from the immunized mice were treated with Tris-buffered ammonium chloride solution to remove red blood cells as described in our previous report [7].

2.8. Bacterial infection

A seed of *L. monocytogenes* EGD strain was cultured overnight in trypticase soy broth (Beckton Dickinson and Company, Cockeysville, MD) at 37 °C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above, or immunized by a single intraperitoneal injection with a sub-lethal dose (1×10^4 CFU) of *L. monocytogenes*. One month later, the mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (Beckton Dickinson and Company).

2.9. Statistics

Data from multiple experiments were expressed as the mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Construction of plasmids for DNA immunization

In order to evaluate vaccination with plasmid DNA encoding murine GM-CSF inserted with a dominant Th epitope, we constructed pGM215m plasmid (Fig. 1A). As a control, we prepared another plasmid, p215m, a minigene plasmid for expression of LLO 215–226 peptide alone (Fig. 1A). In order to confirm expression of the GM-CSF-LLO 215–226 protein (GM215) by transfection of pGM215m, we transiently transfected 293T cells with pGM215m or pGM-CSF control plasmid and prepared the culture supernatants. As shown in Fig. 1B, we were able to detect expression of GM215 protein in the culture supernatant of pGM215m-transfected 293T cells by using GM-CSF-specific ELISA.

3.2. Proliferative responses of spleen cells of mice immunized with pGM215m plasmid and the epitope-specific IFN-γ production by the spleen cells

In order to examine the effect of immunization with pGM215m, we performed lymphocyte proliferation assay after immunization of C3H/He mice with the plasmid by using gene gun bombardment. We chose the immunization method as it is an appropriate vaccination route to evaluate the effect of GM-CSF expression plasmid on Langerhans cells and also it is a reliable and reproducible method from our previous experience [8]. As shown in Fig. 2, immunization with pGM215m plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunized mice. Immunization with p215m plasmid showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naïve mice (data not shown).

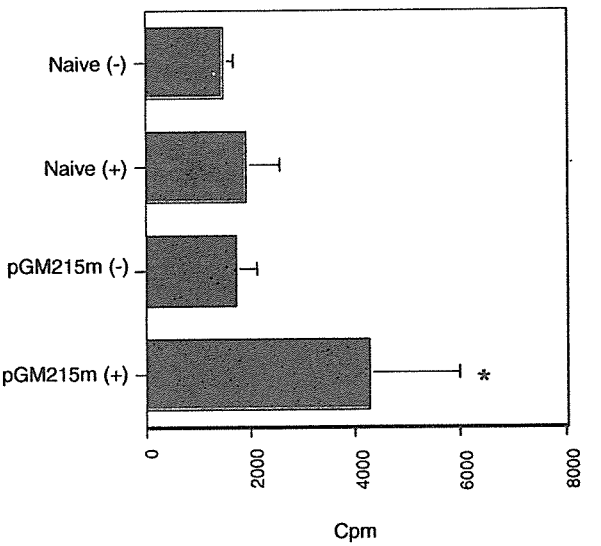

Fig. 2. Specific proliferative responses of spleen cells from pGM215m-immunized mice. C3H/He mice were immunized with pGM215m plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested three weeks after the last immunization and cultured in vitro (5×10^5 cells/well) in the presence or absence of 1 μM of LLO 215–226 peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of naïve C3H/He mice are also shown as a control. The values indicate cpm per well. The mean ± S.D. of cpm of four mice per group are shown. Asterisks indicate statistical significance ($p \leq 0.001$) compared with the value of spleen cells of naïve mice without LLO 215–226 stimulation.

Furthermore, we analyzed IFN-γ amounts in the supernatants of spleen cell culture after 5-day in vitro stimulation with LLO 215–226 peptide. Again, immunization with pGM215m induced higher amounts of IFN-γ than those of mice immunized with p215m after the in vitro stimulation (Table 1). We did not detect IL-4 pro-

Table 1
IFN-γ production by splenocytes from C3H/He mice immunized with pGM215m plasmid

Immunization	Stimulation[a]	IFN-γ (pg/ml)[b]
Naïve	–	85.1
	LLO 215	74.1
pGM215m	–	95.9
	LLO 215	1318.7

[a] Spleen cells of immunized mice (2×10^6 cells/well) were cultured in the absence (–) or presence of 1 μM of LLO 215–226 peptide (LLO 215).

[b] After 4 days, cytokine concentrations in culture supernatants were quantified with sandwich ELISA. The mean of duplicate wells of representative data was shown.

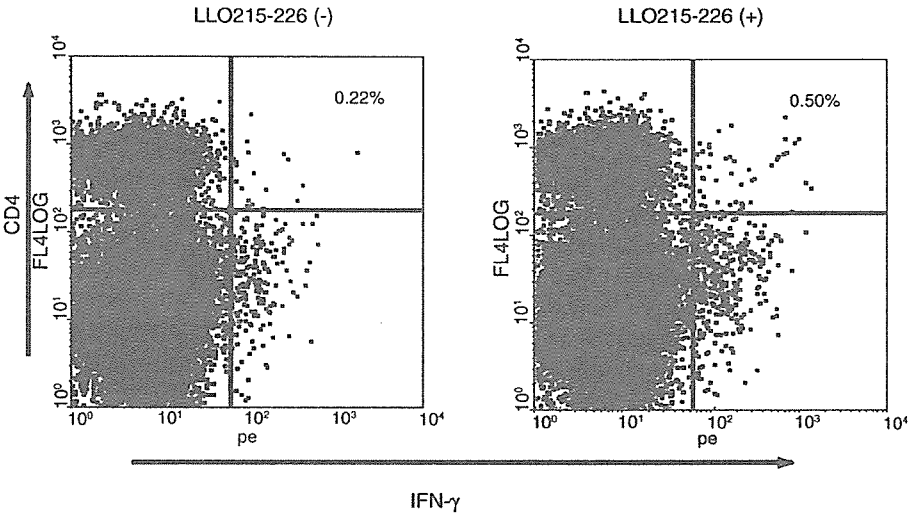

Fig. 3. Induction of LLO 215-specific CD4+ T cells after pGM215m immunization. Intracellular IFN-γ staining on CD4+ T-cell subset was performed using spleen cells after pGM215m immunization in the presence or absence of LLO 215–226 peptide. The percentages of IFN-γ-positive cells in CD4+ T cells are shown.

duction from spleen cells of the immunized mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg/ml; data not shown). In addition, we performed intracellular IFN-γ staining with spleen cells of mice immunized with pGM215m. After in vitro stimulation with LLO 215–226 peptide, CD4+ IFN-γ-producing cells was induced in the immune spleen cells (Fig. 3).

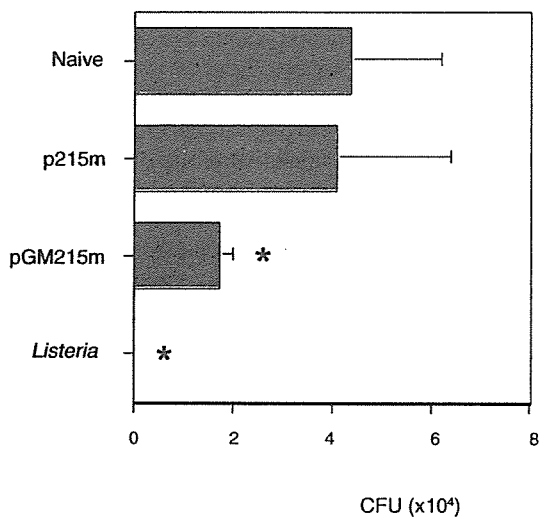

Fig. 4. Protective immunity induced by immunization with pGM215m. Mice were immunized with pGM215m four times at one-week intervals. One month after the last immunization, the immunized mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naïve mice and mice immunized with a sublethal dose of *L. monocytogenes* are also shown as controls. Results are expressed as the means ± S.D. for three mice for each group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of naïve mice.

3.3. Induction of protective immunity against listerial infection after immunization with pGM215m plasmid

In order to examine whether the immunity evoked by immunization with pGM215m plasmid is associated with an increased resistance to infection of virulent *L. monocytogenes*, the in vivo protection experiment was carried out. Seventy-two hours after listerial challenge, mice immunized with pGM215m plasmid were sacrificed and CFU from the spleens were counted. As shown in Fig. 4, immunization with p215m did not show significant protective effects. On the contrary, mice immunized with a sublethal dose of *L. monocytogenes* were able to eliminate challenged *L. monocytogenes* from the spleens. Immunization with pGM215m conferred moderate, but significant protective immunity against lethal listerial challenge.

4. Discussion

DNA vaccination may work through direct transfection of antigen presenting cells (APCs), or by secretion of the encoded protein by muscle or skin cells for the uptake by APCs. Therefore, two different strategies have been considered to induce a particular epitope-specific Th by DNA vaccination. One is an intracellular targeting of antigens [9–11]. Another strategy is taking an advantage of secreted proteins. In general, secreted proteins are phagocytosed by APCs and presented on MHC class II molecules. In this study, we used a plasmid expressing murine GM-CSF, a cytokine which is indispensable for development of APCs such as dendritic

cells and macrophages. GM-CSF is one of most studied cytokines for vaccine adjuvants [12,13]. GM-CSF expression plasmid injected into mouse muscle has been reported to lead to a local infiltration of potential APCs [14]. We therefore reasoned that immunization of a gene for GM-CSF-Th epitope fusion molecule may work well for induction of the epitope-specific Th subset.

Several reports showed that immunization with a DNA vaccine co-expressing both antigen and GM-CSF [15] or a bicistronic plasmid DNA for antigen and GM-CSF [16,17] is superior to co-immunization of DNA vaccines for antigen alone and for GM-CSF in terms of strength of the specific immunity induced by the vaccines. Linking antigen and GM-CSF expression closely in vivo may provide a microenvironment suitable for the uptake and presentation of antigen by dendritic cells or macrophages. Immunization of pGM215m plasmid fits this condition.

It has been reported that $CD4^+$ T cells, especially LLO 215–226-specific T cells, are involved in protective immunity against listerial challenge. Verma et al. [18] demonstrated that induction of $CD4^+$ T cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using *Salmonella* carrier system. They showed one-log order reduction in numbers of the bacterium in spleens and livers of the immunized mice. In another approach, we showed that significant induction of protective immunity to *L. monocytogenes* by immunization with a plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [10] or immunization with a plasmid encoding LLO 215–216 Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 [11]. pGM215m immunization shown here was more effective than LLO215–LAMP fusion DNA immunization [11] in terms of induction of the protective immunity (data not shown).

The immunization strategy shown here, i.e., immunization with GM-CSF gene inserted with a double-stranded oligonucleotide encoding a Th epitope, would be applicable to DNA vaccination for induction of CTL or antibodies as a molecular adjuvant for supplying Th. Investigators add a universal Th epitope such as 13 aminio-acid Pan HLA-DR Epitope (PADRE) [19] in multi-CTL epitope plasmid DNA construction for efficient CTL induction [20]. GM-CSF-Th epitope DNA vaccination would be the alternative strategy for induction of Th.

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Molecular Analysis of RANKL-Independent Cell Fusion of Osteoclast-Like Cells Induced by TNF-α, Lipopolysaccharide, or Peptidoglycan

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Abstract Focusing on the final step of osteoclastogenesis, we studied cell fusion from tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells into multinuclear cells. TRAP-positive mononuclear cells before generation of multinuclear cells by cell fusion were differentiated from RAW264.7 cells by treatment with receptor activator of nuclear factor kappa B ligand (RANKL), and then the cells were treated with lipopolysaccharide (LPS), followed by culturing for further 12 h. LPS-induced cell fusion even in the absence of RANKL. Similarly, tumor necrosis factor (TNF)-α and peptidoglycan (PGN) induced cell fusion, but M-CSF did not. The cell fusion induced by RANKL, TNF-α, and LPS was specifically blocked by osteoprotegerin (OPG), anti-TNF-α antibody, and polymyxin B, respectively. LPS- and PGN-induced cell fusion was partly inhibited by anti-TNF-α antibody but not by OPG. When TRAP-positive mononuclear cells fused to yield multinuclear cells, phosphorylation of Akt, Src, extracellular signal-regulated kinase (ERK), p38MAPK (p38), and c-Jun NH2-terminal kinase (JNK) was observed. The specific chemical inhibitors LY294002 (PI3K), PP2 (Src), U0126 (MAPK-ERK kinase (MEK)/ERK), and SP600125 (JNK) effectively suppressed cell fusion, although SB203580 (p38) did not. mRNA of nuclear factor of activated T-cells c1 (NFATc1) and dendritic cell-specific transmembrane protein (DC-STAMP) during the cell fusion was quantified, however, there was no obvious difference among the TRAP-positive mononuclear cells treated with or without M-CSF, RANKL, TNF-α, LPS, or PGN. Collectively, RANKL, TNF-α, LPS, and PGN induced cell fusion of osteoclasts through their own receptors. Subsequent activation of signaling pathways involving PI3K, Src, ERK, and JNK molecules was required for the cell fusion. Although DC-STAMP is considered to be a requisite for cell fusion of osteoclasts, cell fusion-inducing factors other than DC-STAMP might be necessary for the cell fusion. J. Cell. Biochem. 9999: 1–13, 2006.

Key words: TNF-α; lipopolysaccharide; peptidoglycan; cell fusion; osteoclasts

Abbreviations used: Akt, PKB, protein kinase B; c-Fos, cellular homolog of v-fos; c-Src, cellular homolog of v-src; DC-STAMP, dendritic cell-specific transmembrane protein; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HRP; horse radish peroxidase; IL-1β, interluekine-1β; IL-6, interluekine-6; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MyD88, myeloid differentiation factor 88; NFATc1, nuclear factor of activated T-cells c1; NF-κB, nuclear factor kappa B; OPG, osteoprotegrin ligand; P-, phosphorylated; PBS, phosphate-buffered saline; PGN, peptidoglycan; p38, p38MAPK; p65, 65 kD subunit of NF-κB (RelA); RANKL, receptor activator of nuclear factor kappa B ligand; SDS, sodium dodecyl sulfate; TBST, 150 mM NaCl and 0.1% Tween-20 in 25 mM Tris/HCl, pH 7.6; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase;

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Bone is continuously remodeled by bone formation and resorption, and the cooperative bone metabolism is precisely regulated to maintain homeostasis. Osteoclasts, which are responsible for bone resorption in bone metabolism, are multinucleated cells originating from hematopoietic precursor cells of the monocyte/macrophage lineage. One of the key factors for osteoclastogenesis, which is induced by osteoblasts, is receptor activator of nuclear factor kappa B ligand (RANKL), a member of the tumor necrosis factor (TNF) family [Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998]. RANKL was found to be expressed by T-cells [Wong et al., 1997; Josien et al., 1999; Kong et al., 1999] and B-cells [Li et al., 2000] as well as osteoblastic/stromal cells and to be essential for osteoclast differentiation. Receptor activator of nuclear factor kappa B (RANK), which is one of TNF receptor family members, is expressed in osteoclasts and their precursor cells as the receptor of RANKL [Josien et al., 1999; Li et al., 2000; Choi et al., 2001]. Downstream signaling through RANK is essential for osteoclastogenesis.

Osteoclasts are differentiated through multiple steps that include cell fusion at the latest step of differentiation, yielding multi-nuclear cells. Dendritic cell-specific transmembrane protein (DC-STAMP) was recently found to play a role in the cell fusion of osteoclasts, which seems to support the activity of bone resorption by osteoclasts [Kukita et al., 2004; Yagi et al., 2005]; however, the factors involved in and the precise mechanism of the cell fusion are unknown.

Deviation from the normal conditions of bone resorption results in bone diseases such as osteopetrosis, osteoporosis, and bone resorptive infectious disease. Periodontal disease is the most frequent bone resorptive infectious disease and is thought to be caused mainly by infection with Gram-negative bacteria. Such an infectious and pathological condition induces inflammation, resulting in bone resorption. A major bacterial factor for inflammation has been believed to be lipopolysaccharide (LPS), a main component of the cell surface of Gram-negative bacteria. LPS has the ability to induce proinflammatory cytokines such as TNF-α, interluekine-1β (IL-1β), and interluekine-6 (IL-6) in various kinds of cells [Wang and Ohura, 2002]. Bacterial components such as LPS or various cytokines elicited in infectious lesions may modulate physiological osteoclastogenesis, leading to a pathological bone resorptive condition.

In the present study, we investigated what bacterial components or cytokines affect osteoclastogenesis at the stage of cell fusion, what receptor molecules are involved in the cell fusion, and what signaling pathways are necessary for the cell fusion.

MATERIALS AND METHODS

Reagents

Anti-nuclear factor of activated T-cells c1 (NFATc1) (7A6), anti-phospho extracellular signal-regulated kinase (ERK) (E-4), anti-TRAF6 (H-274) and anti-cellular homolog of v-src (c-Src) were purchased from Santa Cruz (Santa Cruz, CA). TNF-α, anti-TNF-α, osteoprotegrin ligand (OPG), and transforming growth factor-β (TGF-β) were from R&D Systems Inc. (Mineapolis, MN). Anti-ERK, anti-p38 MAPK (p38) (5F11), anti-phospho-p38 (28B10), anti-Akt, anti-phospho-Akt, and anti-phospho-c-Jun NH2-terminal kinase (JNK) antibodies were from Cell Signaling Technology (Beverly, MA). RANKL was from Peprotech EC Ltd (London, United Kingdom). LY294002, PD169316, SB203580, SP600125, PP2, U0126, and PD98059 were purchased from Calbiochem Corp. (La Jolla, CA). M-CSF was from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan). RANKL was from Peprotech (Rocky Hill, NJ). cDNA of mouse kidney was from Clontech. Minimal essential medium α modification (α-MEM) and all other reagents including LPS, PGN, IL-1β, and IL-6 were obtained from Sigma (St. Louis, MO).

Cell Culture

The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C and maintained on uncoated plastic dishes of 9 cm in diameter in α-MEM containing 10% (v/v) heat-inactivated fetal calf serum (FCS) with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For subculture, the cells were resuspended with 10 ml of fresh medium by mild pipetting and transferred to a 15 ml conical tube. After sitting for 5 min, the upper 14.5 ml fraction was removed, and aggregated cells were collected from the bottom and seeded into

a fresh dish containing 12 ml of fresh medium. For osteoclastogenesis experiments, the indicated number of cells was seeded on tissue culture plates in the presence or absence of RANKL and chemical reagents.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for more than 60 min. The cells were then treated with 0.2% Triton X-100 in PBS at room temperature for 5 min, followed by rinsing twice with PBS. Finally, the fixed cells were stained with 0.01% naphthol AS-MX phosphate (Sigma) and 0.05% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0) for 15–60 min and then rinsed twice with PBS.

Measurement of TRAP Intensity

Following TRAP staining, the plates were scanned by a transparent light scanner, and the red color image was extracted from the scanned image using the Photoshop (Adobe Systems Inc., San Jose, CA) computer program. The intensity of the red color image was measured using National Institutes of Health (NIH) Image computer program and is represented as TRAP intensity in this paper.

Cell Proliferation Assays

Cell proliferation was measured using a Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Similar to the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide] assay, this kit measures intracellular mitochondrial dehydrogenase activity in living but not in dead cells by forming water-soluble formazan dye with the tetrazolium compound WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Immunoblotting

Cells were seeded at 5×10^6 cells/well on a 24-well plate and kept in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C for 4 h. The cells were stimulated with RANKL or/and other chemical reagents and incubated for the indicated time. After incubation, the cells in the 24-well plate were rinsed twice with ice-cold PBS, followed by addition of 50 μl of sodium dodecyl sulfate (SDS)-sample buffer [1% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, 0.0025% bromophenol blue, 62.5 mM Tris/HCl, and pH 6.8] containing 1 mM sodium orthovanadate (protein tyrosine phosphatase inhibitor), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1μg/ml chymostatin, and 1 μg/ml leupeptin. The whole cell lysate was then treated in ice-cold water by sonication (two 15-s pulses with a 60-s interval) (Bioruptor UDC-200T, Cosmo Bio, Tokyo, Japan). After boiling for 5 min, 4–7 μl of the lysate (20 μg of protein) was subjected to SDS- polyacrylamide gel electrophoresis (PAGE), and the proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 5% skim milk, 25 mM Tris/HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20 (TBST) at room temperature for 1 h, washed twice with TBST for 5 min, and then incubated with an antibody at 2000–4000 × dilution in TBST at 4°C for 16 h. The membrane was washed three times with TBST for 10 min, incubated with a horse radish peroxidase (HRP)-conjugated second antibody at 4000–8000 × dilution in TBST at room temperature for 1 h, washed vigorously five times for 10 min, and subjected to chemiluminescence (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ) to visualize HRP. In some experiments for reprobing, the membrane was stripped of antibody with Reblot Plus Strong (Chemicon, CA) according to the manufacturer's instructions.

Real-Time PCR

Total RNA was prepared using an RNeasy Mini Spin Column (Quiagen) according to the manufacturer's instructions, and the cDNA was reverse-transcribed by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Using an M × 3005PTM Real-time PCR System and Brilliant SYBR Green QPCR Mastermix (Stratagene), the reverse-transcribed cDNA was amplified and quantifed with specific primers according to the manufacturer's instructions. The primer sequences used for amplification were as follows: mouse GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAACAC-3′; NF-ATc1, 5′-TCATCCTGTCCAACACCAAA-3′ and 5′-TCACCCTGGTGTTCTTCCTC-3′; DC-STA-MP, 5′-GGGCACCAGTATTTTCCTGA-3′ and 5′-TGGCAGGATCCAGTAAAA-

GG-3′; and TRAP, 5′-CAGCAGCCAAGGAGGACTAC-3′ and 5′-ACATAGCCCACACCGTTCTC-3′. The relative amounts of each mRNA were normalized by the GAPDH expression.

RESULTS

LPS-Mediated Inhibition in the Initial Step of Osteoclast Differentiation

LPS, a major component of the cell wall of Gram-negative bacteria, has been reported to strongly induce inflammation, which is thought to cause bone destruction. LPS induces bone resorption when administered in vivo; however, it suppresses osteoclast formation from bone marrow macrophages in vitro cell cultures. In order to determine what step of osteoclastogenesis is influenced by LPS, we examined whether LPS affected RANKL-induced osteoclastogenesis when it was present in RAW264.7 cell culture at different time periods after treatment with RANKL. In a previous study, we found that 1–3 μM U0126, a MAPK-ERK kinase (MEK)/ERK inhibitor, accelerated osteoclastogenesis of RAW264.7 cells in the presence of RANKL but that 10 μM of U0126 suppressed osteoclastogenesis [Hotokezaka et al., 2002]. In culture conditions with or without 2 μM U0126, cells were treated with LPS for different periods of 12 h during the total 48-h culture. In both culture conditions, LPS suppressed osteoclastogenesis when added for the first 12 h, and the

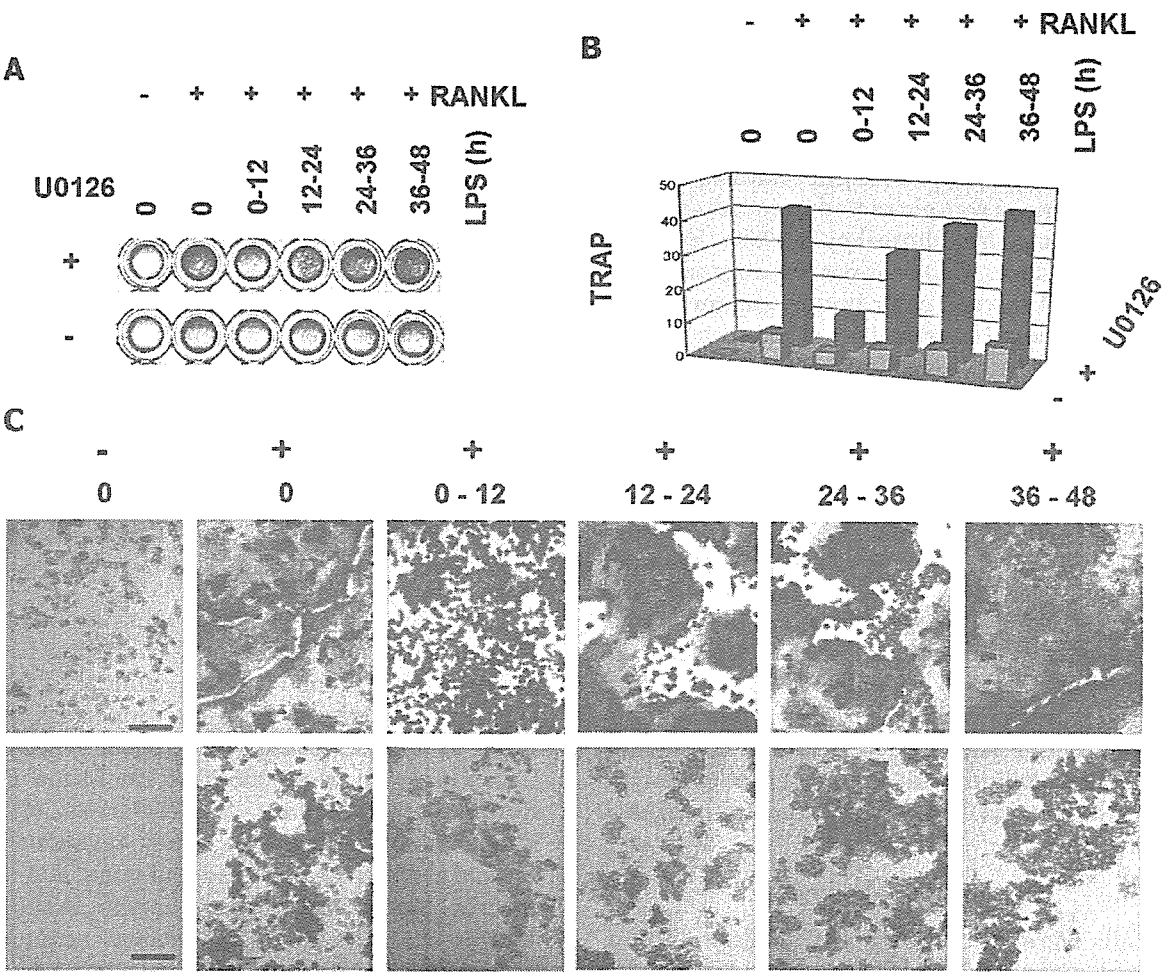


Fig. 1. Effect of LPS on differentiation of RAW264.7 cells into osteoclast-like cells. RAW264.7 cells in 250 μl of α-MEM were cultured in a 96-well tissue culture plate in the presence of 25 ng/ml RANKL with (upper; 16,000 cells) or without (lower; 8,000 cells) 2 μM U0126. The cells were stimulated with 25 ng/ml LPS during the indicated period in the absence of RANKL. TRAP activity was visualized by TRAP staining. **A:** culture plate scanned. **B:** measured TRAP intensity, the mean of three different determinations was plotted. **C:** cell shapes under an optical microscope. Bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

suppression was markedly weaker when LPS was added at a later 12-h period (Fig. 1). Similar to the previous reports [Takami et al., 2002; Zou and Bar-Shavit, 2002; Zou et al., 2002; Hayashi et al., 2003], LPS inhibits osteoclastogenesis only in the early step of differentiation suggests that effects of cytokines or Toll-like receptor (TLR) ligand molecules such as LPS on osteoclastogenesis may vary among steps of differentiation.

Effects of Cytokines or TLR Ligands on the Latest Step of Osteoclastogenesis

In this study, we used an enhanced culture condition in which the cells were cultured in the presence of both RANKL and U0126 since TRAP-positive multinuclear cells were generated in the culture condition within 48 h (Fig. 1). Effects of various cytokines or TLR ligands on the latest step of osteoclastogenesis and fusion of TRAP-positive mononuclear cells were investigated. First, TRAP-positive mononuclear cells were generated from RAW264.7 cells by incubation with RANKL and U0126 for 36 h. The cells were then incubated in culture medium with various cytokines or TLR ligand molecules for 12 h in the absence of RANKL. Similar to the previous reports [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002], LPS, PGN, and TNF-α induced cell fusion as well as RANKL (Fig. 2). Although M-CSF failed to induce cell fusion, it increased the number of TRAP-positive mononuclear cells. IL-1β had no ability to induce cell fusion in this condition. In addition, we examined IL-6 (0.1–50 ng/ml), TGF-β (0.1–50 ng/ml), phorbol myristic acetate (PMA; 0.1–10 μM), concanavalin A (0.1–10 μg/ml), amphotericin B (AmB; 0.1–10 μg/ml), and oxydized low-density lipoprotein (LDL; 0.1–10 μg/ml). None of them induced cell fusion (data not shown) although PMA, AmB, and oxydized LDL are known as inducers of proinflammatory cytokines in macrophages [Stuart and Hamilton, 1980; Cleary et al., 1992; Pollaud-Cherion et al., 1998]. These results suggested that cell fusion in the latest step of osteoclastogenesis might be induced by treatment with RANKL, TNF-α, LPS, and PGN but not by treatment with others.

It is known that IL-1β-induced cell signaling is similar to that of LPS and PGN with respect to involvement of MyD88, an adaptor molecule of IL1R family members [Subramaniam et al., 2004]. We investigated whether the IL-1β receptor IL1RI was expressed in RAW264.7 cells. Real-time RT-PCR analysis revealed that IL1RI was not expressed in RAW264.7 cells, whereas RANK, c-fms, TLR2, and TLR4, receptors for RANKL, M-CSF, PGN, and LPS, respectively, were expressed in the cells (Fig. 2F). Absence of the IL-1β receptor in the cell line may account for the disability of IL-1β to induce cell fusion.

Ability of LPS and PGN to Induce TNF-α-Independent Cell Fusion

LPS and PGN are known to induce TNF-α in RAW264.7 and other cells [Remick et al., 1988; Gupta et al., 1995]. Furthermore, TNF-α was reported to induce osteoclastogenesis in macrophages exposed to permissive levels of RANKL [Lam et al., 2000]. In order to determine whether cytokines or TLR ligands directly or indirectly induced cell fusion, OPG—a neutralizing antibody against TNF-α (anti-TNF-α), and polymyxin B were used to block RANKL, TNF-α, and LPS, respectively (Fig. 3). OPG, anti-TNF-α, and polymyxin B (each 0.3 μg/ml) specifically inhibited the osteoclast cell fusion induced by RANKL, TNF-α, and LPS, respectively. Anti-TNF-α strongly inhibited TNF-α-induced cell fusion and also had some inhibitory effects at 1 μg/ml on LPS- and PGN-induced cell fusion (Fig. 3A). Anti-TNF-α (1 μg/ml) completely suppressed cell fusion induced by TNF-α (10 ng/ml) and the secretion of TNF-α induced by LPS or PGN was less than 1.2 ng/ml, suggesting that LPS and PGN had the ability to induce TNF-α-independent cell fusion (Fig. 3B).

Immunoblot Analysis During the Cell Fusion Process

An intracellular domain of RANK interacts with TNF receptor-associated factor (TRAF) 2 and TRAF 6 [Galibert et al., 1998; Wong et al., 1998, 1999; Darnay et al., 1999; Kim et al., 1999], which appear to be involved in the activation of downstream signaling molecules such as nuclear factor kappa B (NF-κB), Src [Horne et al., 1992; Lowe et al., 1993], phosphatidylinositol 3 (PI3), protein kinase B (PKB/Akt), and mitogen-activated protein kinases (MAPKs) including p38, ERK, and JNK [Xia et al., 1995; Wong et al., 1998, 1999; Matsumoto et al., 2000; Chang and Karin, 2001; Lee et al., 2002; Wei et al., 2002]. In order to study cell signaling during the cell fusion process, we determined the amounts of several

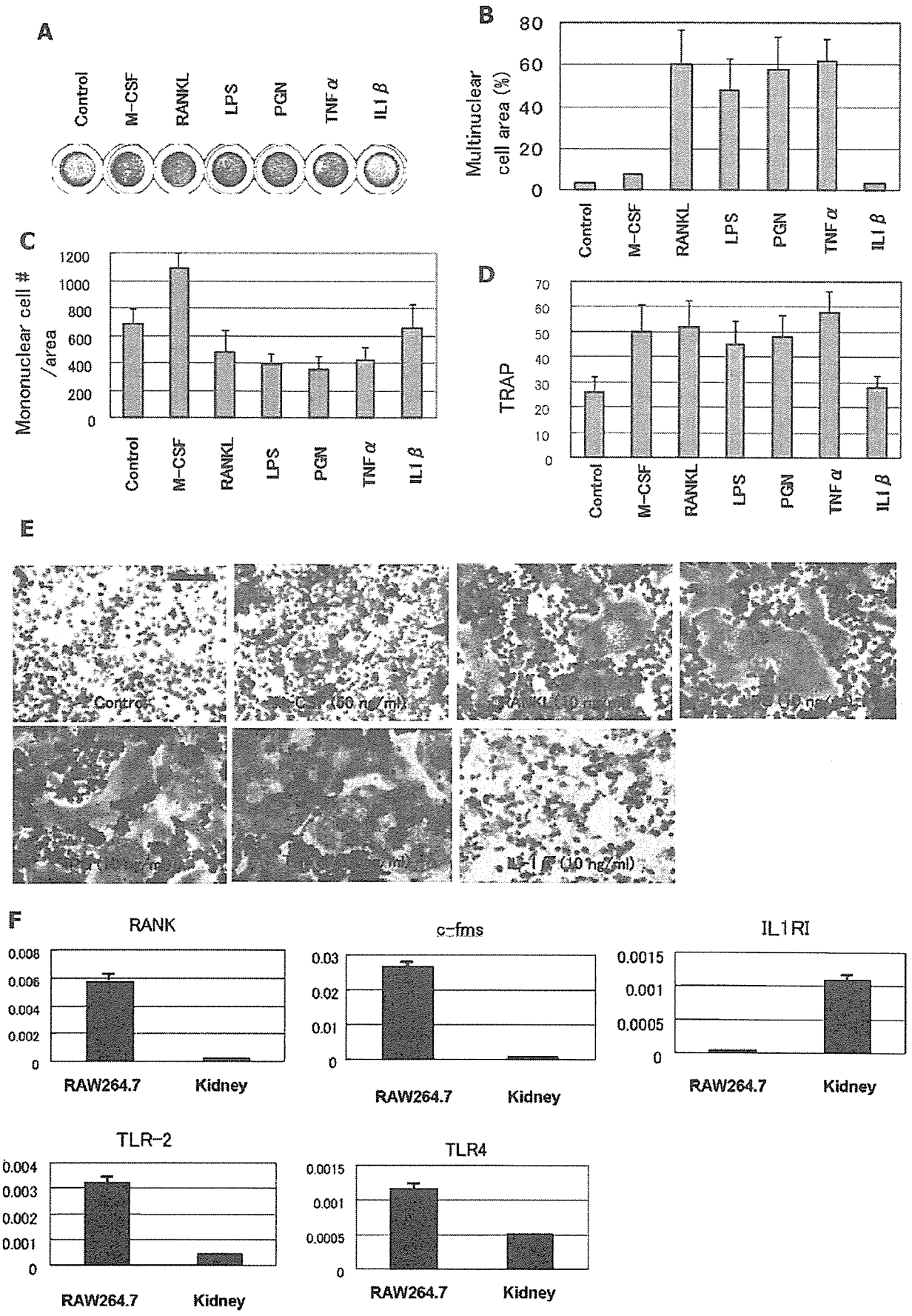

A
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
B
Multinuclear cell area (%)
80
60
40
20
0
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
C
Mononuclear cell # /area
1200
1000
800
600
400
200
0
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
D
TRAP
70
60
50
40
30
20
10
0
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
E
Control
IL-1 β (10 ng/ml)
F
RANK
0.008
0.006
0.004
0.002
0
RAW264.7
Kidney
c-fms
0.03
0.02
0.01
0
RAW264.7
Kidney
IL1RI
0.0015
0.001
0.0005
0
RAW264.7
Kidney
TLR-2
0.004
0.003
0.002
0.001
0
RAW264.7
Kidney
TLR4
0.0015
0.001
0.0005
0
RAW264.7
Kidney

Fig. 2.

transcription factors and phosphorylation of various signaling molecules by immunoblot analysis. In our culture system, TRAP-positive mononuclear cells began to fuse at 8 h, and they were fully fused at 16 h after stimulation. As shown in Figure 2, RANKL, TNF-α, and LPS equally induced fusion of TRAP-positive mononuclear cells, but M-CSF did not. Phosphorylation of Akt, Src, ERK, JNK, and p38 was observed at 480 or 960 min in cells stimulated with RANKL, TNF-α, and LPS (Fig. 4). p38 showed the most prominent phosphorylation by stimulation with RANKL, TNF-α, and LPS throughout the incubation period. Continuous phosphorylation of Akt was observed in cells treated with M-CSF, in contrast to the transient phosphorylation in cells treated with RANKL, TNF-α, or LPS at 8 and 16 h. The continuous or transient phosphorylation of Akt may be involved in cell fate, growth, or fusion. Since phosphorylation of signaling molecules is thought to be involved in the subsequent cellular responses, we determined the importance of the signaling molecules in the cell fusion process by using specific inhibitory compounds. As expected, LY294002 (PI3K inhibitor), PP2 (Src inhibitor), U0126 (ERK inhibitor), and SP600125 (JNK inhibitor) efficiently inhibited the RANKL-induced cell fusion, but SB203580 (p38 inhibitor) had no inhibitory effect (Fig. 5). Another p38 inhibitor, PD169316, also had no effect (data not shown). In order to study whether p38 is involved in an activity of osteoclast, resorption pit formation was measured. However, resorption pit formation induced by RANKL, TNF-α, or LPS was not inhibited by the p38 inhibitor SB203580. The role of p38 during the cell fusion process in osteoclastogenesis remains unclear.

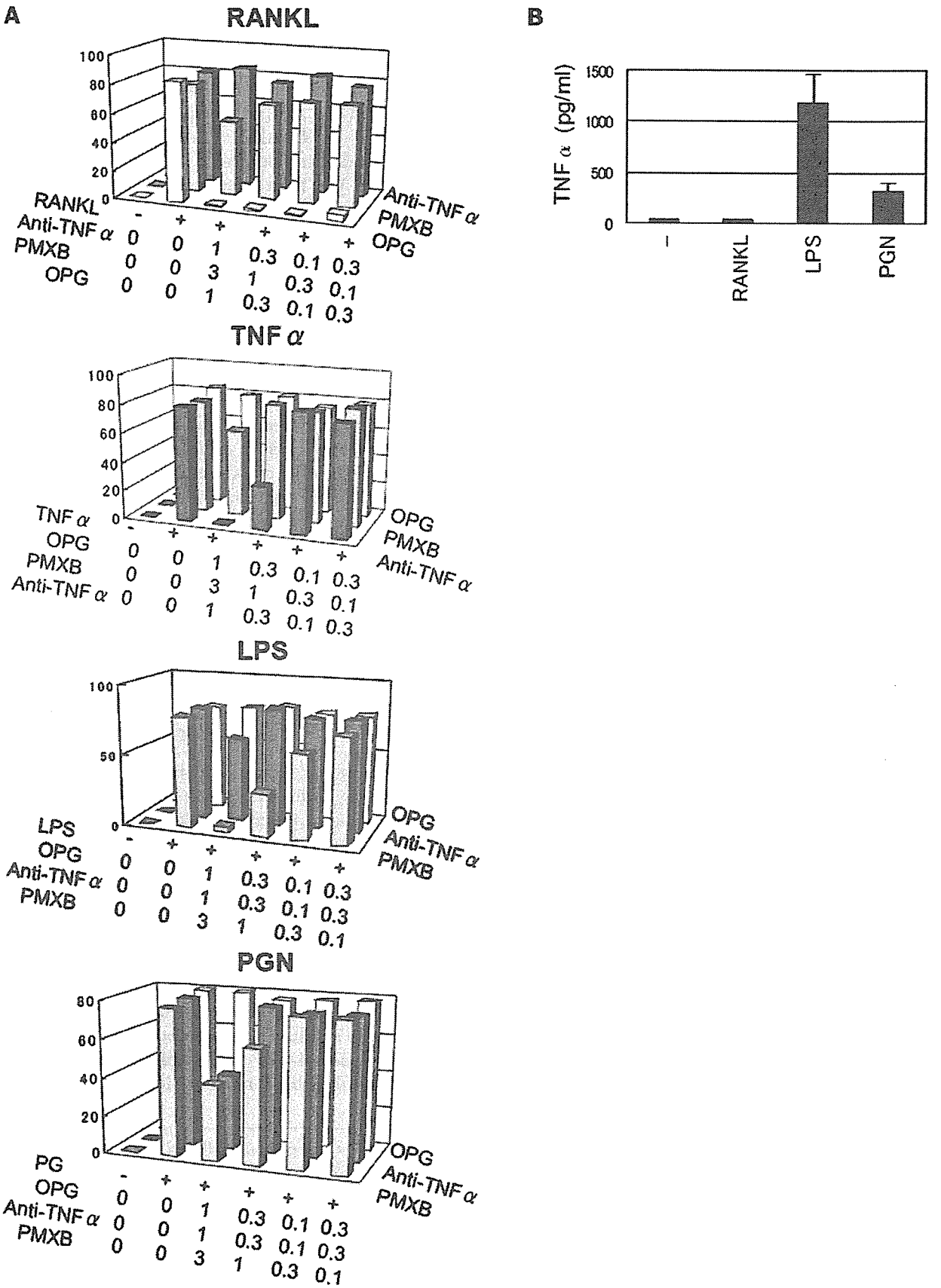
Real-Time PCR Analysis of Expression of NFATc1, DC-STAMP, and TRAP

It was recently found that DC-STAMP participates in the cell fusion of osteoclasts [Kukita et al., 2004; Yagi et al., 2005]. First, we determined levels of mRNA of NFATc1, DC-STAMP, and TRAP in cells treated with RANKL at several time points. The mRNA level of NAFTc1 increased at an earlier time point than did the mRNA levels of DC-STAMP and TRAP. DC-STAMP and TRAP mRNAs increased immediately before cell fusion (Fig. 6). We then investigated the expression of NFATc1, DC-STAMP, and TRAP in the latest step of osteoclastogenesis. RAW264.7 cells that had been treated with RANKL for 36 h were then treated with RANKL, M-CSF, LPS, or TNF-α for 6 h. At this time point, the cells began to fuse. Then the expression of NFATc1, DC-STAMP, and TRAP in the cells was determined by real-time PCR analysis. Expression of DC-STAMP in RANKL- or LPS-treated cells was not significantly different from that in M-CSF-treated cells or non-treated cells. The M-CSF-treated cells and non-treated cells showed no cell fusion, whereas RANKL-, LPS-, and TNF-α-treated cells showed cell fusion. We could not find any significant difference in expression of NFATc1, DC-STAMP, and TRAP between the fused cells and non-fused cells. These results suggest that an essential factor other than DC-STAMP may determine whether TRAP-positive mononuclear cells are fused or not.

DISCUSSION

In an in vitro culture system using osteoclast precursor cells purified from various tissues such as bone marrow, spleen, and liver, it is difficult to avoid contamination with other cell lineages such as T-cells and mesenchymal stromal cells. In this study, we used RAW264.7—a murine macrophage cell line that can differentiate into osteoclast-like cells in the presence of RANKL [Hsu et al., 1999]. There are some characteristic differences between RAW264.7 cells and macrophages; for example, RAW264.7 cells do not respond to IL-1β, and the osteoclast-like cells differentiated from RAW264.7 cells form smaller and shallower

Fig. 2. Effects of factors on cell fusion of osteoclasts. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS and indicated factors. Concentrations used were 50 ng/ml for M-CSF, 10 ng/ml RANKL, 10 ng/ml LPS, 100 ng/ml PGN, 10 ng/ml TNF-α, and 50 ng/ml IL-1β. **A:** scanned culture plate. **B:** area % of multinuclear cells. **C:** number of mononuclear cells. **D:** TRAP intensity. **E:** cell shapes under an optical microscope. **F:** mRNA quantified by real-time RT-PCR. Total RNAs were prepared from RAW264.7 cells treated with RANKL and U0126 for 36 h and kidney cells of mice, and they were subjected to real-time RT-PCR using primers for RANK, c-fms, TLR4, and IL-1R type 1. Bar indicates 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


A
RANKL
RANKL
Anti-TNF α
PMXB
OPG
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PMXB
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OPG
PMXB
Anti-TNF α
LPS
LPS
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PMXB
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TNF α (pg/ml)
1500
1000
500
0
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RANKL
LPS
PGN

Fig. 3.

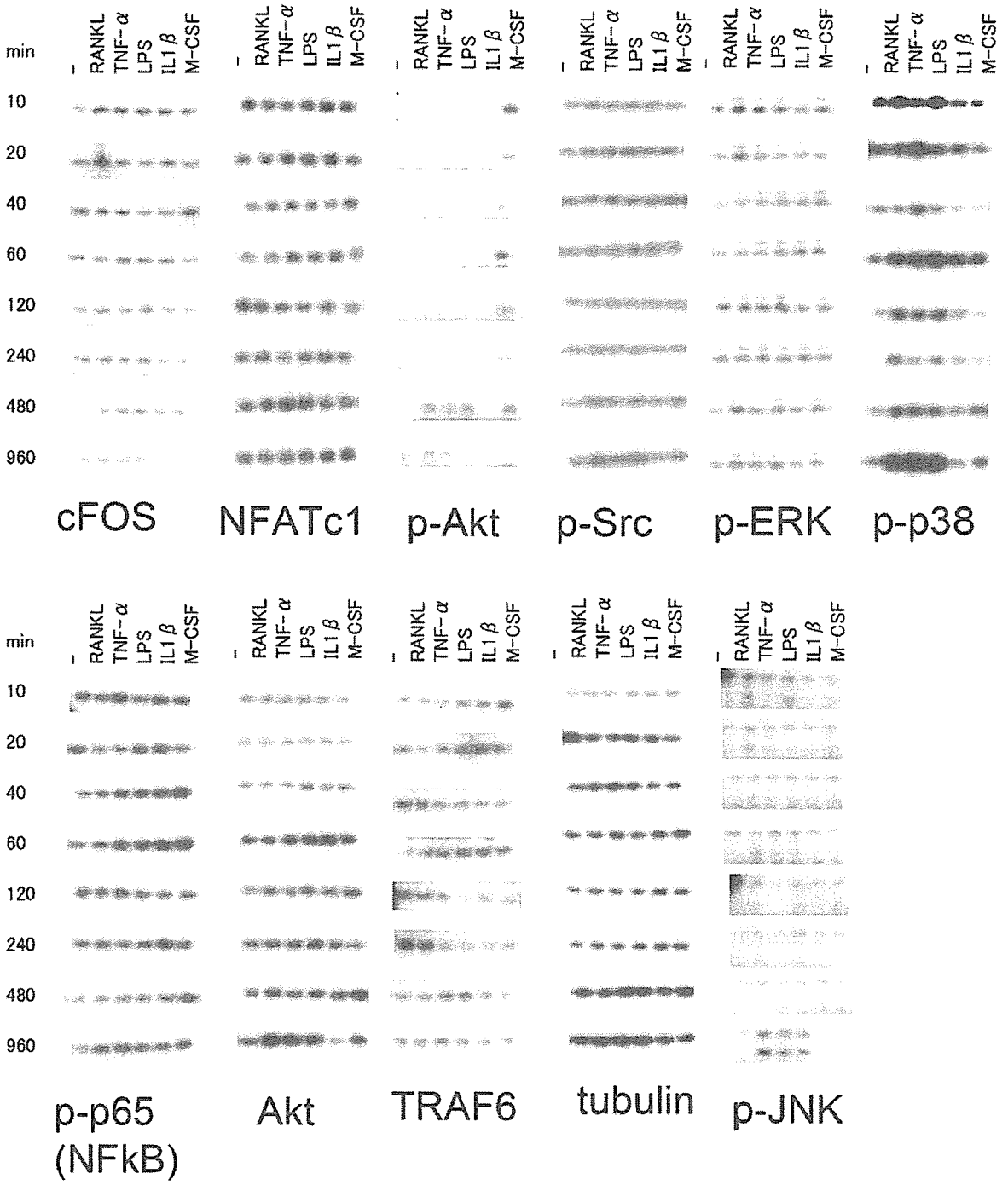
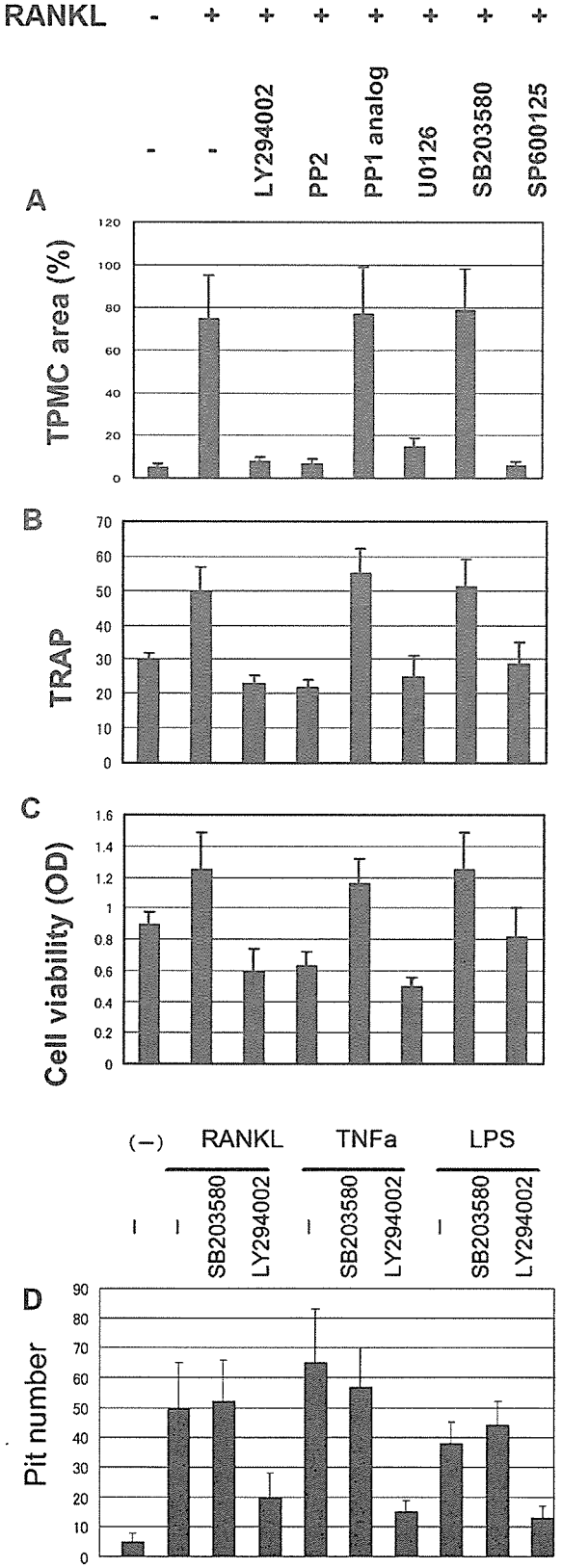


Fig. 4. Western blotting analysis during cell fusion process. The cells were treated with 25 ng/ml RANKL, 25 ng/ml TNF-α, 25 ng/ml LPS, or 100 ng/ml PGN for the indicated time and then subjected to Western blotting analysis probing with antibodies against c-Fos, NFATc1, phosphorylated (P-) Akt, P-Src, P-ERK, P-p38, P-p65 (RelA, 65 kD subunit of NF-κB), Akt, TRAF6, tubulin, and P-JNK.

Fig. 3. RANKL, TNF-α, LPS, and PGN induced cell fusion specifically through their receptors. RAW264.7 cells (1.6 × 105) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, indicated stimulation factors (RANKL, TNF-α, LPS, and PGN), and blocking reagents (OPG, PMXB, and Anti-TNF-α; indicated number μg/ml were used). The fused cell area was measured as percent of total plate area. Concentrations used were 10 ng/ml RANKL, 10 ng/ml TNF-α, 10 ng/ml LPS, and 100 ng/ml PGN (**A**). Amounts of TNF-α in the culture media stimulated with RANKL, LPS, and PGN were measured by ELISA (**B**). The mean of three different determinations was plotted.



resorption pits on dentin slices than do osteoclasts derived from bone marrow macrophages. However, the cell line is useful to analyze the detailed mechanisms of osteoclast differentiation.

In this study, we focused on the cell fusion step of osteoclastogenesis. Several investigators reported the later stage of osteoclastogenesis [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002]. However, this process is not characterized well since it is not easy to isolate cells at each step during the process of osteoclast differentiation. In this study, we used an enhanced culture condition for osteoclastogenesis of RAW264.7 cells. By culturing the cell line in the presence of RANKL and the MEK/ERK inhibitor U0126, the culture period for osteoclastogenesis was shortened to 48 h, less than half of the standard culture period, and almost all the cells are fused to one another at the end of the culture period. Therefore, the TRAP-positive mononuclear cells (preosteoclasts) prepared shortly before cell fusion in the enhanced culture condition appear to be almost homogeneous in terms of differentiation.

In the present study, LPS suppressed RANKL-induced osteoclast formation at the initial step; in contrast, at the latest step it induced cell fusion without RANKL, resulting in osteoclast formation. The suppressive effect of LPS on osteoclastogenesis, which was observed in the present study when LPS and RANKL were simultaneously added at the initial step, has previously been reported for bone marrow macrophages by Takami et al. [2002]. Moreover, it has been reported that LPS promotes the survival of mature osteoclasts via TLR4 [Itoh et al., 2003]. Taken together, the results indicate that the effects of LPS on osteoclastogenesis vary among steps of osteoclast differentiation. In this context, it is notable

Fig. 5. Effects of chemical inhibitors of signal transduction on cell fusion and pit formation. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 µl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 µM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 µl of α-MEM containing 10% FCS, 25 ng/ml RANKL, TNF-α, and 10 of chemical inhibitors, and the cells were cultured for a further 12 h. The fused cell area was measured as percent of total plate area (**A**). The TRAP intensity and cell viability were also measured (**B** and **C**). **D:** Pit formation was measured on a plate coated with calcium phosphate (BD BioCoat Osteologic Bone Cell Culture System, Nippon BD, Tokyo, Japan) stimulated with 25 ng/ml RANKL, TNF-α, or LPS in the presence or absence of 10 µM SB203580 or LY294002.

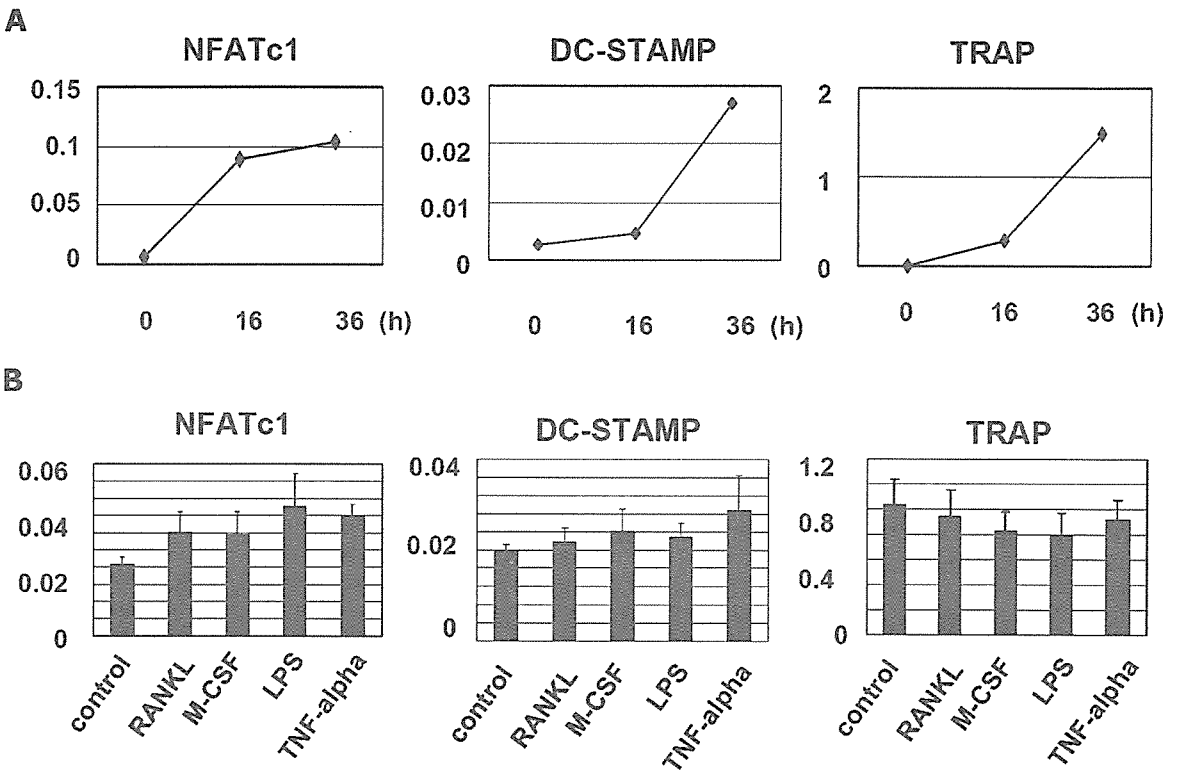


Fig. 6. Real-time PCR for NFATc1, DC-STAMP, and TRAP. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate (**A**). Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, or TNF-α, and the cells were cultured for further 6 h (**B**), followed by RNA preparation and real-time RT-PCR. Each value was normalized by that of GAPDH. The mean of three different determinations was plotted.

that LPS induces bone resorption when injected into bone surfaces of mice [Umezu et al., 1989]. Since osteoclast precursor cells of various steps of differentiation exist together in an in vivo condition, osteoclast precursor cells of the pre-fusion step around bones might differentiate into mature osteoclasts in response to LPS, resulting in bone resorption.

Cell fusion is seen in muscle, nerves, bone in their development, and in the liver in its repair and regeneration [Ogle et al., 2005]. TRAP-positive multinuclear osteoclasts appear in bone. TRAP-positive mononuclear cells fuse to one another because the cell fusion increases cell size and enables the cells to resorb bone to a larger extent [Vignery, 2005]. This explanation is supported by the finding that a DC-STAMP knockout mouse in which TRAP-positive mononuclear cells do not fuse to one another shows an increase in bone density due to a decrease in bone resorption by osteoclasts [Yagi et al., 2005]. Osteoclasts seem to have the same origin in cell lineage as that of macrophages. Macrophages sometimes fuse with one another during infection and tissue repair. The resulting multinucleated macrophages effectively phagocytise pathogens and repair tissues. The mechanism by which macrophages repair tissues seems to be similar to bone resorption of osteoclasts. In this study, inflammatory factors such as TNF-α, LPS, and PGN induced cell fusion, which may be one of mechanisms to repair the inflammatory circumstance of bone, although the inflammation results in an unfavorable bone loss.

Cell fusion-inducing factors include several groups. One is known as fusogens that can directly induce cell fusion. And other groups that include receptors, signaling proteins, transcription factors, and proteins organizing cytoskeleton and membrane, indirectly induce cell fusion [Ogle et al., 2005]. Although EFF-1 of *Caenorhabditis elegans* might be the only one fusogen that have been found in higher eukaryotes so far as we know [Kontani and Rothman, 2005], no fusogens have been found in mammalian cells. Some molecules such as meltrin-α [Harris et al., 1997; Inoue et al.,

1998], CD47, MFR (also reported as SIRP, SHPS-1, BIT, and MyD-1) [Vignery, 2005], and DC-STAMP have been suggested as cell fusion-inducing factors in osteoclasts. In the bones of mice lacking DC-STAMP multinuclear osteoclasts were completely absent, although development of mononuclear osteoclasts was normal. The DC-STAMP-deficient mice suffer from mild osteopetrosis probably because mononuclear osteoclasts can still resorb bones [Yagi et al., 2005]. The expression of DC-STAMP was not significantly different between fused and non-fused cells in this study, suggesting the presence of other cell fusion-inducing factor(s) that directly function at the latest stage of osteoclastogenesis. Further study is needed to understand the molecular mechanisms of cell fusion of osteoclasts.

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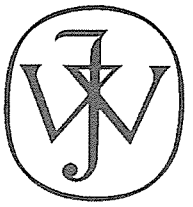

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