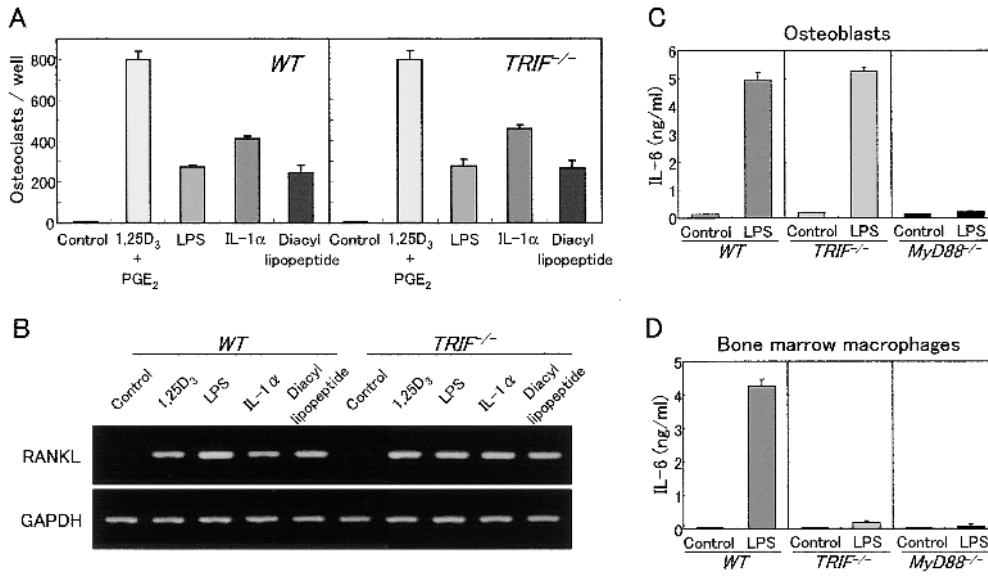
Figure 4. TRIF is not involved in osteoclast differentiation induced by TLR4 ligand. (A) Effects of $1,25(OH)_2D_3$ plus PGE_2, LPS, IL-1α, and diacyl lipopeptide on osteoclast formation in cocultures of osteoblasts and hemopoietic cells prepared from WT and TRIF$^{-/-}$ mice. Calvarial osteoblasts (1.5×10^4 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-
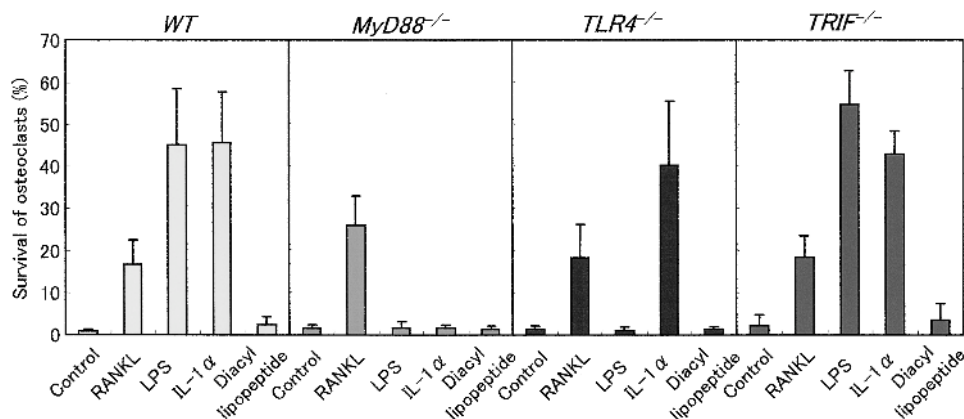
Figure 5. Effects of RANKL, LPS, IL-1α, and diacyl lipopeptide on the survival of osteoclasts prepared from WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and TRIF$^{-/-}$ mice. Purified osteoclasts were prepared in cocultures of osteoblasts and bone marrow cells obtained from WT, MyD88$^{-/-}$, TLR4$^{-/-}$, and 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.

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.
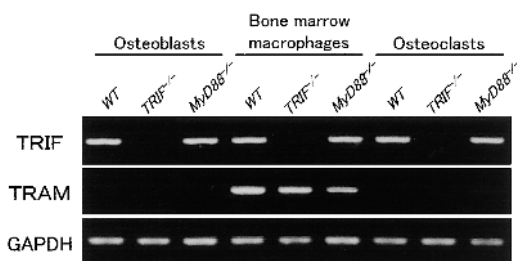

Figure 6. Expression of TRIF and TRAM in osteoblasts, macrophages and mature osteoclasts. Expression of TRIF and TRAM in osteoblasts, macrophages and osteoclasts prepared from WT, TRIF$^{-/-}$, and MyD88$^{-/-}$ mice. Total cellular RNA was extracted from osteoblasts, M-CSF–induced bone marrow macrophages, 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.

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
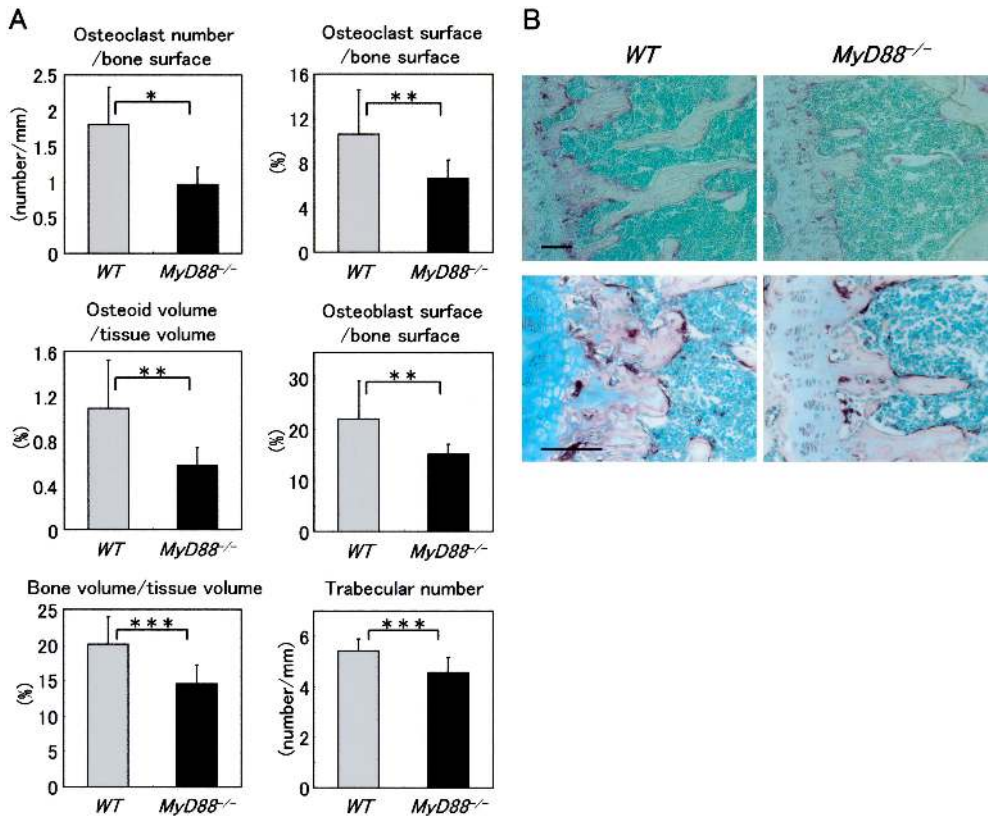
Figure 7. Histomorphometric analysis of vertebrae in WT and MyD88$^{-/-}$ mice. (A) Seven male MyD88$^{-/-}$ and WT (14-wk-old) mice each were killed for bone histomorphometric analysis. Vertebrae were removed from the mice, fixed in 70% ethanol, and embedded in glycol-methacrylate without decalcification. Sections were prepared and stained with Villanueva Goldner to discriminate between mineralized and unmineralized bone and to identify cellular components. Quantitative histomorphometric analysis was done in a double-blind fashion. Values were expressed as the mean ± SD of seven mice. Statistical analysis was performed using Student's t test. Significant difference between WT and MyD88$^{-/-}$ mice (*, $P < 0.005$; **, $P < 0.05$; ***, $P < 0.01$). (B) Histological evaluation (double staining of TRAP and methylgreen) of femoral trabecular bones obtained from male MyD88$^{-/-}$ and WT (12-wk-old) mice. TRAP positive osteoclasts appeared dark red. Arrowheads indicate osteoblasts along the bone surface. Bars, 500 μm.

all stimulated osteoclast formation in cocultures of osteoblasts and hemopoietic cells obtained from WT and TRIF$^{-/-}$ mice, but not from MyD88$^{-/-}$ mice (Figs. 1 and 4). Osteoclast precursors from MyD88$^{-/-}$ mice and WT mice similarly differentiated into osteoclasts in response to RANKL plus M-CSF, but the extent of osteoclast formation induced by 1,25(OH)$_2$D$_3$ plus PGE$_2$ in the MyD88$^{-/-}$ mice was always significantly less than that in wild-type cocultures (Fig. 1). This

suggests that MyD88 is involved in osteoblast function including the support of osteoclasts in response to 1,25(OH)$_2$D$_3$ plus PGE$_2$. LPS, diacyl lipopeptide, and IL-1α stimulated expression of RANKL mRNA in WT and TRIF$^{-/-}$ osteoblasts, but not MyD88$^{-/-}$ osteoblasts (Figs. 2 and 4). These results suggest that RANKL expression in osteoblasts through MyD88-mediated signals is a key step in osteoclast formation induced by LPS, diacyl lipopeptide, and IL-1 (Fig. 8).
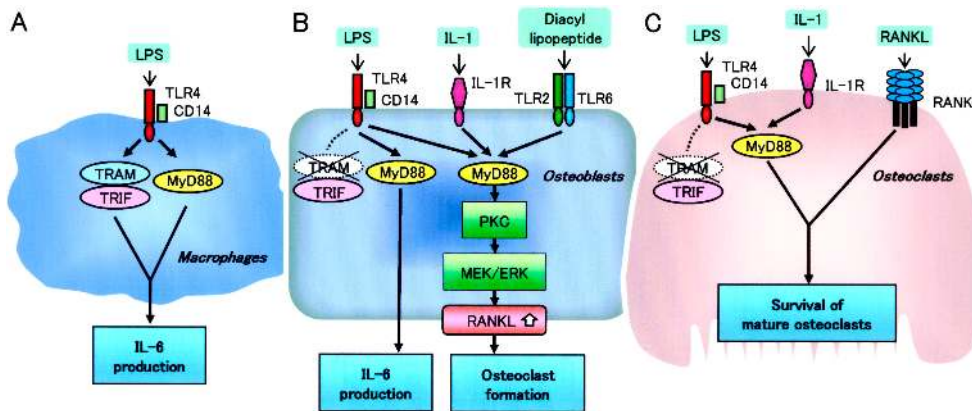

Figure 8. Roles of MyD88 and TRAM–TRIF signaling pathways in macrophages, osteoblasts, and osteoclasts exposed to LPS, IL-1, diacyl lipopeptide, and RANKL. (A) Role of MyD88- and TRAM–TRIF-mediated signaling in IL-6 production in macrophages. Macrophages express CD14 and TLR4. Both MyD88-dependent and TRAM–TRIF-dependent pathways mediated by TLR4 were essential for IL-6 production in macrophages. (B) Roles of MyD88-mediated signals in IL-6 production and osteoclast formation in osteoblasts. Osteoblasts express CD14, TLR2, TLR4, TLR6, and IL-1R. LPS stimulates IL-6 production through MyD88 signaling. LPS, IL-1, and diacyl lipopeptide stimulate RANKL expression in osteoblasts through the respective receptor systems. TLR- and IL-1R–induced RANKL mRNA expression in osteoblasts is mediated through MyD88 signaling followed by PKC and MEK/ERK signaling. (C) Role of MyD88-mediated signals in osteoclast function. Mature osteoclasts express CD14, TLR4, and IL-1R as well as RANK. LPS, IL-1, and RANKL stimulate the survival of osteoclasts through TLR4, IL-1R, and RANK, respectively. MyD88 is involved in the survival of osteoclasts supported by LPS and IL-1, but not by RANKL.

LPS and IL-1α stimulated phosphorylation of ERK1/2 in WT osteoblasts, but not MyD88$^{-/-}$ osteoblasts (Fig. 3). The elevated calcium concentration (5 mM) in the culture medium stimulated both phosphorylation of ERK1/2 and RANKL mRNA expression in WT and MyD88$^{-/-}$ osteoblasts. This calcium concentration did not appear to be toxic to the cells. A serine threonine kinase, Cot, also known as tumor progression locus 2 (Tpl2), has been shown to be an essential kinase in LPS-induced TNFα production in mouse macrophages (34, 35). Cot/Tpl2 activated ERK, but not JNK and p38 MAPK, in the LPS-treated macrophages. It was reported that both RANKL mRNA induction and ERK activation by LPS were markedly reduced in osteoblasts prepared from Cot/Tpl2-deficient mice (36). These results suggest that LPS-induced RANKL mRNA expression is mediated through MyD88 followed by PKC and MEK/ERK signals rather than JNK and p38 MAPK signals (Fig. 8).

We reported previously that osteoclasts formed in vitro expressed TLR4 and CD14, and LPS directly supported the survival and stimulated the dentine-resorbing activity of osteoclasts (32). Takami et al. (33) showed that mouse bone marrow macrophages expressed all known TLRs (TLR1–TLR9), but mouse osteoclasts expressed only TLR2 and TLR4. Consistent with these findings, LPS and IL-1α but not diacyl lipopeptide (a ligand for the TLR2 plus TLR6 complex) stimulated the survival of osteoclasts derived from WT and TRIF$^{-/-}$ mice (Fig. 5). The survival of osteoclasts from MyD88$^{-/-}$ mice was supported by RANKL, but not by LPS and IL-1α. These results suggest that MyD88 but not TRIF is involved in IL-1R– and TLR4-mediated signaling in the survival of osteoclasts. MyD88 and RANK are shown to associate with TNF receptor-associated factor 6 to induce their signals in target cells. TNF receptor-associated factor 6 appears to be a common signaling molecule downstream of MyD88 and RANK in osteoclasts.

Recent studies using TRIF$^{-/-}$ mice showed the essential role of TRIF in the MyD88-independent pathways of TLR3 and TLR4 signaling (17, 18). TRAM was shown to be involved in the TLR4-mediated TRIF-signaling pathway in the innate immune response to LPS (19, 20). Consistent with previous findings, TRIF$^{-/-}$ macrophages abolished the response to LPS in IL-6 production (Fig. 4). However, surprisingly, TRIF$^{-/-}$ osteoblasts and TRIF$^{-/-}$ osteoclasts responded to LPS as those from WT mice did. Osteoblasts and osteoclasts expressed TRIF but not TRAM, suggesting that TRAM expression is required for TRIF-mediated action in osteoblasts and osteoclasts (Fig. 8). Our results also suggest that TRAM may be an important key adaptor in the TLR4-mediated pathway of cell-specific functions.

At present, it is unknown why immune cells such as macrophages and B cells required both MyD88 and TRIF signaling in response to LPS. We reported previously that LPS stimulated the production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in bone marrow macrophages but not in osteoclasts (32). Thus, osteoclasts respond to LPS through TLR4, but the characteristics of osteoclasts are quite different from those of their precursors, bone marrow macrophages. These results suggest that TRIF is important for the function of immune cells, but not that of nonimmune cells such as osteoblasts and osteoclasts. Loss of immune responsiveness to LPS in osteoclasts must be a requirement for performing essential roles in physiological bone turnover. Further studies will elucidate the significance of the requirement of TRAM–TRIF signals in immune cells.

MyD88$^{-/-}$ mice exhibited a significant decrease in trabecular bone volume and trabecular number in vertebrae, although no significant differences in body size and shape were observed between MyD88$^{-/-}$ and WT mice. Not only bone resorption–related parameters but also bone formation–related parameters were significantly decreased in MyD88$^{-/-}$ mice in comparison with WT mice (Fig. 7). Mice deficient in bone matrix proteins such as osteonectin and biglycan similarly developed profound osteopenia with a decrease of bone formation and resorption (37–39). Deficiency of OPG in mice induced severe osteoporosis caused by enhanced bone resorption, but accelerated bone formation was also observed in these mice (27, 40). These findings suggest that bone formation is tightly coupled with bone resorption.

In conclusion, MyD88 but not TRIF plays essential roles in RANKL expression in osteoblasts in response to IL-1 and TLR ligands. MyD88 is also a key molecule for osteoclast function induced by IL-1 and LPS. MyD88$^{-/-}$ mice exhibit osteopenia with reduced bone resorption and bone formation. Thus, MyD88-mediated signaling plays important roles not only in bone resorption induced by inflammatory diseases but also in ordinary bone metabolism. Further studies are necessary to clarify the physiological and pathological significance of MyD88 signals in bone resorption and bone formation.

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