Bacterial infection sometimes impairs bone metabolism. In this study, we infected the osteoblastic cell line MC3T3-E1 with Mycobacterium bovis bacillus Calmette-Guérin (BCG) and identified genes that were up-regulated in the BCG-infected cells by the suppression subtractive hybridization method. A gene encoding 4-1BB (CD137), a member of the tumor necrosis factor-α receptor family, was found to be one of the up-regulated genes. Up-regulation of 4-1BB was also observed by infection with Escherichia coli, Salmonella typhimurium, and Staphylococcus aureus, and by treatment with lipopolysaccharides and heat-killed BCG. Bone marrow cells and the macrophage-like cell lines J774 and RAW264.7 were found to express 4-1BB ligand (4-1BBL). Recombinant 4-1BB (r4-1BB) that was immobilized on culture plates strongly inhibited macrophage colony stimulating factor (M-CSF)/receptor activator of nuclear factor-κB ligand (RANKL)-induced in vitro osteoclast formation from bone marrow cells. Anti-4-1BBL antibody also inhibited osteoclast formation to a lesser extent, indicating involvement of reverse signaling through 4-1BBL during inhibition of osteoclast formation. A casein kinase I (CKI) inhibitor markedly suppressed the inhibitory effect of r4-1BB on M-CSF/ RANKL-induced osteoclast formation, suggesting that CKI might be involved in 4-1BB/4-1BBL reverse signaling. r4-1BB showed no effects on M-CSF- or RANKL-induced phosphorylation of I-κB, ERK1/2, p38, or JNK, whereas RANKL-induced phosphorylation of Akt, a downstream target of phosphatidylinositol 3-kinase (PI3K), was completely abolished by r4-1BB, suggesting that 4-1BB/4-1BBL reverse signaling may interfere with PI3K/Akt pathway. r4-1BB also abolished RANKL-mediated induction of nuclear factor of activated T cells-2. This study may elucidate a novel role of 4-1BB in cell metabolism, especially osteoclastogenesis.

Tuberculosis is an ancient disease but still ranks as one of the foremost killers of the 21st century. About one-third of the world’s population is infected with Mycobacterium tuberculosis and more than two million people die annually from tuberculosis (1). Most tuberculosis cases are pulmonary, but the microorganism sometimes invades the central nervous system, the lymphatic system, and the musculoskeletal system, or is sometimes disseminated throughout the whole body. Mycobacterium infection in bones and joints presents ~10% of the extra-pulmonary cases (2). Vertebral osteomyelitis and osteitis also occur as a complication of vaccination with bacillus Calmette-Guérin (BCG) or intravesical use of BCG (3, 4). Osteomyelitis and osteitis caused by M. tuberculosis or BCG infection sometimes lead to vertebral caries in some patients as a result of increased bone resorption.

Bone is maintained by dynamic equilibrium between osteoblasts and osteoclasts in bone marrow cells. M. tuberculosis and BCG infection of bone alters this equilibrium, resulting in the loss of extracellular matrix and collapse of bone, especially vertebrae. Whether this bone resorption is attributable to direct effects of the bacteria on bone cells or infection-induced activation of inflammatory cells has not been clarified. However, a few studies have shown that mycobacterial antigens interfere with bone metabolism. Wax D, a mycobacterial cell wall peptidoglycan fragment-arabinogalactan-mycolic acid complex, induced reactive bone formation accompanied with osteomyelitis in Buffalo rats (5). Heat shock protein 10 of M. tuberculosis stimulates bone resorption in bone explanting cultures and induces osteoclast recruitment but inhibits proliferation of an osteoblast bone-forming cell line (6). The secreted protein MPB70 of BCG has significant homology with four repeat domains of osteoblast-specific factor 2/peristin (7). Epidemiological study suggests that MPB70-overproducing strains of BCG seem to be associated with an increased incidence of osteitis after BCG vaccination of neonates (7).

Osteoblasts express various enzymatic markers such as alkaline phosphatase (ALP) and produce collagenous and noncollagenous proteins.
lagenous bone matrix proteins, including osteocalcin (OCN) and osteopontin (OPN) (8). Osteoblasts also express receptors for various hormones, including parathyroid hormone (9, 10), 1α,25-dihydroxyvitamin D3 (11), estrogen (12, 13), and glucocorticoids (14, 15). In addition, osteoblasts have the ability to produce cytokines and augment an inflammatory response. Some cytokines produced by osteoblasts modulate proliferation and differentiation of osteoclasts. Osteoclasts are multinuclear cells with bone-resorbing activity. They play a crucial role in bone modeling (see Refs. 16 and 17 for review). Two molecules, macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANKL), are essential and sufficient for differentiation to osteoclasts (18–20). M-CSF, which is indispensable for macrophage maturation, binds to its receptor in early osteoclast precursors, thereby providing signals required for their survival, proliferation, and differentiation to osteoclasts (21, 22). RANKL, belonging to the tumor necrosis factor-α (TNF-α) family, binds to their receptor, receptor activator of NF-κB (RANK) and activates several intracellular signaling pathways, leading to osteoclastic differentiation and activation (18).

In the present study, to clarify the effect of *Mycobacterium* infection on bone metabolism, genes up-regulated in BCG-infected osteoblastic cells were screened by the suppression subtractive hybridization (SSH) technique (23). 4-1BB, a costimulatory molecule of the TNF receptor (TNFR) family expressed on activated T cells, was identified as one of the up-regulated genes. Up-regulation of 4-1BB was not limited to BCG-infected osteoblastic cells but observed in osteoblastic cells by infection with *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*, and by treatment with lipopolysaccharides (LPS) and heat-killed BCG. We also report that 4-1BB has the ability to suppress M-CSF/RANKL-induced *in vitro* osteoclastogenesis.

### Experimental Procedures

**Antibodies and Chemicals—**Recombinant human soluble RANKL was purchased from PeproTech EC Ltd. (London, England), and recombinant human M-CSF (Leukoprol) was purchased from Yoshitomi Pharmaceutical Co. (Osaka, Japan). An inhibitor of caspase 1 (C1), C1, H2O, S, C1, was purchased from Seikagaku Co. (Tokyo, Japan). *Escherichia coli* LPS was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho-IκB, phosho-MAPK, phospho-p38, phospho-JNK, Akt, and Phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). Anti-TRAF6 and nuclear factor of activated T cells-2 (NFAT2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-fos was purchased from Oncogene Research Products (Cambridge, MA). Anti-phosphotyrosine was purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

**Mouse anti-4-1BB** was purchased from Calbiochem (San Diego, CA) and monoclonal antibody (TKS-1) against 4-1BB ligand (4-1BBL) was from Molecular Innovations (Beverly, MA). Anti-phosphotyrosine was purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-IκB, phospho-MAPK, phospho-p38, phospho-JNK, Akt, and Phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). Anti-TRAF6 and nuclear factor of activated T cells-2 (NFAT2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-fos was purchased from Oncogene Research Products (Cambridge, MA). Anti-phosphotyrosine was purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell Cultures—**Mouse osteoblasts were obtained from newborn mouse calvaria (24). MC3T3-E1 and RAW264.7, NIH3T3 and J774 cells were maintained in α-MEM, Dulbecco’s modified Eagle’s medium, and RPMI 1640 (Invitrogen, Carlsbad, CA), respectively, containing 10% fetal bovine serum (FBS; Invitrogen) and 100 units/ml of penicillin-streptomycin (G) at 37 °C in a humidified atmosphere of 5% CO2 in air. Bone marrow cells were obtained from tibias and femurs of 4-week-old male *C57Bl/6J*, and seeded in a new dish. Adherent cells were removed by vigorously washing with PBS and adherent cells were harvested by pipetting with 0.02% EDTA in PBS and seeded in a new dish. Adherent cells were further cultured with osteoclast differentiation medium (α-MEM with 10% FBS containing 10 ng/ml M-CSF and 20 ng/ml RANKL) for 3 days. The purity of TRAP-positive cells in this preparation was >95%. BMM cells were cultured 12 h to 6 days in various medium conditions indicated.

**Bacterial Infection of Osteoblastic Cells—**BCG was grown in sterile Middlebrook 7H9 medium (BD Biosciences, Microbiology Systems, Cockeysville, MD) supplemented with albumin-dextrose-catalase (BD Biosciences) and 0.05% Tween 80 at 37 °C with shaking (160 rpm). Prior to the assays, bacteria cultured up to an A600 of 0.6 were washed with Dulbecco’s phosphate-buffered saline (DPBS) and passed through an 18-gauge needle 10 times. The bacterial cell suspension was placed in a 50-ml plastic tube and agitated for 2 min. MC3T3-E1 cells were mixed with BCG suspension in cell-bacteria ratios of 1:100. After 12 h, the infected cells were rinsed three times with DPBS, followed by

![Fig. 1. RT-PCR analysis.](http://www.jbc.org/)}
addition of fresh α-MEM containing 50 μg/ml gentamicin. After 12 h, the gentamicin-containing medium was replaced by gentamicin-free α-MEM. The period of infection varied from 12 h to 6 days and was stipulated for each experiment as described under "Results." Colony forming units were determined as follows. The infected cells were lysed in 1 ml of DPBS containing 0.5% Nonidet P-40, and the resulting lysates were passed 10 times through an 18-gauge needle. The lysates were diluted in Middlebrook 7H9 broth, and a 100-μl aliquot of the dilutions was spread in duplicate onto Middlebrook 7H10 agar plates and incubated for 21 days.

MC3T3-E1 cells were also infected with E. coli strain MC1400 in cell-bacteria ratios of 1:50. After 2 h, the infected cells were rinsed and incubated in fresh α-MEM containing 50 μg/ml gentamicin. After 22 h, the infected cells were rinsed three times with DPBS, followed by addition of fresh α-MEM containing 50 μg/ml gentamicin. After 24 h, the gentamicin-containing medium was replaced by gentamicin-free α-MEM.

Results. Probes were chemically labeled using the ECL direct (Amersham Biosciences). The membrane was hybridized with RsaI-digested DR4-harboring bone marrow (Clontech) and dot-blotted onto a nylon membrane (Hybond-N) from the recombinant plasmid DNA using NP-1 and NP-2 primers (Clontech) and synthesized from mRNAs of MC3T3-E1 cells infected with BCG for 6 days, respectively. The amplified DNA fragment was cloned into the NdeI-XhoI site of pET-22b (Novagen), allowing expression of the extracellular form of 4-1BB protein fused to the poly histidine tag at the C terminus. Recombinant 4-1BB (r4-1BB) was expressed in E. coli BL21 harboring the plasmid by treatment with isopropylthio-D-β-D-thiogalactopyranoside. r4-1BB that was formed into insoluble inclusion bodies was solubilized in 20 mM sodium phosphate buffer supplemented with 0.5 mM NaCl and 6 mM guanidine hydrochloride. r4-1BB were purified by Ni2+-chelate affinity chromatography over a ProBond™ resin (Invitrogen) column under denaturing conditions as described in the manufacturer's instructions. The peak fractions were then dialyzed against 10 mM Tris, pH 8.0, containing 0.1% Triton X-100 to remove urea. Removal of lipopolysaccharide (LPS) was accomplished by fractionation with a Trition X-114 (Sigma) column (29). LPS concentrations in r4-1BB samples used in this study were less than 1 pg/μg of protein. To immobilize r4-1BB on culture plates, the 96-well plate (IWAKI glass, Tokyo, Japan) was incubated with r4-1BB at 37°C for 1 h and washed with PBS. Plates were blocked by α-MEM containing 10% FBS at 37°C for 1 h and washed with PBS.

TRAP Staining—At 3 or 5 days of culture, cells were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. After treatment with 0.2% Triton X-100 in PBS for 5 min at room temperature, the cells were stained for tartrate-resistant acid phosphatase (TRAP) as described previously (30). TRAP-positive mono- or multinuclear cells were counted under a light microscope. Cells containing three or more nuclei were considered a multinucleated cell.

Analysis of Cell Proliferation—G-10 column-eluted bone marrow cells were cultured at 6.5 × 10^5 cells/ml in 96-well plates with or without immobilized r4-1BB. Cells were cultured in α-MEM with 10% FBS in the presence of 10 ng/ml M-CSF. Medium was changed to remove non-adherent cells after 4 days. CCK-7 was used at the concentration of 20 μM during incubation when indicated. For determination of cell viability, adherent cells were stained with trypan blue and counted under a light microscope. Alternatively, viability of adherent cells was determined by the WST-1 quantitative colorimetric assay for cell survival (Cell counting kit; Dojindo Laboratory, Kumamoto, Japan).

Reverse Transcript-PCR—Total RNA was extracted from mammalian cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and poly(A)+ mRNA was affinity-purified from the total RNA with an oligo(dT) cellulose column (Takara Biomedicals, Kyoto, Japan). SSH was performed using the PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol. Teste cDNA was synthesized from mRNAs of MC3T3-E1 cells infected with BCG for 6 days, and driver cDNA was from mRNAs of uninfected MC3T3-E1 cells. At 3 or 5 days of culture, cells were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. After treatment with 0.2% Triton X-100 in PBS for 5 min at room temperature, the cells were stained for tartrate-resistant acid phosphatase (TRAP) as described previously (30). TRAP-positive mono- or multinuclear cells were counted under a light microscope. Cells containing three or more nuclei were considered a multinucleated cell.

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Immunoblot Analysis—Mammalian cells were washed three times with PBS containing 1 mM Na2VO4, and solubilized in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.5% Nonidet P-40, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin). Lysed cells were centrifuged at 12,000 × g for 30 min, and the protein concentration of each sample was determined by the Lowry method (31). Proteins in the lysates were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk and 0.2% Tween 20 in Tris-buffered saline (TBS) at 4°C overnight, the membranes were incubated with anti-4-1BB, anti-β-

| Name                                      | Accession ID | Redundancy in screening |
|-------------------------------------------|--------------|-------------------------|
| Serum amyloid A                           | NM_011315    | 7                       |
| Haptoglobin                               | NM_017370    | 3                       |
| Complement component C3                   | XM_125743    | 12                      |
| Factor heavy chain                         | BE_321514    | 1                       |
| Coagulation factor XIII                    | BC011073     | 1                       |
| Monocyte chemotactic protein-3 (MCP-3)    | S71251       | 1                       |
| LPS-induced CXC chemokine (LIX)           | AF349465     | 6                       |
| 24p3                                      | S82469       | 6                       |
| Nuclear body-associated kinase 1b (NBAK1) | AF170302     | 1                       |
| 4-1BB/CD137                               | AL670143     | 1                       |

TABLE I

BCG infection-up-regulated gene products identified from subtraction, RT-PCR, and sequence analysis

4-1BB Induction in Osteoblasts and Osteoclastogenesis

Fig. 2. Induction of 4-1BB protein in osteoblastic cells infected with BCG. Proteins in BCG-infected or uninfected MC3T3-E1 cells were separated by SDS-PAGE, transferred to a PVDF membrane, and immunostained with anti-4-1BB or anti-β-actin antibody.
actin, anti-phospho-IκB, anti-phospho-MAPK, anti-phospho-p38, anti-phospho-JNK, or anti-phosphotyrosine antibodies in TBS containing 1% bovine serum albumin for 1 hour at room temperature. Unless otherwise stated, antibodies were used at a dilution of 1:2000 to 1:5000. Anti-phospho-Akt was used at a dilution of 1:500. Anti-c-fos, TRAF6, and NFAT2 were used at a dilution of 1:200. The membranes were washed five times with TBS containing 0.2% Tween 20 (TBST) and then incubated with secondary antibodies at a dilution of 1:5000 in TBS with 1% bovine serum albumin for 1 hour at room temperature. The membranes were washed five times with TBST, and signals were detected using an ECL plus kit (Amersham Biosciences).

Statistical Analysis—Differences between data were analyzed with the Student's t test.

RESULTS

Expression of Osteoblastic Markers and Analysis of Suppression Subtraction Library Genes after BCG Infection—Expression of ALP, OCN, and OPN, typical markers of initial differentiation of osteoblasts, was analyzed at the transcriptional level in MC3T3-E1 cells with or without BCG infection. After 6 days culture, non-infected cells reached confluence and their expression of OCN mRNA was clearly increased (Fig. 1A). However, the BCG-infected cells showed significant reduction of ALP and OCN mRNA expression as previously reported (32). On the other hand, the expression of OPN mRNA was highly increased in the BCG-infected cells.

To identify genes specifically up-regulated by BCG infection, the SSH technique was applied using the mRNA extracted from BCG-infected or -uninfected MC3T3-E1 cells. Differential screening of 400 cDNA clones resulted in 40 positive clones. Sequencing analysis classified the 40 clones into 10 genetic loci. RT-PCR was performed with the same mRNA samples using specific primers for the 10 genes, revealing that all of them were actually up-regulated by BCG infection. BLAST analysis of their sequences revealed that they shared homology with known genes as listed in Table I. As TNF-α family proteins have recently been reported as important signaling molecules to regulate bone remodeling, especially bone resorption (16), we focused on the increased expression of 4-1BB of BCG-infected MC3T3-E1 cells in this study.

Increased Expression of 4-1BB Protein in BCG-infected Osteoblastic Cells—As described above, the amount of 4-1BB mRNA was increased during BCG infection (Fig. 1B). To determine whether 4-1BB was increased at the protein level in BCG-infected cells. We performed immunoblot analysis using anti-4-1BB antibody. As shown in Fig. 2, 4-1BB protein ap-
Because human 4-1BB induces the expression of M-CSF in peripheral blood mononuclear cells (33), we examined whether 4-1BB induced the expression of M-CSF in bone marrow cells. Expression of M-CSF was clearly increased 12 h after addition of r4-1BB to G-10 column-eluted bone marrow cell culture (Fig. 1D). However, in MC3T3-E1 cells, expression of M-CSF was not affected by treatment with r4-1BB (data not shown).

Immobilized r4-1BB Inhibits M-CSF/RANKL-induced Osteoclastogenesis—Based on the fact that the macrophage-like cell lines and G-10 column-eluted bone marrow cells had the ability to express 4-1BBL, we determined whether 4-1BB could influence osteoclast differentiation. First, we added r4-1BB to G-10 column-eluted bone marrow cell culture in the presence of M-CSF and RANKL and measured the numbers of TRAP-positive mononuclear cells after 3 days and TRAP-positive multinuclear cells after 5 days. In the presence of M-CSF and RANKL, the bone marrow cells could differentiate into TRAP-positive mononuclear cells and after that formed TRAP-positive multinuclear giant cells. r4-1BB failed to inhibit the M-CSF/RANKL-induced osteoclast formation even at the concentration of 1 μg/ml (Fig. 3, A–C). Then, we used cell culture plates with an immobilized extracellular domain of r4-1BB for this experiment. FBS-coated plates were used as a negative control. In the r4-1BB-immobilized plates, formation of TRAP-positive mononuclear and multinuclear cells was markedly inhibited at the concentration of 100 ng/ml (Fig. 3, A–C). The inhibitory effect of immobilized r4-1BB on osteoclastogenesis was observed in a dose-dependent manner, and the numbers of TRAP-positive mononuclear and multinuclear cells were drastically decreased at concentrations of 0.1–1 ng/ml and 1–10 ng/ml of r4-1BB, respectively (Fig. 3, D and E). These results suggested that 4-1BB might be a strong inhibitor of osteoclastogenesis.

4-1BB Inhibits Osteoclast Formation through 4-1BBL—To examine whether the inhibitory effect of 4-1BB on osteoclast formation was mediated by interaction of 4-1BB with 4-1BB ligand (4-1BBL), we determined the effect of anti-4-1BBL antibody on M-CSF/RANKL-induced osteoclast formation. Addition of the antibody to G-10 column-eluted bone marrow cell culture at the presence of 10 ng/ml M-CSF and 20 ng/ml RANKL showed decrease of TRAP-positive cells in a dose-dependent manner (Fig. 4), suggesting that the 4-1BBL antibody, like immobilized 4-1BB, might bind to 4-1BBL on the surface of osteoclast precursor cells, transduce intracellular signals, and suppress osteoclast formation.

r4-1BB Accelerates Proliferation of Bone Marrow Cells—Recently, it was reported that 4-1BB accelerates proliferation of human peripheral blood mononuclear cells via reverse signaling through 4-1BBL (34). To determine whether 4-1BB also accelerates proliferation of bone marrow cells, G-10 column-eluted bone marrow cells were cultured in r4-1BB-immobilized plates with M-CSF, and the number of adherent cells was determined periodically by counting under a light microscope (Fig. 5A). r4-1BB did not affect cell proliferation during the first 2 days. But after 3 days, the number of r4-1BB-treated cells was 1.5- to 2-fold larger than that of r4-1BB-ununtreated cells. Cell proliferation was also monitored by WST-1 assay described under “Experimental Procedures.” Increased optical density at 450 nm was observed in the r4-1BB-treated cells even at a concentration of 10 pg/ml (Fig. 5B).

CKI Is Involved in 4-1BB/4-1BBL Reverse Signaling—Phosphorylation of a recognition site of CKI in the cytoplasmic domain of TNF is implicated in reverse signaling, and the CKI motif is conserved in the 4-1BBL molecule (35). A CKI inhibitor, CKI-7, suppressed the inhibitory effect of r4-1BB on M-CSF/RANKL-induced osteoclast formation (Fig. 6, A and B).
The CKI inhibitor also cancelled r4-1BB-mediated increase of cell proliferation of BMM cells (Fig. 6C). These results suggest that CKI is involved in 4-1BB/4-1BBL reverse signaling.

Effects of r4-1BB on Known Signaling Pathways Induced by RANKL and M-CSF—RANKL induces recruitment of TNFR-associated factors 2 and 6 (TRAF2 and TRAF6) to the cytoplasmic tail of RANK with activation of I-κB kinase and phosphorylation of I-κB. RANKL also activates the MAPK and the phosphatidylinositol 3-kinase (PI3K) cascades (36–38). These signals have been suggested to be important for osteoclast differentiation and survival. Because 4-1BB is a receptor for the TNF-α family ligand 4-1BBL, it may be possible that 4-1BBL directly binds to RANKL as a decoy receptor like osteoprotegerin (OPG) and interrupts RANKL-RANK signaling. To determine the effect of 4-1BB on RANKL-mediated signal transduction, we examined the phosphorylation of IκB and ERK1/2 of RANKL-treated bone marrow cells in the presence or absence of r4-1BB. IκB phosphorylation was observed 15 min after addition of RANKL to G-10 column-eluted bone marrow cell culture, and then decreased (Fig. 7A). r4-1BB failed to affect the transient phosphorylation of IκB. In addition, no difference was observed in the phosphorylation of ERK1/2, p38, and JNK between r4-1BB-treated and untreated cells. Phosphorylation of ERK1/2 was observed after addition of M-CSF to G-10 column-eluted bone marrow cell culture; however, r4-1BB failed to suppress the phosphorylation (Fig. 7B). In contrast, r4-1BB effectively suppressed the Akt phosphorylation that was observed 15 min after addition of RANKL to G-10 column-eluted bone marrow cell culture (Fig. 7A). A similar result was obtained in BMM cells treated with RANKL in the presence or absence of r4-1BB. These results suggested the possibility that 4-1BB/4-1BBL reverse signaling might interfere with PI3K/Akt pathway.

NFAT2 was increased after culturing BMM cells with M-CSF and RANKL for 3 days. Addition of immobilized 4-1BB markedly suppressed the NFAT2 induction (Fig. 7C). On the other hand, expression of TRAF6 and c-Fos of BMM cells cultured with M-CSF and RANKL was not affected by immobilized r4-1BB.

4-1BB-induced Responses of BMM Cells—We examined levels of tyrosine phosphorylation of proteins of BMM cells in the presence or absence of 4-1BB (Fig. 8A). Levels of tyrosine phosphorylation of several proteins, especially proteins with molecular masses of more than 140 kDa, were markedly decreased 5 min after r4-1BB treatment. Akt phosphorylation was also decreased by addition of r4-1BB.

Increased expression of M-CSF in monocytes treated with 4-1BB was reported (33). As shown in Fig. 8B, the amounts of M-CSF and TNF-α mRNA were increased in BMM cells that were cultured in the plates immobilized with r4-1BB. BMM cells expressed little or no TNF-α or M-CSF mRNA in medium without r4-1BB. Up-regulation of TNF-α was also observed in mouse macrophage cell lines (J774 and RAW264.7 by treatment with immobilized 4-1BB; supplementary data).

DISCUSSION

Our previous study (32) showed that internalization of BCG into osteoblasts reduced proliferation and ALP activity of the
cells; however, interleukin-6 production in the cells was increased after BCG infection. In this study, ALP and OCN mRNA expression in BCG-infected osteoblasts was markedly reduced. These results suggest that BCG infection of osteoblasts suppresses their proliferation and differentiation. In contrast, expression of OPN mRNA, another commonly used marker of initial osteoblast differentiation, was increased by BCG infection. In this connection, Nau et al. (39, 40) found that *M. tuberculosis*, but not *E. coli*, infection of macrophages causes a substantial increase in OPN gene expression and that OPN augments the protective host response against a mycobacterial infection.

To identify the molecules associated with bone remodeling, we performed the subtraction method using BCG-infected and uninfected osteoblastic cell line MC3T3-E1, because *M. tuberculosis* and BCG infection showed significant alteration of bone metabolism (3, 4). From the results of the subtraction experiments, one of the TNF-α/H9251 receptor family proteins, 4-1BB, was identified as an up-regulated gene in the BCG-infected cells. Up-regulation of 4-1BB was also observed in osteoblastic cells by infection with *E. coli*, *S. typhimurium*, and *S. aureus*, and by treatment with heat-killed BCG and LPS, suggesting that 4-1BB in osteoblasts is generally up-regulated by bacterial infection. 4-1BB is a co-stimulatory molecule expressed on activated T cells (see Refs. 41 and 42 for review) and natural killer cells (43). Its ligand, 4-1BBL, has been detected on activated antigen-presenting cells, including macrophages, B cells, and dendritic cells (44–46). Interaction of 4-1BB with 4-1BBL provides costimulatory signaling leading to CD4 and CD8 T cell expansion, cytokine production, promotion of effector function of cytotoxic T lymphocytes, and increased cell survival (41, 47–50).
Recently, members of the TNF-TNFR superfamily have been shown to play critical roles in regulating cellular activation, differentiation, and apoptosis (51). In osteoclastogenesis, one of the TNF family members, RANKL, is an essential factor for differentiation of monocyte/macrophage precursors to osteoclasts (18–20). Binding of RANKL to its cognate receptor, RANK, which is expressed on osteoclast precursors, elicits osteoclast formation. TNF-α also induces osteoclast differentiation in bone marrow macrophages in vitro (52, 53) and activates osteoclasts through a direct action independent of RANKL (54).

In this study, we determined whether 4-1BB belong to the TNF receptor family affected osteoclastogenesis and found that r4-1BB immobilized on culture plates inhibited M-CSF/RANKL-induced osteoclast formation from G-10 column-eluted bone marrow cells. As far as we know, the present study is the first description of the inhibitory effect of the 4-1BB on osteoclastogenesis.

OPG plays a role as a regulatory decoy receptor for RANKL (19). OPG has a similar sequence of extracellular domain of TNF-α receptor, binds to RANKL, and blocks osteoclast differentiation induced by RANKL (19). Inhibition of osteoclast formation by 4-1BB may result from binding of 4-1BB to RANKL as a decoy receptor. However, several lines of evidence suggest that it is not feasible. First, a very low amount of r4-1BB strongly inhibited osteoclast formation even in the presence of an excess amount of RANKL. Second, soluble r4-1BB failed to inhibit M-CSF/RANKL-induced osteoclast formation. Third, r4-1BB did not bind to RANKL.

Anti-4-1BBL antibody also inhibited M-CSF/RANKL-induced osteoclast formation when relatively large amounts of the antibody were added to this system, suggesting that direct interaction of 4-1BB with 4-1BBL elicits this inhibitory effect. Reverse signaling via 4-1BBL induces a widespread and profound proliferation of human peripheral monocytes in the presence of M-CSF and/or granulocyte-macrophage colony-stimulating factor (34). Binding of 4-1BB to B cells in the presence of anti-IgM antibodies also increases proliferation of mouse spleen B cells (45). Moreover, 4-1BB induces the expression of M-CSF in monocytes (33). In connection with these findings, we found in this study that G-10 column-eluted bone marrow cells expressed 4-1BBL and that r4-1BB was internalized in M-CSF in bone marrow cells. We also found that 4-1BB induced proliferation of G-10 column-eluted bone marrow cells and BMM cells. These results strongly indicate that reverse signaling via 4-1BBL takes place in those cells.

A CKI-recognized phosphorylation site is present in the cytoplasmic domains of all TNF family members known to utilize reverse signaling (35). This motif in transmembrane TNF-α has been shown to be a target for phosphorylation by CK. The cytoplasmic domain of 4-1BBL also contains this CKI recognition site and phosphorylation of this site is implicated in 4-1BBL signaling. The CKI inhibitor CKI-7 markedly suppressed the inhibitory effect of 4-1BB on RANKL/M-CSF-induced osteoclast formation and the 4-1BB-induced cell proliferation, suggesting that CKI may play an important role in 4-1BB/4-1BBL reverse signaling leading to inhibition of osteoclastogenesis.

IκB, MAPK12/ERK1/2 (ERK1/2), p38 MAPK, and Src are clearly activated after the engagement of RANKL (37, 55–57). ERK1/2 are also activated by M-CSF (58). These signals are believed to contribute to differentiation, resorption, and survival responses of osteoclasts. r4-1BB failed to affect RANKL-induced activation of IκB, ERK1/2, and p38, whereas it markedly suppressed RANKL-induced activation of Akt. Akt is located in the signaling pathway from Src and PI3K, activation of which is considered to contribute to cell survival and differentiation (see Refs. 59 and 60 for review). Wortmannin and LY294002, inhibitors of PI3K, strongly inhibit RANKL-induced osteoclast formation and affect proliferation and/or survival of preosteoclasts (37, 61), suggesting that inhibition of RANKL-induced Akt activation by 4-1BB might account for the inhibitory effect of 4-1BB on osteoclast formation. We also found that tyrosine phosphorylation of several proteins were down-regulated by 4-1BB, suggesting that such proteins might be involved in 4-1BB-induced inhibition of osteoclastogenesis. This finding may be related with the fact that tyrosine phosphatases, Src homology 2 domain-containing inositol-5-phosphatase and Src-2 homology 2 domain-containing phosphatase-1, regulate PI3K/Akt signaling pathway (62, 63).

NFAT2 has been found to be a key transcriptional factor in osteoclastogenesis (64, 65). NFAT2-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL. We found in this study that r4-1BB had the ability to cancel RANKL-induced increase of NFAT2 expression. The result is not inconsistent with the inhibitory effect of 4-1BB on RANKL-induced osteoclast formation and may account for it, at least in part.

Both 4-1BBL-deficient mice and 4-1BB-deficient mice have been established (48, 66). Both mice fail to show abnormalities in the organs, including major skeletons upon gross necropsy or histopathologic examination. However, 4-1BBL transgenic mouse that overexpress 4-1BB/4-1BBL signaling show splenomegaly. In spleens of the transgenic mice, cells expressing a marker of macrophage lineage, Mac1, were markedly increased (67). This result suggests that 4-1BB/4-1BBL signaling is important for in vivo proliferation of the macrophage lineage, which is consistent with our finding that r4-1BB accelerated proliferation of G-10 column-eluted bone marrow cells.

Bone is the site of maturation of certain types of immune cells, and osteoclasts are derived from the same progenitor cells as those of monocyte/macrophage lineage cells. In addition, T cells have the ability to secrete RANKL and several immune responses involve RANKL-related signaling pathways (67). Moreover, 4-1BB has been found as a co-stimulatory surface molecule of T cells (68). Considering these observations, one can imagine that the ability of 4-1BB to inhibit osteoclast formation may be one of the cross-talks between the immune system and bone metabolism. Further study will elucidate the biological significance of 4-1BB in this cross-talk.

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Infection-induced Up-regulation of the Costimulatory Molecule 4-1BB in Osteoblastic Cells and Its Inhibitory Effect on M-CSF/RANKL-induced in Vitro Osteoclastogenesis

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