ARTICLE
Journal of Cellular Biochemistry 105:896–904 (2008)

Journal of Cellular Biochemistry

Lipopolysaccharide Suppresses RANK Gene Expression in Macrophages by Down-Regulating PU.1 and MITF

Junko Ishii,[1] Riko Kitazawa,[1] Kiyoshi Mori,[1] Kevin P. McHugh,[2] Eiichi Morii,[3] Takeshi Kondo,[1] and Sohei Kitazawa[1*]

[1]*Department of Pathology, Kobe University Graduate School of Medicine, Kobe, Japan*
[2]*Beth Israel Deaconess Medical Center, Orthopaedic Research, Boston, Massachusetts*
[3]*Department of Pathology, Osaka University Medical School, Osaka, Japan*

ABSTRACT

Receptor activator of NF-κB (RANK) is a receptor for RANK ligand (RANKL), and signals transduced by RANK–RANKL interaction are prerequisite for the differentiation and activation of osteoclasts. We cloned and characterized a 6-kb fragment containing the 5′-flanking region of the mouse RANK gene. A fragment of 1-kb from the transcription start sites containing four Sp-1 sites and putative binding sites for MITF, CRE/AP-1, and PU.1 was ligated to the pGL3-basic vector, and the promoter activity was confirmed by transfection studies. By electrophoretic gel motility shift assay, both PU.1 and proximal MITF binding site showed specific DNA-protein binding. Co-transfection studies with MITF- and PU.1-expression vectors revealed that MITF and PU.1 increased RANK promoter activity three- and twofold, respectively, and sixfold synergistically. Taken together, these results show that RANK transcription is positively regulated by both PU.1 and MITF. The effect of lipopolysaccharide (LPS) on RANK gene expression, analyzed by in situ hybridization using mouse bone tissue, showed that LPS decreased RANK transcripts of both precursor and mature osteoclasts. Furthermore, LPS treatment of RAW.264.7 cells decreased their RANK mRNA expression by 70%, mirroring the decrease of PU.1 and MITF mRNA. Short-term treatment with LPS decreased the promoter activity of pGL3-WT by 70%. Although LPS has been reported to promote osteoclastogenesis in chronic and local pyogenic inflammation, we speculate that LPS per se may directly suppress RANK expression in the osteoclastic cell lineage by down-regulating the expression of PU.1 and MITF genes in acute and systemic severe endotoxemia, such as in septic shock. J. Cell. Biochem. 105: 896–904, 2008.

KEY WORDS: RANK; PROMOTER; PU.1; MITF; OSTEOCLASTS

Osteoclasts, derived from the committed monocyte-macrophage lineage in response to the action of colony-stimulating factor 1 (CSF-1) and of receptor activator of NF-κB (RANK) ligand (RANKL), are highly specialized multinucleated giant cells capable of bone resorption upon stimulation of bone-seeking steroids, cytokines and prostaglandins (PGs) [Tanaka et al., 1993; Teitelbaum and Ross, 2003; Ross and Teitelbaum, 2005]. Osteoclasts and bone-forming cells, osteoblasts, play reciprocal roles in the bone remodeling process requisite for maintaining bone integrity and function, imbalances of which are known to cause many human bone disorders, including osteoporosis [Kaji et al., 1997], Paget's disease [Layfield, 2007], and rheumatoid arthritis [Mizuno et al., 2002]. In the osteoclast differentiation program, some key transcription factors are essential [Takayanagi et al., 2002]. Among these, nuclear factor of activated T cells (NFAT) c1 is, as the master transcription factor, involved in the transcriptional regulation of many of the osteoclast-specific genes, such as tartrate-resistant acid phosphatase (TRACP) [Ikeda et al., 2004], calcitonin receptor [Day et al., 2004], and cathepsin K [Matsumoto et al., 2004]. Furthermore, that both PU.1- and MITF-deficient animals are osteopetrotic due to the

Abbreviations used: RANK, receptor activator of NF-κB; EMSA, electrophoretic gel motility shift assay; LPS, lipopolysaccharide; CSF-1, colony-stimulating factor 1; RANKL, receptor activator of NF-κB (RANK) ligand; NFAT, nuclear factor of activated T cells; TRAP, tartrate-resistant acid phosphatase; TLR, toll-like receptor; PG, producing prostaglandin; TNF, tumor necrosis factor; IL, interleukin; BAC, bacterial artificial chromosome; RACE, rapid amplification of cDNA end; TRACP, tartrate-resistant acid phosphatase; OSCAR, osteoclast-associated receptor.

Grant sponsor: Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant numbers: 16590278, 19390100, 20790283, 18590372.

*Correspondence to: Dr. Sohei Kitazawa, Sohei Kitazawa at the Division of Pathology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. E-mail: kitazawa@med.kobe-u.ac.jp

Received 13 June 2008; Accepted 17 July 2008 • DOI 10.1002/jcb.21886 • 2008 Wiley-Liss, Inc.
Published online 29 August 2008 in Wiley InterScience (www.interscience.wiley.com).

deficiency of differentiated functional osteoclasts [Boyce et al., 1999] clearly shows that PU.1 and MITF also play essential roles in osteoclast differentiation.

Lipopolysaccharide (LPS), on the other hand, one of the major bacterial factors of inflammation expressed as the main component on the cell surface of Gram-negative bacteria [Zou and Bar-Shavit, 2002] exerts its biological effect mainly through the toll-like receptor (TLR)-4 expressed on host cells [Necela et al., 2008]. Upon bacterial infection, in response to LPS, hosts activate their immune systems to defend against infection by producing prostaglandin (PG)E_2 [Suda et al., 2004; Yang et al., 2005] and several kinds of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 from macrophages, lymphocytes and endothelial cells [Kunkel et al., 1988; Itoh et al., 2003; Wada et al., 2004]. In inflammation-induced bone remodeling, especially in periodontal disease, LPS per se or through these inflammatory cytokines modulates pathological as well as physiological osteoclastogenesis [Lerner, 2006], where LPS can affect osteoclasts and their precursors directly [Kikuchi et al., 2001; Suda et al., 2004; Gao et al., 2007] or indirectly through osteoblastic cells [Gao et al., 2007].

In this study, focusing on the transcriptional regulation mechanism of RANK by PU.1 and MITF, we cloned and characterized the promoter of the mouse RANK gene, an essential receptor for RANKL expressed on osteoclasts and their precursors. Using a reporter gene and quantitative RT-PCR assays, we demonstrate that PU.1 and MITF cooperatively transactivate the RANK gene. We also tested the direct effect of LPS on RANK expression in macrophages, and found that LPS per se directly suppresses RANK expression in the macrophage/osteoclastic cell lineage, at least in part, by down-regulating the expression of PU.1 and MITF.

MATERIALS AND METHODS

CLONING AND CHARACTERIZATION OF MOUSE RANK PROMOTER

The 5′-flanking region of the mouse RANK gene was subcloned from the mouse bacterial artificial chromosome (BAC) clone by polymerase chain reaction (PCR) and restriction enzyme digestion. The cloned 5′-flanking region of the RANK gene was sequenced by the dideoxy nucleotide termination method with an ABI PRISM 310 automated sequence analyzer (Applied Biosystems, Foster City, CA). The transcription start site of the mouse RANK gene was determined with the use of CapFishing™ Full-length cDNA Premix Kit (Seegene, Seoul, South Korea) according to the manufacturer's instructions. Briefly, the cDNA for 5′-rapid amplification of cDNA end (RACE) was derived from murine macrophage cell line RAW264.7 (RIKEN, Tsukuba, Japan) and generated using the CapFishing™ adaptor. The PCR was done using the CapFishing™ 5′-RACE Primer (5′-GTCTACCAGGCATTCGCTTCAT-3′) and the gene-specific antisense primer (5′-AGATCTGGCGCGGTGCGGGCGGGCTG-3′). The resulting PCR products of interest were gel-purified and cloned into pCR2.1 by TA cloning (Invitrogen, San Diego, CA), and the clones were sequenced using the M13R primer.

CELL CULTURE

RAW264.7 cells (RIKEN) and mouse bone marrow stromal cell line ST2 (RIKEN) were cultured and maintained in α-MEM (Sigma, St. Louis, MO) supplemented with 10% FBS (Sigma), 50 IU/ml-50 μg/ml penicillin/streptomycin (ICN Biomedicals, Inc., Aurora, OH) and 2 mM of L-glutamine (ICN Biomedicals, Inc.) and subjected to dual luciferase assay (Promega, Madison, WI). RAW264.7 cells were subjected to in vitro osteoclast formation assay and quantitative real-time reverse transcription (RT)-PCR analysis. Primary mouse bone marrow mononuclear cells, obtained from the femora and tibiae of 8-week-old male mice, were cultured in α-MEM supplemented with 10% FBS, 50 IU/ml-50 μg/ml penicillin/streptomycin and 2 mM of L-glutamine with or without 500 U/ml of M-CSF, prepared as previously described [Kitazawa et al., 1995]. M1 cells (RIKEN) were cultured in RPMI 1640 (ICN Biomedicals, Inc.) supplemented with 10% FBS and subjected to quantitative real-time RT-PCR. All cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

MICE AND TISSUE PREPARATION

All animal handling was conducted in accordance with guidelines established by Kobe University Graduate School of Medicine. Female BALB/c mice (10 weeks of age), obtained from Charles River Japan, Yokohama, Japan, were housed 3–4 per cage on a 12 h light/dark cycle, provided with standard rodent chow and water ad libitum, and acclimated to their environment for at least one week before the start of experiments. The mice were intraperitoneally injected with lipopolysaccharide (LPS, *Escherichia coli* (0111: B4) (Sigma)) at 1 mg/kg in 100 μl of H_2O 24 h before the tibia were collected. Control (vehicle-treated) mice received 100 μl of H_2O i.p. The bone specimens were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C for 4 days, decalcified with 20% EDTA in 0.1 M PB for 3–7 days, and embedded in paraffin. Serial sections 5 μm thick were used for hematoxylin and eosin (HE) and TRACP staining, and for in situ hybridization studies.

IN SITU HYBRIDIZATION

In situ hybridization with a single-stranded DNA probe was carried out as previously described [Mori et al., 2002]. Briefly, for probe preparation, total RNA extracted from the mouse thymus was used as a template for RT-PCR of RANK. The cDNA fragments from the coding region of mouse RANK (712–990) were amplified by RT-PCR with rTth reverse transcriptase (Perkin-Elmer, Norwalk, CT) using the following pairs of oligonucleotide primers.

RANK (sense): 5′-GTGGTAGTAGTGGCTGCCATCATC-3′ (694–717)
RANK (nested sense): 5′-ATCATCTTCGGCGTTTACTACAGG-3′ (712–735)
RANK (antisense): 5′-GACCAGTGTGAACGTCCTAGAATC-3′ (979–1,002)
RANK (nested antisense): 5′-CGTCCTAGAATCTCTGACTTCTGC-3′ (967–990).

Antisense primer-primed cDNA was synthesized, purified and digoxigenin (DIG)-labeled. The conditions of pretreatment and hybridization of bone tissue sections were optimized as previously described [Mori et al., 2002]. The tissue sections were incubated in a hybridization medium [10 mM of Tris–HCl (pH 7.3), 1 mM of EDTA,

600 mM of NaCl, 0.25% sodium dodecyl sulfate, 1× Denhardt's Medium, 50% (v/v) deionized formamide/1 μg/ml of each antisense DNA probe (RANK), 10% dextran sulfate] at 50°C in a moist chamber for 12 h. Negative controls were prepared with DIG-labeled sense DNA probes. The slides were then washed and incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 60 min after blocking with 1.5% non-fat dry milk in PBS for 30 min to visualize the hybridized probe. The specimens were washed with 100 mM of Tris–HCl (pH 7.5) containing 150 mM of NaCl, and immersed in 100 mM of Tris–HCl (pH 9.5) containing 100 mM of NaCl and 50 mM of $MgCl_2$. The colorimetric reaction was done with nitroblue tetrazolium salt and bromo-4-chloro-3-indolyl phosphate solution (Roche) in the dark for 6 h, then stopped with 10 mM of Tris–HCl (pH 8.0) containing 1 mM of EDTA. The slides were mounted with Crystal/Mount (Biomeda, Foster City, CA) and analyzed under a light microscope without counterstaining.

PLASMID CONSTRUCTION AND TRANSFECTION STUDIES

The 5′-flanking region of the mouse RANK gene was cloned as described above, and the 1 kb and the 5 kb upstream from the transcription start site were ligated into a pGL3 Basic vector (Promega) yielding a pGL3-RANK-1 vector and a pGL3-RANK-5 vector, respectively. For site-directed mutagenesis of the putative MITF binding sites (−511/−506, −262/−257, −106/−101) and/or the PU.1 binding site (−483/−478), the MITF core elements (CAGGTG: MITF-1 (−511/−506), MITF-2 (−262/−257), MITF-3 (−106/−101)) in pGL3-RANK-1 were replaced with ACTTGT; the PU.1 core element (GAGGAA) was replaced with GTCGAC with the use of QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The constructs with mutated PU1, MITFs, and both were named pGL3-PU.1m, pGL3-MITFm, and pGL3-PU.1mMITFm, respectively. The sequence of the three mutant constructs was confirmed by direct sequencing to eliminate PCR-related mutations. Each construct was transfected into RAW264.7 cells or ST2 cells using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The phRG-TK vector (Promega) was co-transfected to standardize transfection efficiency. The cells were harvested 48 h after transfection, and the luciferase activity of firefly and Renilla from cell lysates was determined with a luminometer (Model ATP-3010; Advantec, Tokyo, Japan). To assess the effect of LPS on RANK gene expression 8 h after transfection, the cultured transfected RAW264.7 cells were pretreated with 10 nM soluble RANKL (sRANKL) or the vehicle for 16 h, and then treated with 20 ng/ml LPS from *Escherichia coli* (0111: B4) (Sigma) or the vehicle for 8 h with or without 10 nM of sRANKL, then lysed and subjected to dual luciferase assay (Promega) with an ATP-3010 luminometer (Advantec). For co-transfection studies, pEF-BOS-MITF and pcDNA3-PU.1 were used. The latter was kindly provided by Dr. Harinder Singh (Howard Hughes Medical Institute and Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, USA). The transfection study was repeated three times; the representative data are expressed as the means ± SD of relative luciferase activity standardized by TK promoter activity obtained from four culture-wells.

RNA INTERFERENCE EXPERIMENT

The RNA molecules (small interfering RNA (siRNA)) against MITF (#16704–63142) and PU.1 (#16704–151628) were synthesized by Ambion (Austin, TX), with the target mRNA sequences of

sense (MITF): 5′-GGCAUUUUAUAAGUUUGAGTT-3′,
antisense (MITF): 5′-CUCAAACUUAUAAAAUGCCTC-3′;
sense (PU.1): 5′-GCCAUAGCGAUCACUACUGTT-3′,
antisense (PU.1): 5′-CAGUAGUGAUCGCUAUGGCTT-3′.

RAW 264.7 cells were transfected with siRNA for 48 h using SilencerTM siRNA Transfection Kit II (Ambion) and total RNA was extracted for quantitative real-time RT-PCR. For the control, non-specific siRNA (contained in the commercial kit) was transfected.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION (RT)-PCR

Total RNA was extracted from mouse bone marrow mononuclear cells, M1 cells, and RAW 264.7 cells treated with or without 10^{-6} M of TPA (Sigma), 20 ng/ml of LPS, 10 nM recombinant murine sRANKL (PeproTech House, London, UK), 2 ng/ml of hTGFβ or 1 mg/ml of BMP-2, and/or 500 U/ml of M-CSF or with the vehicle by standard methods using an RNeasy Protect Mini kit (Qiagen KK, Tokyo, Japan). To assess the amount of mRNA of RANK, MITF, PU.1, NFAT, TRAP, and c-fms, 1 μg of total RNA was reverse transcribed to produce cDNA that was then amplified and quantified by the ABI PRISM 7300 Real Time PCR system (Applied Biosystems) using a set of primers and probes (Assay ID; RANK, Mm00437135_m1, MITF, Mm00434954_m1, PU.1, Mm00488140_m1, NFAT, Mm00479445_m1, TRAP, Mm00475698_m1, c-fms, Mm00432689_m1) (Applied Biosystems). For standardization of relative mRNA expression, rodent GAPDH primers and a probe (Assay ID; Mm99999915_g1)(Applied Biosystems) were used.

ELECTROPHORETIC GEL MOTILITY SHIFT ASSAY (EMSA)

Nuclear protein extracts were prepared from RAW 264.7 cells as previously described [Dyer and Herzog, 1995] and those from Hela cells were purchased from Promega. For EMSA, the following double-stranded oligonucleotides spanning the MITF and PU.1 binding sites of the mouse RANK gene promoter were used: MITF-1(−519/−498) 5′-GGCAGAGACAGGTGCGGTGACT-3′, MITF-2(−270/−249) 5′-CCTGCACGCAGGTGCTGCACCC-3′, MITF-3(−114/−93) 5′-CGCGGGGGCAGGTGCCGGGCGG-3′, PU.1 (−497/−463) 5′-CGGAGAACAGCCAAGAGGAAGCGGTTGTTGGAGGC-3′. Oligonucleotides were 5′-end labeled with γ^{32}P-ATP (3,000 Ci/mmol) by T4 polynucleotide kinase (Promega). The binding reaction was carried out, and samples were electrophoresed and analyzed as previously described [Kitazawa and Kitazawa, 2007].

For the competition assay, the oligonucleotide with the mutated PU.1 binding site (−483/−478) was used: PU.1 mut (−497/−463) 5′-CGGAGAACAGCCAAGTCGACGCGGTTGTTGGAGGC-3′.

STATISTICAL ANALYSIS

Data are expressed as means ± SEM. Statistical analyses were carried out by Student's *t*-test. The level of significance was taken to be $P < 0.05$ (Figs. 3, 7).

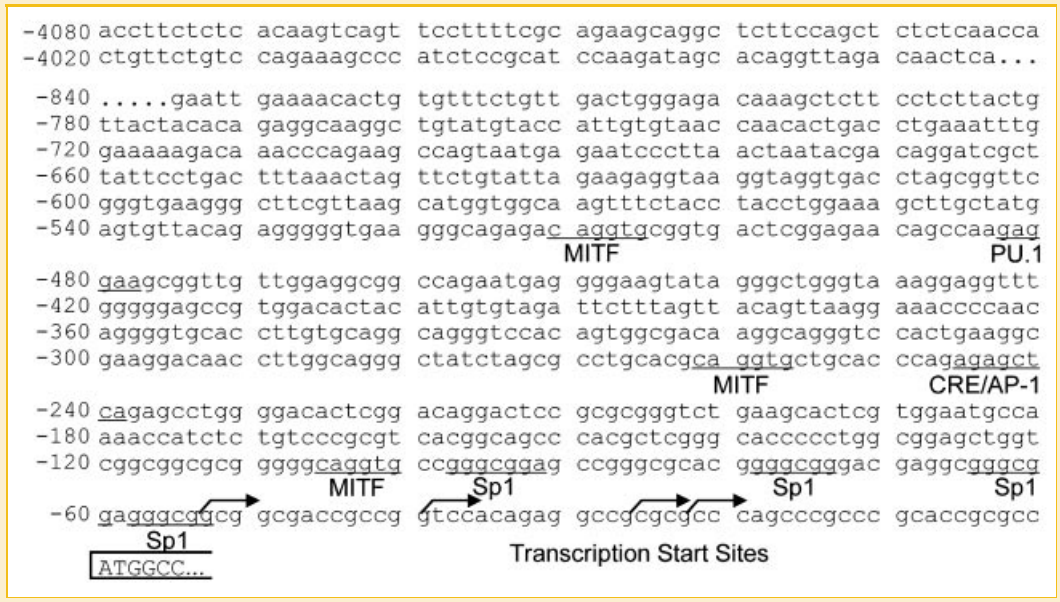

Fig. 1. The 5′-flanking region (6 kb) of the mouse RANK gene was isolated from the mouse BAC clone. Sequencing of the cloned insert shows that mouse RANK lacks canonical TATA-box but has four continuous Sp1-binding sites located at −98, −79, −65, and −58. Four transcription start sites, identified by primer extension experiment, are located at −54, −41, −27, and −23 upstream of the translation start site. Putative binding sites for PU.1 (−480), CRE/AP-1 (−240), three E-boxes (−510, −260, −100) are located within the 1-kb of the transcription start sites.

RESULTS

CLONING AND CHARACTERIZATION OF MOUSE RANK PROMOTER

The 5′-flanking region (6 kb) of the mouse RANK gene was isolated from the mouse BAC clone. Sequencing of the cloned insert revealed that mouse RANK lacked canonical TATA-box but had four continuous Sp1-binding sites located at −98, −79, −65, and −58, and four transcription start sites located at −54, −41, −27, and −23 upstream of the translation start site. In addition, putative binding sites for PU.1 (−480), CRE/AP-1 (−240), three E-boxes (−510, −260, −100), and three NFAT (−720, −550, −370) were located within the 1-kb of the transcription start sites (Fig. 1). In vitro protein-DNA binding analyzed by EMSA revealed that among three probes (−519/−498, −270/−249, −114/−93) containing the putative MITF binding site, only one probe (−114/−93) showed specific protein-DNA binding (Fig. 2A, arrowhead) that was partially blockshifted by the addition of the anti-MITF antibody. Specific protein-DNA binding was also observed (Fig. 2B, arrowhead) by the use of the WT probe (−497/−463) containing the putative PU.1 binding site that was inhibited by the addition of excess amounts of cold competitors and blockshifted by the anti-PU.1 antibody. No specific protein-DNA binding was observed in the mutant oligo-DNA where the putative PU.1-binding site was totally replaced by random sequences.

TRANSIENT TRANSFECTION STUDIES WITH RANK PROMOTER-LUCIFERASE REPORTER GENE CONSTRUCT

The 6 and 1 kb fragments upstream from the transcription start site were ligated into a pGL3 Basic vector. Both fragments showed significant promoter activity when transfected into RAW 264.7 cells. A construct containing a 1 kb fragment, designated as pGL3-WT (pGL3-RANK-1), was used for the following reporter assays. The promoter-reporter gene constructs with mutation of the PU.1 and/or the MITF site were generated and designated as pGL3-PU.1m, pGL3-MITFm, and pGL3-PU.1mMITFm, respectively (Fig. 3, upper panel). Co-transfection studies with the use of expression vectors for MITF and PU.1 revealed that MITF and PU.1 increased the promoter

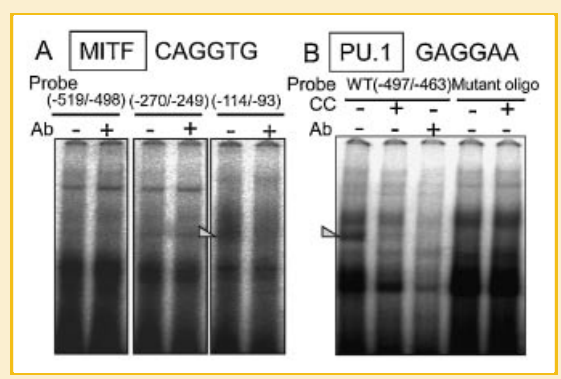

Fig. 2. In vitro protein-DNA binding was analyzed by EMSA. Among three probes (−519/−498, −270/−249, −114/−93) containing the putative MITF binding site, only one probe (−114/−93) shows specific protein-DNA binding (A, arrowhead) that is partially blockshifted by the addition of the anti-MITF antibody. Specific protein-DNA binding is also observed (B, arrowhead) by the use of the WT probe (−497/−463) containing the putative PU.1 binding site that is inhibited by the addition of excess amounts of cold competitors (CC) and blockshifted by the anti-PU.1 antibody. No specific protein-DNA binding is observed in the mutant oligo-DNA where the putative PU.1-binding site is totally replaced by random sequences.

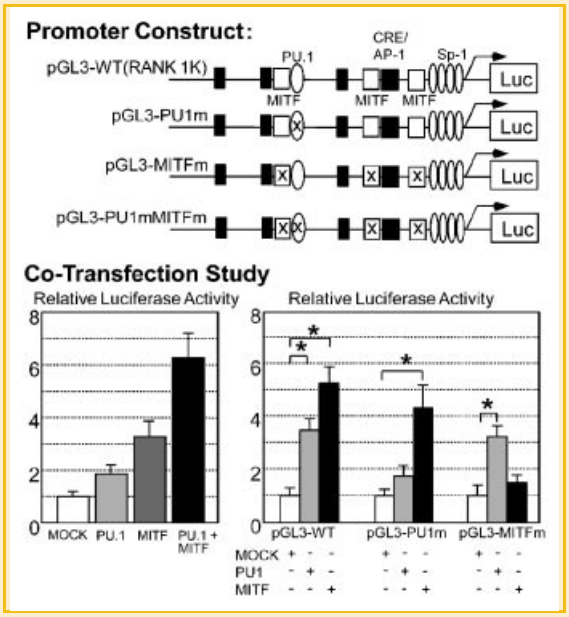

Fig. 3. A construct containing a 1 kb intact RANK promoter fragment, pGL3-WT (pGL3-RANK-1), and three promoter-reporter gene constructs with mutation of the PU.1 and/or the MITF site, designated as pGL3-PU.1m, pGL3-MITFm, and pGL3-PU.1mMITFm, respectively, are shown (upper panel). Co-transfection studies with the use of expression vectors for MITF and PU.1 revealed that MITF and PU.1 increased the promoter activity of pGL3-WT three- and twofold, respectively, and sixfold synergistically (lower panel, left). On the other hand, while MITF increased the promoter activity of pGL3-PU.1m, MITF did not affect that of pGL3-MITFm ($^{*}P < 0.05$ vs. vehicle). Similarly, PU.1 increased the promoter activity of pGL3-MITFm but did not affect that of pGL3-PU.1m ($^{*}P < 0.05$ vs. vehicle) (lower panel, right).

activity of pGL3-WT three- and twofold, respectively, and sixfold synergistically (Fig. 3, lower panel, left). On the other hand, MITF increased the promoter activity of pGL3-PU.1m but did not affect that of pGL3-MITFm ($^{*}P < 0.05$ vs. vehicle). Similarly, PU.1 increased promoter activity of pGL3-MITFm but did not affect that of pGL3-PU.1m ($^{*}P < 0.05$ vs. vehicle) (Fig. 3, lower panel, right).

RNA INTERFERENCE EXPERIMENT

The effect of MITF and PU.1 knock-down on RANK expression in RAW 264.7 cells, tested by the RNA interference experiment, revealed that MITF knock-down decreased MITF mRNA expression to 63% of its steady state expression and, concurrently, PU.1 mRNA expression to 74% (Fig. 4, middle). PU.1 knock-down decreased PU.1 mRNA expression to 64% of its steady state expression and MITF mRNA expression to 92% (Fig. 4, right). RANK mRNA expression in RAW 264.7 cells was significantly suppressed by MITF and by PU.1 siRNA to 82% and to 49% of its steady state expression, respectively, and by both to 44% (Fig. 4, left).

THE EFFECT OF LPS ON RANK EXPRESSION IN TIBIAE OF MICE

Serial sections of the epiphyseal spongiosa of the tibiae of 10-week-old normal and LPS-treated mice were stained for tartrate-resistant

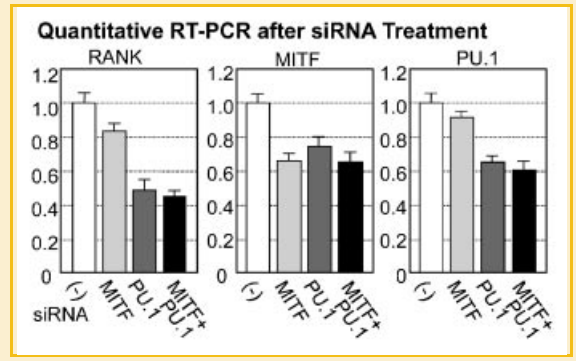

Fig. 4. The effect of MITF and PU.1 knock-down on RANK expression in RAW 264.7 cells was tested by RNA interference experiment. RANK mRNA expression in RAW 264.7 cells was significantly suppressed by MITF and by PU.1 siRNA to 82% and 49% of its steady state expression, respectively, and by both to 44% (left). MITF knock-down itself decreased MITF mRNA expression to 63% of its steady state expression and, at the same time, PU.1 mRNA expression to 74% (middle). PU.1 knock-down efficiently decreased PU.1 mRNA expression to 64% of its steady state expression, but did not have much effect on MITF mRNA expression (right).

acid phosphatase (TRACP), and for RANK by in situ hybridization. TRACP-stained sections of normal controls showed TRACP-positive osteoclasts and osteoclast precursors on the surface of the tibiae (Fig. 5A, arrows, 400×), and RANK mRNA was detected exclusively on these osteoclasts and osteoclast precursors (Fig. 5B, arrows, 400×). In the TRACP-stained sections of short-term LPS treated mice, while TRACP-positive osteoclasts and osteoclast precursors

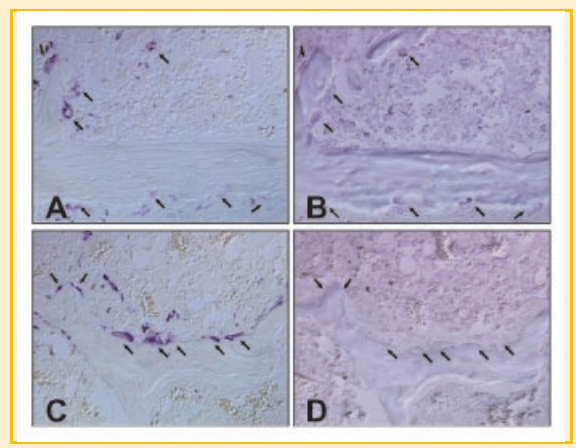

Fig. 5. Serial sections of the epiphyseal spongiosa of the tibiae of 10-week-old normal and LPS-treated mice were stained for tartrate-resistant acid phosphatase (TRACP), and for RANK by in situ hybridization. TRACP-stained sections of normal controls show TRACP-positive osteoclasts and osteoclast precursors on the surface of the tibiae (A, arrows, 400×). RANK mRNA is detected exclusively on these osteoclasts and osteoclast precursors (B, arrows, 400×). In TRACP-stained sections of short-term LPS treated mice, while the number of TRACP-positive osteoclasts and osteoclast precursors is not changed after LPS treatment (C, arrows, 400×), RANK signals are significantly weak on these osteoclasts and osteoclast precursors (D, arrows, 400×), indicating that LPS treatment rapidly decreased RANK transcripts on both mature and committed osteoclasts.

were similarly observed on the surface of the tibiae (Fig. 5C, arrows, 400×), RANK signals were, significantly weak on these osteoclasts and osteoclast precursors (Fig. 5D, arrows, 400×).

THE EFFECT OF LPS ON RANK mRNA EXPRESSION

LPS treatment decreased RANK mRNA expression in RAW 264.7 cells to 55% in the absence of sRANKL, and from 172% to 119% in the presence of sRANKL (Fig. 6, upper, left). During the period between 24 and 48 h of LPS treatment, LPS similarly suppressed RANK mRNA expression in RAW264.7 cells both in the presence and absence of sRANKL (data not shown). sRANKL increased TRACP mRNA expression in RAW 264.7 cells 196-fold, and LPS treatment suppressed the sRANKL-induced TRACP mRNA expression from 196- to 46-fold of its steady state expression (Fig. 6, upper, right). The expression of MITF and PU.1, as well as of RANK mRNA, was suppressed by LPS treatment in the presence of sRANKL from 250% to 155% (Fig. 6, lower, left) and from 198% to 134% (Fig. 6, lower, right), respectively. LPS treatment did not affect the expression of MITF and PU.1 mRNA in the absence of sRANKL.

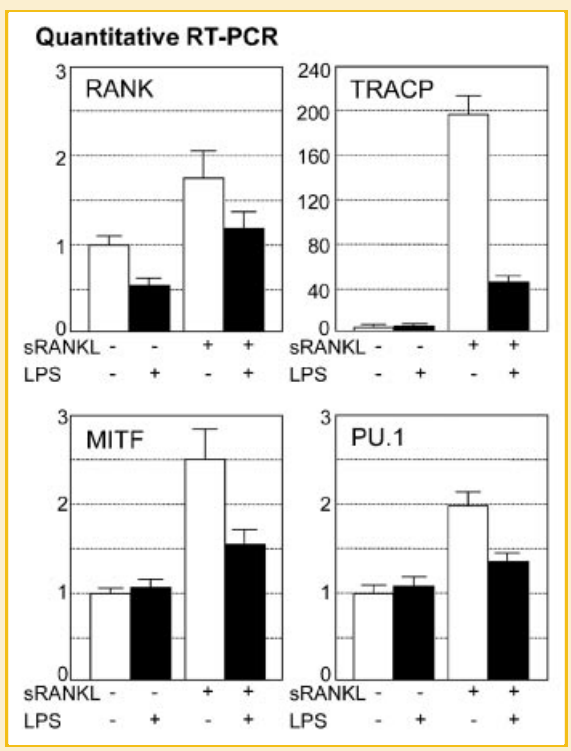

Fig. 6. Mirroring in vivo LPS treatment, in vitro LPS treatment (48 h) decreased RANK mRNA expression in RAW 264.7 cells to 55% in the absence of sRANKL, and from 172% to 119% in the presence of sRANKL (upper, left). sRANKL increased TRACP mRNA expression in RAW 264.7 cells 196-fold, and LPS treatment suppressed the sRANKL-induced TRACP mRNA expression from 196- to 46-fold of its steady state expression (upper, right). The expression of MITF and PU.1, as well as of RANK mRNA, was suppressed by LPS treatment in the presence of sRANKL from 250% to 155% (lower, left) and from 198% to 134% (lower, right), respectively. LPS treatment did not affect the expression of MITF and PU.1 mRNA in the absence of sRANKL.

THE EFFECT OF LPS ON RANK PROMOTER ACTIVITY ASSESSED BY TRANSIENT TRANSFECTION STUDIES WITH RANK PROMOTER-LUCIFERASE REPORTER GENE CONSTRUCT AND ITS MUTATED CONSTRUCT

Eight hours after transfection of pGL3-WT, the RAW264.7 cells were pretreated with 10 nM of sRANKL or with the vehicle for 16 h and then treated with 20 ng/ml of LPS or with the vehicle for 8 h. LPS treatment decreased the promoter activity to 70% in the presence or the absence of sRANKL (Fig. 7A). LPS decreased the RANK promoter activity of pGL3-WT to 58% but did not affect that of mutated constructs (pGL3-PU.1m, pGL3-MITFm, or pGL3-PU.1mMITFm) (Fig. 7B, $^{*}P < 0.05$ vs. vehicle).

DISCUSSION

Osteoclasts are highly specialized bone-resorbing cells derived from the monocyte-macrophage lineage in the presence of both a TNF-related factor (RANKL) and CSF-1 (M-CSF) [Lacey et al., 1998; Yasuda et al., 1998; Teitelbaum and Ross, 2003]. Since both RANKL and CSF-1 are produced by bone-forming cells (osteoblasts), communication between osteoblasts and osteoclasts plays a central

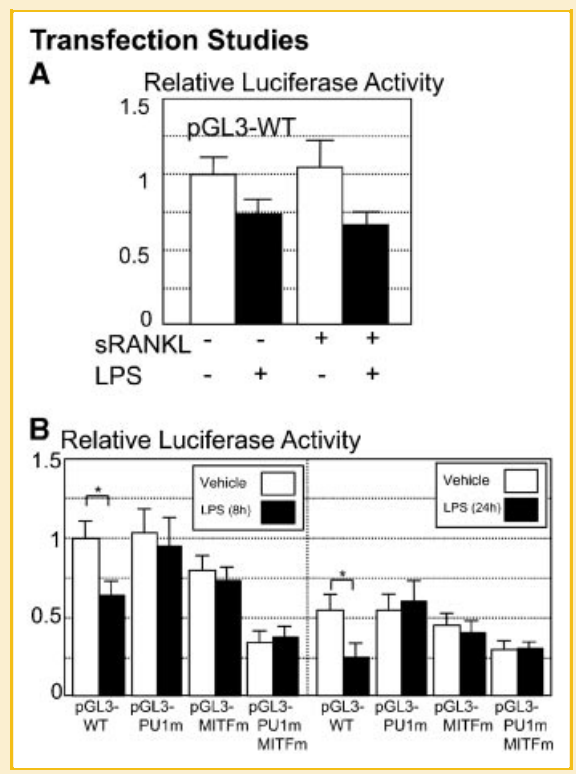

Fig. 7. Eight hours after transfection of pGL3-WT, the RAW264.7 cells were pretreated with 10 nM soluble RANKL (sRANKL) or with the vehicle for 16 h and then treated with 20 ng/ml of LPS or with the vehicle for 8 and 24 h. LPS treatment decreased the promoter activity to 70% in the presence or the absence of sRANKL (A). LPS decreased the RANK promoter activity of pGL3-WT to 58% by 8 h (B, left) and 40% by 24h (B, right) of LPS treatment but did not affect that of mutated constructs (pGL3-PU.1m, pGL3-MITFm, or pGL3-PU.1mMITFm) (B, $^{*}P < 0.05$ vs. vehicle).

role in the recruitment, differentiation, maintenance, and activation of osteoclasts from their hematopoietic precursors. Recently, some of the crucial interactions between receptors expressed on osteoclasts and their precursors, and ligands expressed on osteoblasts, have been identified: between c-Fms and CSF-1, RANK and RANKL [Lacey et al., 1998; Yasuda et al., 1998], and between immunoglobulin (Ig)-like receptors including osteoclast-associated receptor (OSCAR) and yet unidentified ligands [Kim et al., 2002; So et al., 2003]. Among these interactions, the binding of RANKL to its receptor RANK leads to receptor trimerization and the activation of multiple intracellular signaling pathways that, in turn, specifically transactivate many osteoclast-specific genes leading to terminal differentiation. Thus the sequential and coordinated expression of these receptors on osteoclasts and their precursors must be a highly sophisticated and strictly regulated process that maintains bone and mineral homeostasis within a narrow range.

Tissue-residing phagocytes are, on the other hand, also derived from the monocyte-macrophage lineage and, thus, share the same precursors with osteoclasts [Chambers, 1978]. The roles of these two types of the cells are, however, totally different from each other: phagocytes, containing many lysosomes that digest foreign material, are useful as an initial immune system response to infection by engulfing pathogens, debris, dead or dying cells and extracellular matrix. Since RANK–RANKL signaling is one of the essential processes of osteoclastogenesis, we explored the promoter structure of mouse RANK gene on the assumption that the molecular mechanism modulating RANK gene expression holds some important key for directing the monocyte-macrophage population to the final recruitment and commitment of osteoclast precursors or to non-specific phagocytes. The sequence of the mouse basic promoter region is highly homologous to that of the human promoter [Kwon et al., 2005]. By gelshift assay and site-directed mutagenesis studies, two important transcription factors, PU.1 and MITF, were found to positively and synergistically regulate RANK gene expression. We also noticed that the features of the RANK gene basic promoter structure with PU.1 and MITF response elements, but lacking canonical TATA-box, were functionally similar to the previously characterized OSCAR [So et al., 2003] and cathepsin K [Li and Chen, 1999] gene promoters, indicating that a group of target genes sharing the same trend for osteoclast differentiation are controlled by the sequential and overlapping expression of PU.1 and MITF. While MITF has been implicated in a wide range of differentiation and survival of developmentally unrelated cell types other than osteoclasts, but including mast cells, melanocytes, and pigmented retinal epithelial cells [Hodgkinson et al., 1993], interaction with ETS family transcription factor PU.1 may partly account for the ability of MITF to selectively regulate target genes during osteoclast differentiation [Hu et al., 2007; Sharma et al., 2007]. This, however, poses the next question of how osteoclast-specific gene expression patterns are selectively maintained in these cell lineages especially that MITF and PU.1 are expressed in phagocytes and osteoclasts, as well as in the common mononuclear precursor for both of these cell types [Zhao et al., 2007]. As a cue to resolving this question, recently identified corepressor Eos [Hu et al., 2007], a zinc finger protein capable of forming a complex with MITF and PU.1, may partly be involved in suppressing RANK gene in committed myeloid progenitors. Further study is necessary to identify the transcriptional machinery that specifically controls RANK gene expression in osteoclasts and their precursors.

As is evident from the fact that periodontitis due to gingival bacterial infection is a major cause of tooth decay, the net effect of bacterial LPS on bone is bone loss [Miyauchi et al., 2001; Wada et al., 2004; Taubman et al., 2005]. In vitro studies have also revealed that bacterial LPS promotes osteoclast formation in bone marrow and in co-cultures of bone marrow macrophages and osteoblastic stromal cells [Suda et al., 2002]. These bone-resorbing actions of LPS are achieved mostly indirectly either through the modulation of RANKL-OPG axis on osteoblastic stromal cells [Hotokezaka et al., 2007] or by the production of PGE2 and bone-seeking cytokines such as TNF-α, IL-1, and IL-6 [Abu-Amer et al., 1997]. While LPS supports survival and final fusion of already committed osteoclast precursors independent of TNF-α, IL-1, and RANKL [Suda et al., 2002; Itoh et al., 2003], it acts directly on osteoclast precursors to inhibit osteoclastogenesis through TLRs [Takami et al., 2002]. Indeed, in our in vitro experiments with sRANKL-treated RAW264.7 cells and the in situ hybridization study for the LPS-treated in vivo mouse model of endotoxemia, the level of RANK mRNA decreased rapidly (Figs. 5 and 6) by LPS treatment. Thus, LPS exerts two opposite actions on osteoclasts: it indirectly induces osteoclastogenesis and, at the same time, directly limits the survival of mature osteoclasts and their committed precursors within a limited period of time by down-regulating RANK expression. This somewhat perplexing phenomenon is explained by the favorable outcome for host defense by preventing the pathogenic effects of bacterial invasion of bone, especially during the acute phase of sepsis or endotoxemia. Furthermore, even during chronic phases of inflammatory bone diseases such as bone abscess and periodontitis, the dual action of LPS on bone is the rationale for inflammation-induced bone remodeling. Hosts may defend themselves against bacterial bone damage first by activating local committed osteoclasts to accelerate scavenging the damaged bone or sequestrum rapidly, and second, by limiting a number of local authentic osteoclasts to enclose the infected area.

In conclusion, LPS per se directly suppresses RANK expression in the osteoclastic cell lineage, at least in part, by down-regulating the expression of PU.1 and MITF genes, which may be important during both acute and chronic phases of inflammation-induced bone remodeling.

ACKNOWLEDGMENTS

The authors thank Mr. Shuichi Matsuda, Ms. Noriko Sakamoto and Ms. Miki Zenigami for excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, (16590278, 19390100 and 19659086 to S.K., 20790283 to T.K. and 18590372 to R.K.).

REFERENCES

Abu-Amer Y, Ross FP, Edwards J, Teitelbaum SL. 1997. Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor. J Clin Invest 100:1557–1565.

Boyce BF, Hughes DE, Wright KR, Xing L, Dai A. 1999. Recent advances in bone biology provide insight into the pathogenesis of bone diseases. Lab Invest 79:83–94.

Chambers TJ. 1978. Multinucleate giant cells. J Pathol 126:125–148.

Day CJ, Kim MS, Stephens SR, Simcock WE, Aitken CJ, Nicholson GC, Morrison NA. 2004. Gene array identification of osteoclast genes: Differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. J Cell Biochem 91:303–315.

Dyer RB, Herzog NK. 1995. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. Biotechniques 19:192–195.

Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, Weitzmann MN, Pacifici R. 2007. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. J Clin Invest 117:122–132.

Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA, Arnheiter H. 1993. Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 74:395–404.

Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K, Fujimura Y, Yoshida N, Nakayama K. 2007. Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan. J Cell Biochem 101:122–134.

Hu R, Sharma SM, Bronisz A, Srinivasan R, Sankar U, Ostrowski MC. 2007. Eos, MITF, and PU.1 recruit corepressors to osteoclast-specific genes in committed myeloid progenitors. Mol Cell Biol 27:4018–4027.

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, Hata K, Yamashita K, Hiraga T, Watanabe T, Kukita T, Yoshioka K, Rao A, Yoneda T. 2004. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest 114:475–484.

Itoh K, Udagawa N, Kobayashi K, Suda K, Li X, Takami M, Okahashi N, Nishihara T, Takahashi N. 2003. Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. J Immunol 170:3688–3695.

Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Chihara K. 1997. Dexamethasone stimulates osteoclast-like cell formation by directly acting on hemopoietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E2. J Bone Miner Res 12:734–741.

Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, Yamamoto G, Hishikawa T, Noguchi T, Yoshikai Y. 2001. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol 166:3574–3579.

Kim N, Takami M, Rho J, Josien R, Choi Y. 2002. A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 195:201–209.

Kitazawa R, Kitazawa S. 2007. Methylation status of a single CpG locus 3 bases upstream of TATA-box of receptor activator of nuclear factor-kappaB ligand (RANKL) gene promoter modulates cell- and tissue-specific RANKL expression and osteoclastogenesis. Mol Endocrinol 21:148–158.

Kitazawa S, Ross FP, McHugh K, Teitelbaum SL. 1995. Interleukin-4 induces expression of the integrin alpha v beta 3 via transactivation of the beta 3 gene. J Biol Chem 270:4115–4120.

Kunkel SL, Spengler M, May MA, Spengler R, Larrick J, Remick D. 1988. Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. J Biol Chem 263:5380–5384.

Kwon OH, Lee CK, Lee YI, Paik SG, Lee HJ. 2005. The hematopoietic transcription factor PU.1 regulates RANK gene expression in myeloid progenitors. Biochem Biophys Res Commun 335:437–446.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Layfield R. 2007. The molecular pathogenesis of Paget disease of bone. Expert Rev Mol Med 9:1–13.

Lerner UH. 2006. Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. J Dent Res 85: 596–607.

Li YP, Chen W. 1999. Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. J Bone Miner Res 14:487–499.

Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, Nogi Y. 2004. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem 279:45969–45979.

Miyauchi M, Sato S, Kitagawa S, Hiraoka M, Kudo Y, Ogawa I, Zhao M, Takata T. 2001. Cytokine expression in rat molar gingival periodontal tissues after topical application of lipopolysaccharide. Histochem Cell Biol 116: 57–62.

Mizuno A, Kanno T, Hoshi M, Shibata O, Yano K, Fujise N, Kinosaki M, Yamaguchi K, Tsuda E, Murakami A, Yasuda H, Higashio K. 2002. Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. J Bone Miner Metab 20:337–344.

Mori H, Kitazawa R, Mizuki S, Nose M, Maeda S, Kitazawa S. 2002. RANK ligand, RANK, and OPG expression in type II collagen-induced arthritis mouse. Histochem Cell Biol 117:283–292.

Necela BM, Su W, Thompson EA. 2008. Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor gamma and nuclear factor-kappaB in macrophages. Immunology [Epub ahead of print].

Ross FP, Teitelbaum SL. 2005. alphavbeta3 and macrophage colony-stimulating factor: Partners in osteoclast biology. Immunol Rev 208:88–105.

Sharma SM, Bronisz A, Hu R, Patel K, Mansky KC, Sif S, Ostrowski MC. 2007. MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation. J Biol Chem 282:15921–15929.

So H, Rho J, Jeong D, Park R, Fisher DE, Ostrowski MC, Choi Y, Kim N. 2003. Microphthalmia transcription factor and PU.1 synergistically induce the leukocyte receptor osteoclast-associated receptor gene expression. J Biol Chem 278:24209–24216.

Suda K, Woo JT, Takami M, Sexton PM, Nagai K. 2002. Lipopolysaccharide supports survival and fusion of preosteoclasts independent of TNF-alpha, IL-1, and RANKL. J Cell Physiol 190:101–108.

Suda K, Udagawa N, Sato N, Takami M, Itoh K, Woo JT, Takahashi N, Nagai K. 2004. Suppression of osteoprotegerin expression by prostaglandin E2 is crucially involved in lipopolysaccharide-induced osteoclast formation. J Immunol 172:2504–2510.

Takami M, Kim N, Rho J, Choi Y. 2002. Stimulation by toll-like receptors inhibits osteoclast differentiation. J Immunol 169:1516–1523.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901.

Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, Stanley ER, Kurokawa T, Suda T. 1993. Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. J Clin Invest 91:257–263.

Taubman MA, Valverde P, Han X, Kawai T. 2005. Immune response: The key to bone resorption in periodontal disease. J Periodontol 76:2033–2041.

Teitelbaum SL, Ross FP. 2003. Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649.

Wada N, Maeda H, Yoshimine Y, Akamine A. 2004. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha. Bone 35:629–635.

Yang S, Takahashi N, Yamashita T, Sato N, Takahashi M, Mogi M, Uematsu T, Kobayashi Y, Nakamichi Y, Takeda K, Akira S, Takada H, Udagawa N, Furusawa K. 2005. Muramyl dipeptide enhances osteoclast formation induced by lipopolysaccharide, IL-1 alpha, and TNF-alpha through nucleotide-binding oligomerization domain 2-mediated signaling in osteoblasts. J Immunol 175:1956–1964.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95: 3597–3602.

Zhao Q, Shao J, Chen W, Li YP. 2007. Osteoclast differentiation and gene regulation. Front Biosci 12:2519–2529.

Zou W, Bar-Shavit Z. 2002. Dual modulation of osteoclast differentiation by lipopolysaccharide. J Bone Miner Res 17:1211–1218.