Inhibition of Hormone and Cytokine-stimulated Osteoclastogenesis and Bone Resorption by Interleukin-4 and Interleukin-13 Is Associated with Increased Osteoprotegerin and Decreased RANKL and RANK in a STAT6-dependent Pathway*

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Interleukin (IL)-4 and IL-13 are cytokines that inhibit bone resorption. Data showing an inhibitory effect of IL-4 and IL-13 on RANK mRNA in mouse calvariae were first reported at the 22nd American Society for Bone and Mineral Research Meeting (Lerner, U.H., and Conaway, H. H. 2000) J. Bone Min. Res. 15, Suppl. 1, Abstr. SU 230). In the present study, release of 45Ca from cultured mouse calvarial bones stimulated by different cytokines, peptides, and steroid hormones was inhibited by IL-4 and IL-13. IL-4 and IL-13 decreased receptor activator of nuclear factor-κB ligand (RANKL) and RANK mRNA and increased osteoprotegerin (OPG) mRNA in calvariae. Additionally, the cytokines decreased RANKL protein and increased OPG protein in calvarial bones. In osteoblasts isolated from calvariae, both an increase in RANKL mRNA and a decrease in OPG mRNA and protein elicited by vitamin D3 were reversed by IL-4 and IL-13. IL-4 and IL-13 decreased the number of tartrate-resistant acid phosphatase positive multicellular cells and the mRNA expression of calcitonin receptor, tartrate-resistant acid phosphatase, and cathepsin K in mouse spleen cells and bone marrow macrophages (BMM) treated with macrophage colony-stimulating factor and RANKL. Inhibition of mRNA for RANK and the transcription factor NFAT2 was also noted in spleen cell and BMM cultures treated with IL-4 and IL-13. In addition, RANK mRNA and RANK protein were decreased by IL-4 and IL-13 in RAW 264.7 cells. Osteoblasts, spleen cells, and BMM expressed mRNA for the four proteins making up the IL-4 and IL-13 receptors. No effects by IL-4 on bone resorption and osteoclast formation or on RANKL and RANK mRNA expression were seen in Stat6−/− mice. The data indicate that IL-4 and IL-13, via a STAT6-dependent pathway, inhibit osteoclast differentiation and bone resorption by activating receptors on osteoblasts and osteoclasts that affect the RANKL/RANK/OPG system.

Immune cells are thought to play an important role in bone loss caused by inflammation (1, 2). Disabling joint destruction in rheumatoid arthritis and the loss of teeth in periodontal disease are examples of skeletal loss that can occur with inflammation. Resorption of bone by osteoclasts represents the primary mechanism responsible for the loss of bone caused by inflammatory disease.

Key factors regulating osteoclastogenesis include macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). M-CSF is a secreted product of stromal cells/osteostats that enhances colony expansion of monocyte/osteoclast progenitor cells. RANKL exists both as a transmembrane protein in osteoblasts/stromal cells and as a soluble protein (3–6). RANKL directs the expanded progenitor cell population to the osteoclast lineage by activation of receptor activator of nuclear factor-κB (RANK). The interaction between RANKL and RANK can be inhibited by osteoprotegerin (OPG), a decoy receptor released from stromal cells/osteoblasts.

RANKL stimulation of RANK causes receptor trimerization and recruitment of tumor necrosis factor receptor-associated factors (TRAFs). TRAF1, 2, 3, and 5 bind to the carboxyl-terminal end of the RANK trimer, whereas TRAF6 binds more closely to the membrane. Downstream intracellular signaling mediated by RANK in osteoclast progenitor cells includes TRAF6-dependent activation of NF-κB, mitogen-activated protein kinases (MAP kinases) and AP-1, and activation of c-Src and the phosphatidylinositol 3-kinase/Akt pathway (5–7). In addition, immunoreceptor tyrosine-based activation motif-mediated costimulatory signals have been shown to be required for expression of nuclear factor of activated T-cells 2 (NFAT2), the transcription factor believed to be crucial for osteoclast differentiation (8, 9). Several of these intracellular signaling molecules, including p50/p65, c-Fos, NFAT2, Fc receptor common γ subunit (FcγRII)/DAX-α/DAX-α1 receptor 12 protein; DAP12, receptor activator of nuclear factor-κB (RANK). The interaction between RANKL and RANK can be inhibited by osteoprotegerin (OPG), a decoy receptor released from stromal cells/osteoblasts.

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2 The abbreviations used are: M-CSF, macrophage colony-stimulating factor; ALP, alkaline phosphatase; α-MEM, α-modification of minimum essential medium; BMM, bone marrow macrophages; CT, calcitonin; CTR, calcitonin receptor; DAP12, DNAX-activating protein 12; D3, 1,25(OH)2-vitamin D3; ERK, extracellular signal-regulated kinase; FcγRII, Fc receptor common γ subunit; iNOS, inhibitor of iNOS; IL, interleukin; IL-4Rα, interleukin-4 receptor α; IL-13Rα1, interleukin-13 receptor α1; JNK, c-Jun NH2-terminal kinase; MAP kinase, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; OSM, oncostatin M; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PBS, phosphate-buffered saline; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; RT, reverse transcriptase; STAT, signal transducers and activators of transcription; TAPRA, 6-carboxytetramethylrhodamine; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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IL-4/IL-13 Regulates RANKL/RANK/OPG

In inflammatory diseases, increased osteoclast activity can be stimulated by bone resorbing proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-11, IL-17, tumor necrosis factor-α (TNF-α), and kinins released in the kallikrein-kinin system (15–18). Several of these compounds have been shown to increase RANKL expression (19, 20). The bone resorbing effects of stimulatory cytokines can be inhibited by IL-4 and IL-13 (16). IL-4 and IL-13 are 19- and 10-kDa proteins, respectively; they are secreted by Th2-type lymphocytes and mast cells and have pleiotropic immune functions. Although the amino acid sequences of IL-4 and IL-13 exhibit only 25% homology, the two cytokines have many overlapping functions, including the utilization of closely related receptors.

Type 1 and Type 2 IL-4 receptors are formed by heterodimerization of IL-4 receptor α (IL-4Rα) protein to either the γ common chain (γc), a receptor component that is also found in receptors for IL-2, IL-7, IL-9, IL-15, and IL-21, or to the IL-13 receptor α1 (IL-13Rα1) protein, respectively (21, 22). Receptor dimerization, either because of binding of IL-4 to IL-4Rα in Type 1 or 2 receptors or because of binding of IL-13 to IL-13Rα1 in IL-13 receptors, causes binding to the Box1 region in IL-4Rα of JAK-1 in the Janus kinase family, which leads subsequently to phosphorylation of tyrosine residues in the cytoplasmic tail of IL-4Rα. Tyrosine residues 2–4 of IL-4Rα act as docking sites for the transcription factor STAT6 (signal transducers and activators of transcription), which homodimerizes and translocates to the nucleus following tyrosine phosphorylation (21). Activation of IL-4 and IL-13 receptors also results in binding of Tyk2, another member of the Janus kinase family, to the Box region of IL-13Rα. Ligand binding to IL-4Rα additionally causes phosphorylation of the tyrosine 1 residue in the IL-4Rα protein, which then acts as a docking site for insulin receptor substrate-1 and insulin receptor substrate-2, which subsequently become phosphorylated and can activate the phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase pathways. Although much is known about the events following IL-4 receptor activation, downstream intracellular signaling by IL-13 receptors has not been characterized as well. Interestingly, IL-13Rα2 binds IL-13 with high affinity, but this has not been observed to result in a biological response, suggesting the possibility that IL-13Rα2 might function as a decoy receptor.

IL-4 has been shown to inhibit in vitro bone resorption in neonatal mouse calvariae stimulated by IL-11 (23) and in mouse long bones stimulated by parathyroid hormone (PTH), 1,25(OH)2-vitamin D3 (D3), IL-1, and prostaglandin E2 (24). Administration of IL-4 in vivo to mice inhibits hypercalcemia induced by parathyroid hormone-related peptide (PTHrP; 25) and bone loss caused by ovariectomy (26) and by transplanted tumors secreting PTHrP and IL-1 (27). Juxtaarticular bone resorption in mice with collagen type II-induced arthritis can be decreased by local production of IL-4 introduced by an adenovirus vector (28) or by intraarticular injection of IL-4 (29). Similarly, bone resorption in adjuvant-induced arthritis in rats can be decreased by injection of an adenovirus producing IL-4 (30). In an ex vivo model of bone resorption in rheumatoid arthritis, IL-4 has been found to inhibit the appearance of osteoclasts (31). In organ culture studies, the inhibition by IL-4 of the stimulatory effect of IL-1 on bone resorption has been suggested to be because of inhibition of cyclooxygenase-2 expression (32, 33).

The inhibitory effect of IL-4 on bone resorption in vitro is thought to involve decreased osteoclastogenesis. IL-4 has been shown to inhibit osteoclast formation in mouse bone marrow cultures, in co-cultures of mouse spleen cells and ST-2 stromal cells stimulated by D3 (34), and in mouse bone marrow cultures stimulated by PTH, D3, and IL-1α (35, 36). It has recently been shown that IL-4 can inhibit osteoclast formation not only by acting directly on cells in the myelomonocytic lineage but also by acting indirectly on T-cells to induce the expression of an hitherto unknown membrane-associated inhibitor (37). Several molecular mechanisms have been suggested for the inhibition of osteoclast formation by IL-4. These include decreased RANKL stimulation of osteoclast progenitor cells by activation of peroxisome proliferator-activated receptor γ1 (38), inhibition of inhibitor of kB (IkB) phosphorylation, and decrease of nuclear factor κB (NF-κB) nuclear translocation (39) and inhibition of signaling through c-Jun NH2-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) (40). Interestingly, IL-4 is not believed to affect M-CSF signaling in osteoclast progenitor cells through the MAP kinase (JNK) and ERK pathways (40). The present study was undertaken to determine whether IL-4 and IL-13 might inhibit bone resorption by initiating responses in both stromal cells/osteoblasts and osteoclast progenitor cells that alter the RANKL/RANK/OPG system.

EXPERIMENTAL PROCEDURES

Materials—Synthetic bovine parathyroid hormone (PTh(1–34)) was obtained from Bachem, Bubendorf, Switzerland; recombinant mouse M-CSF, recombinant murine RANKL, mouse OPG fused to human IgG, Fc (OPG/Fc chimera), recombinant human TNF-α, recombinant human IL-1β, recombinant human IL-6, recombinant human IL-6 soluble receptor, recombinant murine IL-4, recombinant murine IL-13, recombinant murine oncostatin M (OSM), immunosass kits for mouse OPG, and mouse RANK and goat RANK-specific IgG purified by mouse RANK affinity chromatography were from R&D Systems, Abingdon, UK; essentially fatty acid-free albumin, trans-retinoic acid, dexamethasone, and the kit for leucocyte acid phosphatase staining were from Sigma; fetal bovine serum (FBS) from ICN Pharmaceuticals Inc., Costa Mesa, CA; [3H]Cl, from Amersham Biosciences; Thermo Sequenase-TM II DYEnamic ET® terminator cycle sequencing kit from Amersham Biosciences; HotStar Taq polymerase kit and the QiAquick PCR Purification kit from Qiagen Ltd., Crawley; England; culture dishes and multiwell plates from Costar, Cambridge, MA; α-modification of minimum essential medium (α-MEM), TRIzol LS Reagent, deoxyxynucleoside I (DNase I; amplification grade), and oligonucleotide primers from Invitrogen; fluorescent labeled probes (reporter fluorescent dye VIC at the 5’ end and quencher fluorescent dye TAMRA at the 3’ end) and TaqMan Universal PCR Master Mix were from Applied Biosystems, Warrington, UK; the 1st strand cDNA synthesis Kit and PCR Core Kit from Roche Diagnostics; RNAAqueous®-4PCR kit for isolation of DNA-free mRNA from Ambion Inc., Austin, TX; fluorescein isothiocyanate-labeled affinity pure rabbit antibodies to goat IgG from Jackson ImmunoResearch, West Baltimore Pike, PA. Synthetic salmon calcitonin (CT) was generously provided from Sandoz AG, Basle, Switzerland; 1,25(OH)2-vitamin D3 from Hoffmann-La Roche, Basle, Switzerland; and indomethacin from Merck Sharp and Dohme, Haarlem, The Netherlands. Other chemicals and reagents were from Sigma-Aldrich Co., St. Louis, MO, and were of the highest grade commercially available.

Animals—CsA mice from our own inbred colony were used for most experiments. Mice homozygous for the Stat6tm1Gru mutation in a BALB/c background (C.129S2-Stat6tm1Gru/J; stock number 002826), mice homozygous for the Stat4tm1Gru mutation in a BALB/c background (C.129S2-Stat4tm1Gru/J; stock number 002828) and their corre-
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...sponding wild type mice BALB/cJ (stock number 000651) JAX® MICE, were purchased from The Jackson Laboratory, and were bred in our animal facility unit. Calvarial bones and spleen cells from Stat6<sup>-/-</sup> mice did not express STAT6 mRNA as assessed by reverse transcription-polymerase chain reaction (RT-PCR) analyses (data not shown). Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

**Bone Resorption Bioassay**—Bone resorption was assessed by culturing calvarial bones dissected from 6–7-day-old mice (CsA, Stat6<sup>-/-</sup>, Stat4<sup>-/-</sup>, BALB/cJ) prelabeled 4 days previously with an injection of 1.5 μCi of 45Ca. The bones were dissected in four pieces (41) and preincubated overnight in α-MEM containing 0.1% albumin and 1 μmol/liter indomethacin (42). In one set of experiments, calvarial bones were incubated in α-MEM with or without IL-4 or IL-13 for 24 h after the initial pre-culture period. This was followed by a 96-h incubation in the absence and presence of hormones and cytokines stimulating bone resorption, with or without IL-4 or IL-13. In another set of experiments, subsequent to the 24-h preculture in indomethacin the calvarial bones were cultured in the absence and presence of hormones and cytokines stimulating bone resorption, with and without the continuous presence of indomethacin. At the end of the cultures, 45Ca in bones and media was analyzed using liquid scintillation counting. Release of isotope was expressed as the percentage release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture) (42). In some experiments, the data were recalculated and the results expressed as percent of control, which was set at 100%. This allowed for accumulation of data from several experiments. In time course experiments, mice were injected with 12.5 μCi of 45Ca and the kinetics of the release of 45Ca was analyzed by withdrawal of small amounts of medium at the stated time points.

**Gene Expression in Mouse Calvarial Bone**—Calvarial bones were dissected from 6–7-day-old mice (CsA, Stat6<sup>-/-</sup>, Stat4<sup>-/-</sup>, BALB/cJ), divided into halves along the sagittal suture and preincubated in α-MEM with 0.1% albumin and 1 μM indomethacin overnight. Following the pre-culture period, calvarial bones in one set of experiments were incubated either in control medium or medium containing IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 24 h. In another set of experiments, calvariae were incubated in control medium or medium containing D3 (10 nmol/liter), with or without IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 24 h.

For semiquantitative RT-PCR, bones were homogenized and the RNA extracted from five bones per treatment group was pooled for subsequent analyses. When using quantitative real-time RT-PCR, bones were homogenized and RNA was extracted from individual bones (five/group) and used for analyses.

**Gene Expression in Mouse Calvarial Osteoblast Cultures**—Bone cells were isolated from calvariae of 2–3-day-old CsA mice using a modified time sequential enzyme digestion technique (43). Cells from populations 6 to 10 were used. These cells showed an osteoblastic phenotype as assessed by their cyclic AMP responsiveness to PTH, expression of alkaline phosphatase, osteocalcin, and bone sialoprotein, and the capacity to form mineralized bone noduli (data not shown). The cells were seeded in culture flasks containing α-MEM supplemented with 10% FBS, l-glutamine, and antibiotics at 37 °C in humidified air containing 5% CO₂.

After 4 days in flasks, the cells were seeded in culture dishes. Osteoblasts were plated at a density of 10⁴ cells/cm² in culture dishes containing α-MEM, 10% FBS. After attachment overnight, medium was changed to α-MEM, 10% FBS containing D3 (10 nmol/liter) with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml). In addition, cells were cultured in α-MEM, 10% FBS without test substances as a control group. Control and treatment groups each contained four dishes. After 8 and 48 h of culture, RNA was extracted and used for quantitative real-time RT-PCR analyses.

**Gene Expression during Osteoclastogenesis in RAW 264.7 Cell Cultures**—The murine macrophage cell line RAW 264.7 was seeded at a density of 10⁵ cells/cm² in culture dishes containing α-MEM, 10% FBS. After attachment overnight, cells were incubated in α-MEM, 10% FBS containing RANKL (100 ng/ml) without or with IL-4 (50 ng/ml), IL-13 (50 ng/ml), or OPG (300 ng/ml). In addition, cells were cultured in α-MEM, 10% FBS without test substances as a control group. After 2, 4, and 6 days, RNA was extracted and used for semiquantitative RT-PCR analyses.

**Gene Expression during Osteoclastogenesis in Mouse Spleen Cell Cultures**—Spleen cells were isolated from 5–7-week-old mice (CsA, Stat6<sup>-/-</sup>, Stat4<sup>-/-</sup>, BALB/cJ) and seeded in 0.5 ml of α-MEM with 10% FBS on coverslips in 24-well plates at a density of 1 × 10⁶ cells/cm². After attachment overnight, the medium was changed and the cells were cultured in the absence and presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml), with and without IL-4 (50 ng/ml), IL-13 (50 ng/ml), or OPG (300 ng/ml). The cells were cultured in the presence of test substances for 4–5 days with medium changed after 3 days. At the end of the experiments, the cells were stained for tartrate-resistant acid phosphatase (TRAP) using the Sigma Diagnostics Acid Phosphatase kit by following the manufacturer’s instruction. Cells positive for TRAP and having three or more nuclei were considered osteoclasts, and the number of multinucleated osteoclasts counted. Osteoclasts formed in the spleen cell cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone (data not shown). Osteoclast formation was associated with increased mRNA expression of calcitonin receptor (CTR), TRAP, and cathepsin K. No osteoclasts were formed when cells were treated with M-CSF or RANKL alone. Osteoclasts were also not formed in the presence of PTH or D3, demonstrating the lack of stromal cells in the spleen cell cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by OPG (data not shown).

For semiquantitative RT-PCR and quantitative real-time RT-PCR analyses, spleen cells were seeded at a density of 1 × 10⁶ cells/cm² in culture dishes. After attachment overnight, cells were incubated in α-MEM, 10% FBS in the absence and presence of M-CSF (25 ng/ml)/RANKL (100 ng/ml), with or without IL-4 (50 ng/ml), IL-13 (50 ng/ml), or OPG (300 ng/ml) for 4–5 days.

**Gene Expression during Osteoclastogenesis in Mouse Bone Marrow Macrophage Cultures**—The femurs and tibiae from 5–7-week-old male mice were dissected and cleaned from adhering tissues. The cartilage ends were cut off and the cells in the marrow cavity were flushed out using α-MEM in a syringe with a sterile needle. The marrow cells were collected in α-MEM, 10% FBS and the erythrocytes lysed in red blood cell lysis buffer (0.16 M NH₄Cl, 0.17 M Tris, pH 7.65). The remaining bone marrow cells were then washed and suspended in α-MEM, 10% FBS containing l-glutamine (0.7 mM), antibiotics (100 units/ml benzylpenicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamycin sulfate), and 100 ng/ml M-CSF. The cells were seeded at a density of 8 × 10⁴ cells/cm² in a 60-cm² culture dish, to which stromal cells and lymphoid cells cannot adhere. After 3 days, the cultures were vigorously washed with PBS twice and the cells attached to the bottom were then detached using 0.002% EDTA in PBS. These cells were resuspended in α-MEM, 10% FBS with 100 ng/ml M-CSF and seeded at a density of 0.5 × 10⁴ cells/cm² in 60-cm² dishes. After another 3 days, the cells were washed and detached as described for the initial 3-day culture period.
and used as bone marrow macrophages (BMM). For further details, see Takeshita et al. (44). These cells did not express alkaline phosphatase, RANKL, OPG, or CTR mRNA, but mRNA for RANK, c-Fms, cathepsin K, and TRAP, as assessed by quantitative real-time PCR (data not shown). Using flow cytometry we could demonstrate cell surface expression of the typical macrophage markers F4/80 and c-Fms, but not the lymphoid cell markers CD3 and B 220.3

For osteoclastogenesis experiments, BMM were seeded on 0.8-cm² glass chamber slides at a density of 10⁴ cells/cm² in culture dishes and incubated in MEM, 10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 50 ng/ml of RANKL, with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml). After 4 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer. Subsequently, cells were stained for TRAP, and cells positive for TRAP and having three or more nuclei were considered osteoclasts, and the number of multinucleated osteoclasts counted. Osteoclasts were not formed in the presence of M-CSF and either PTH or D3, indicating the lack of stromal cells and the absence of OPG (data not shown). Osteoclasts were not formed in the presence of M-CSF and either PTH or D3, indicating the lack of stromal cells and the absence of OPG (data not shown). Osteoclast formation caused by M-CSF and RANKL was abolished by OPG (data not shown).

For quantitative real-time PCR analyses, BMM were seeded at a density of 10⁴ cells/cm² in culture dishes and incubated in α-MEM, 10% FBS in the presence of M-CSF (100 ng/ml; control), M-CSF (100 ng/ml)/RANKL (50 ng/ml), with or without IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 3 days.

**RNA Extraction and cDNA Synthesis**—Total RNA was extracted from calvarial bones, mouse calvarial osteoblasts, RAW 264.7 cells, spleen cells, and BMM with TRIzol LS reagent or by the RNAsqueezé™-4PCR kit by following the manufacturer’s protocol. The RNA was quantified spectrophotometrically and the integrity of the RNA preparations was examined by agarose gel electrophoresis. Extracted total RNA with the TRIzol technique was treated with DNase I to eliminate genomic DNA according to the instructions supplied by the manufacturer. One microgram of total RNA, following DNase I treatment, was reverse transcribed into single-stranded cDNA with a 1st Strand cDNA Synthesis Kit using random primers (for semi-quantitative RT-PCRs) or oligo-p(dT)₁₅ primers (for quantitative real-time PCRs). After incubation at 25 °C for 10 min and at 42 °C for 60 min, the avian myeloblastosis virus reverse transcriptase was denaturated at 99 °C for 5 min. The cDNA was kept at −20 °C until used for PCR.

**Semi-quantitative RT-PCR**—1st Strand cDNA mixture was amplified by PCR using a PCR Core Kit and PC-960G Gradient Thermal Cycler (Corbett Research, Australia) or Mastercycler Gradient (Eppendorf). The PCRs for RANK, RANKL, OPG, CTR, TRAP, cathepsin K, NFAT2, γc, IL-4Ra, IL-13Ra1, IL-13Ra2, and GAPDH were performed using 1 μl of template, 0.2 μM of each primer, 2.5 units of Taq DNA polymerase, 1× PCR buffer, 0.2 mM dNTPs, and 1.5 mM MgCl₂ (100 μl total volume), with the exception of those for CTR, which were performed with 1.25 mM MgCl₂. The conditions for PCR were: denaturing at 94 °C for 2 min, annealing for 40 s at 65 °C (RANKL, RANK, and OPG), 64°C (CTR), 61°C (IL-4Ra, IL-13Ra1, and IL-13Ra2), 60 (NFAT2), 59 (cathepsin K), 58 (TRAP), 57 (GAPDH), and 56 °C (γc) followed by elongation at 72 °C for 90 s; in subsequent cycles denaturing was performed at 94 °C for 40 s. The PCRs for NFAT2, γc, IL-4Ra, RANKL, RANK, and OPG were initiated with hot start using HotStar Taq polymerase. The PCRs of RANKL, RANK, and OPG were performed with a step down technology in which the primer annealing temperature was decreased by 5 °C every 5 cycles down to 45 °C. The sequences of primers, the GenBank® accession numbers, and the positions of the 5′ and 3′ ends of the nucleotides for the predicted PCR products and the estimated size of the PCR products are given in Table 1. The expression of these factors was compared at the logarithmic phase of the PCR. No amplification was detected in samples in which the RT reaction had been omitted (data not shown). The PCR products were electrophoretically size fractionated in 1.5% agarose gel and visualized using ethidium bromide. The identity of the PCR products was confirmed using a QIAquick purification kit and a Thermo Sequenase-TM II DYEnamic ET® terminator cycle sequencing kit with sequences analyzed on an ABI 377 XL DNA Sequencer.

**Quantitative Real-time RT-PCR**—Quantitative real-time RT-PCR analyses of RANKL, RANK, OPG, CTR, cathepsin K, NFAT2, TRAF6, and β-actin mRNA were performed using the TaqMan Universal PCR Master Mix kit and the ABI PRISM 7900 HT Sequence Detection System and software (Applied Biosystems, Foster City, CA) with fluorescence labeled probes (reporter fluorescent dye VIC at the 5′ end and quencher fluorescent dye TAMRA at the 3′ end) as described previously (23). In each reaction, cDNA diluted 20-fold with nuclelease-free water was amplified using a TaqMan Universal PCR Master Mix, 300 nM of each primer, and 100 nM probe with the sequences of primers and probes listed in Table 1. The reaction conditions were an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. No amplification was detected in samples where the RT reaction had been omitted (data not shown). For TRAF6, an assay on Demand kit from Applied Biosystems was used. To control for variability in amplification because of differences in starting mRNA concentrations, β-actin was used as an internal standard. The relative expression of target mRNA was computed from the target C values and β-actin C values using the standard curve method (user bulletin number 2, Applied Biosystems).

**Fluorescence-activated Cell Sorter Analysis of RANK Protein**—The expression of RANK protein was assessed by analyzing cell surface expression of RANK in RAW 264.7 cells using fluorescein isothiocyanate-labeled anti-RANK antibodies. RAW 264.7 cells were seeded in 35-mm culture dishes at a density of 2×10⁶ cells/dish and incubated in α-MEM with 10% FBS for 24 h. Then, cells were incubated for 48 h in α-MEM, 10% FBS with or without 50 ng/ml IL-4. At the end of the incubation period, the adherent cells were treated with 0.2% EDTA and detached with a rubber cell scraper. All cells were subsequently fixed with 2% paraformaldehyde in PBS, pH 7.2, for 10 min at room temperature. The cells were washed once with PBS containing 1% bovine serum albumin (PBS-BSA) and harvested by centrifugation at 300 × g for 5 min. The cell pellet was re-suspended in PBS-BSA containing goat RANK-specific IgG (5 μg/ml) and incubated at room temperature for 1 h under gentle agitation. Thereafter, the cells were washed once in PBS-BSA and re-suspended in PBS-BSA containing fluorescein isothiocyanate-labeled rabbit antibodies to goat IgG (2 μg/ml). Following incubation in the dark, the cells were washed once in PBS-BSA and suspended in PBS to a final concentration of 10⁶ cells/ml before being analyzed with a fluorescence-activated cell sorter (Facsstar+) equipped with LYSIS II software (BD Biosciences). The effect of IL-4 on the expression of RANK was calculated in relation to the control cells that had not been exposed to IL-4. The mean fluorescence of each cell population was compared and expressed in relation to fluorescence of control cultures.

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IL-13 (50 ng/ml) for 48 h. Calvarial osteoblasts were seeded in presence of 10 nmol/liter D3 with and without IL-4 (50 ng/ml) or ittal suture. Following preincubation, a total of 8 calvarial halves per 6–7-day-old mice (CsA) and divided into two halves along the sag-

TABLE 1
Sequences of primers and probes, GenBank® accession numbers, and the numbers of the 5’ and 3’ ends of nucleotides for the predicted PCR products

| mRNA species               | Sequences                                   | 5’ and 3’ ends | GenBank   | Base pair |
|----------------------------|---------------------------------------------|----------------|-----------|-----------|
| RANKL (real-time)          | 5’-TGGAAAGCTTCATGTGGAT-3’                   | 606–680        | AF053713  |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-CATGATGAGGCTGTTGCA-3’                    |                |           |           |
| Probe                     | 5’-AGGGCGCTGTCGGCCAC-3’                     |                |           |           |
| RANKL (RT-PCR)             | 5’-GGTTGGCAGATTCGTGAA-3’                   | 957–1769       | 810       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGGAAGTATCTGACCTAT-3’                    |                |           |           |
| Probe                     | 5’-GGGAAATACGCTGACCT-3’                     |                |           |           |
| RANKL (real-time)          | 5’-TGCTCTCTGGGATGAGG-3’                    | 1422–1593      | AF019046  |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-AGGATGATGACCTGTGAC-3’                    |                |           |           |
| Probe                     | 5’-AGGATGATGACCTGTGAC-3’                    |                |           |           |
| RANKL (RT-PCR)             | 5’-GGGAGTATCTGACCTGAC-3’                   | 522–921        | 400       |           |
| Sense primer               |                                             |                |           |           |
| OPG (real-time)            | 5’-GGCCATGCTGAGCTGAC-3’                    | 845–958        | U94331    |           |
| sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-AGGCTGCTGAGCTGAC-3’                     |                |           |           |
| Probe                     | 5’-AGGCTGCTGAGCTGAC-3’                     |                |           |           |
| OPG (RT-PCR)               | 5’-GGGAGTATCTGACCTGAC-3’                   | 428–1147       | 720       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGGAGTATCTGACCTGAC-3’                   |                |           |           |
| Probe                     | 5’-GGGAGTATCTGACCTGAC-3’                   |                |           |           |
| Cathepsin K (real-time)    | 5’-CTGTTTGAGCTGTGACT-3’                    | 771–847        | BC 019160 |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-AACACTCTTGAGCTGACT-3’                   |                |           |           |
| Probe                     | 5’-AACACTCTTGAGCTGACT-3’                   |                |           |           |
| Cathepsin K (RT-PCR)       | 5’-CTGTTTGAGCTGTGACT-3’                    | 1072–1384      | 313       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-TATGTTGAGCTGACTGAC-3’                   |                |           |           |
| Probe                     | 5’-TATGTTGAGCTGACTGAC-3’                   |                |           |           |
| TRAP (real-time)           | 5’-CGACCCTCGAGCTGACTGAC-3’                 | 689–764        | NM 007588 |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-AACACTCTTGAGCTGACT-3’                   |                |           |           |
| Probe                     | 5’-AACACTCTTGAGCTGACT-3’                   |                |           |           |
| TRAP (RT-PCR)              | 5’-CGACCCTCGAGCTGACTGAC-3’                 | 1072–1384      | 313       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-TATGTTGAGCTGACTGAC-3’                   |                |           |           |
| Probe                     | 5’-TATGTTGAGCTGACTGAC-3’                   |                |           |           |
| CTR (real-time)            | 5’-AGTGGCCCTTCTTTATAGGAGAAG-3’             | 1483–1560      | 167       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGAGTGTGCTGCCAGCACAT-3’                 |                |           |           |
| Probe                     | 5’-GGAGTGTGCTGCCAGCACAT-3’                 |                |           |           |
| CTR (RT-PCR)               | 5’-TCTGTGACTCGAAGCTGCCTTGGAGGA-3’          | 1072–1384      | 313       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-TCTGTGACTCGAAGCTGCCTTGGAGGA-3’          |                |           |           |
| Probe                     | 5’-TCTGTGACTCGAAGCTGCCTTGGAGGA-3’          |                |           |           |
| NFAT2 (RT-PCR and real-time) | 5’-TGAGCTGGTGCTTGAGA-3’                  | 1483–1560      | 167       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| NFAT2 (RT-PCR)             | 5’-TGAGCTGGTGCTTGAGA-3’                    | 1685–1776      | 91        |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| β-Actin (real-time)        | 5’-TGAGCTGGTGCTTGAGA-3’                    | 1685–1776      | 91        |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| β-Actin (RT-PCR)           | 5’-TGAGCTGGTGCTTGAGA-3’                    | 1483–1560      | 167       |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| IL-4Ra (RT-PCR)            | 5’-TGAGCTGGTGCTTGAGA-3’                    | 1685–1776      | 91        |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| IL-13Ra1 (RT-PCR)          | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 | 1685–1776      | 91        |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| IL-13Ra2 (RT-PCR)          | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 | 1685–1776      | 91        |           |
| Sense primer               |                                             |                |           |           |
| Antisense primer           | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Probe                     | 5’-GGCCCTTCAGGCTTTGCCAG-3’                 |                |           |           |
| Analysis of RANKL and OPG Protein—The protein synthesis of RANKL and OPG was assessed by measuring the levels of RANKL and OPG in calvarial bones and calvarial osteoblasts using commercially available ELISA kits (20). Calvarial bones were dissected from 6–7-day-old mice (CsA) and divided into two halves along the sagittal suture. Following preincubation, a total of 8 calvarial halves per group were individually incubated in 24-well plates in the absence or presence of 10 nmol/liter D3 with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 48 h. Following treatment with 0.2% Triton X-100, extracted bone samples and osteoblasts were analyzed using the manufacturer’s protocols for the ELISAs. The sensitivities of the immunoassays were 5 pg/ml. D3 was used as a positive control and, as expected, resulted in an increased protein level of RANKL, and a decreased protein level of OPG in calvarial bones.
TABLE 2
Comparison of the effects of interleukin-4, interleukin-13, and indomethacin on 45Ca release from neonatal mouse calvariae stimulated by parathyroid hormone, 1,25-(OH)2-vitamin D3, interleukin-1β, tumor necrosis factor-α, interleukin-6 + soluble interleukin-6 receptor, osteoclast marker, RANKL, and receptor activator of nuclear factor-κ B ligand.

| Stimulator | Amount | + IL-4 | + IL-13 | + Indo |
|------------|--------|--------|--------|--------|
| PTH        | 10 nm  | 275 ± 13 | 217 ± 10 | 174 ± 7 | 324 ± 42 | 297 ± 58 |
| D3         | 10 nm  | 371 ± 7  | 217 ± 26 | 187 ± 25 | 272 ± 46 | 326 ± 54 |
| IL-1β      | 300 pg/ml | 205 ± 11 | 123 ± 9  | 126 ± 4  | 315 ± 30 | 196 ± 31 |
| TNF-α      | 100 ng/ml | 160 ± 8 | 101 ± 3  | 102 ± 4  | 177 ± 20 | 161 ± 13 |
| IL-6 + IL-6R | 100/100 ng/ml | 165 ± 7 | 118 ± 3  | 211 ± 13 | 163 ± 7  | |
| OSM        | 10 ng/ml | 214 ± 9  | 124 ± 7  | 138 ± 4  | 217 ± 37 | 133 ± 11 |
| RANKL      | 100 ng/ml | 279 ± 10 | 172 ± 9  | 106 ± 5  | 246 ± 21 | 236 ± 49 |

4 In each experiment, the release of 45Ca from untreated control bones was set to 100%. Data shown were obtained in 13 different experiments and are mean ± S.E. for 6–24 bones/group. The average release of 45Ca from untreated control bones varied in the 13 experiments from 6.9 to 15.6% of initial 45Ca.

5 Bones were preincubated in the absence or presence of IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 24 h. Preincubation was followed by treatment with or without PTH, D3, IL-1β, TNF-α, IL-6 + IL-6R, OSM, and RANKL in the absence or presence of IL-4 or IL-13 for 96 h. The release of 45Ca, compared to untreated control bones, during the 96-h period is shown.

Statistics—Statistical analysis of multiple treatment groups was performed using one-way analysis of variance with Levene’s homogeneity test and Bonferroni, Dunn’s 2-sided or Dunn’s T3 post-hoc test. In Fig. 10 the non-parametric Kruskal-Wallis and Mann-Whitney tests were used. Results are expressed as mean ± S.E. S.E. is shown when the height of the error bar is larger than the radius of the symbol. All experiments were repeated at least twice with comparable results. The semi-quantitative RT-PCR analyses from one individual experiment was repeated at least once with comparable results.

RESULTS

Effects of IL-4 and IL-13 on Bone Resorption in Mouse Calvariae—When either IL-4 (50 ng/ml) or IL-13 (50 ng/ml) was added to mouse calvarial bones, it was found that 45Ca release stimulated by a variety of agents: PTH (10 nmol/liter), D3 (10 nmol/L), IL-1β (300 pg/ml), TNF-α (100 ng/ml), IL-6 (100 ng/ml), in combination with the soluble IL-6 receptor (100 ng/ml), OSM (10 ng/ml), and RANKL (100 ng/ml) was significantly decreased in every instance (Table 2). IL-4 and IL-13 also inhibited 45Ca release stimulated by trans-retinoic acid (100 nmol/liter) and dexamethasone (1 μmol/liter). IL-4 and IL-13 were more effective inhibitors of cytokine-stimulated 45Ca release than 45Ca release caused by peptide and steroid hormones. The inhibitory effects of IL-4 and IL-13 were concentration-dependent and the cytokines were found to be equipotent in bones stimulated by either D3 (10 nmol/liter; Fig. 1A) or RANKL (100 ng/ml; Fig. 1B).

In bones stimulated by D3 (10 nmol/liter), 45Ca release was inhibited by IL-4 (50 ng/ml) from 24 to 120 h (Fig. 1C). In contrast, the initial inhibition of 45Ca release caused by CT (1 nmol/liter) in D3-stimulated bones was lost over time (data not shown). When IL-4 (50 ng/ml) or CT (1 nmol/liter) was added to bones prestimulated by D3 (10 nmol/liter) for 72 h, 45Ca release was subsequently inhibited by CT, but not by IL-4 (Fig. 1D).

Stimulation of 45Ca release caused by PTH (10 nmol/liter), D3 (10 nmol/liter), TNF-α (100 ng/ml), and RANKL (100 ng/ml) was unaffected by simultaneous addition of indomethacin (1 μmol/liter; Table 2). In contrast, 45Ca release stimulated by IL-1β (300 pg/ml), IL-6 (100 ng/ml) in combination with soluble IL-6 receptor (100 ng/ml), and OSM (10 ng/ml) was partially reduced by indomethacin (Table 2).

Effects of IL-4 and IL-13 on Gene Expression in Mouse Calvariae—When the effects of IL-4 (50 ng/ml) and IL-13 (50 ng/ml) on mRNA expression of osteoclastic markers were compared with expression in control bones, semiquantitative RT-PCR analysis revealed that TRAP and CTR mRNA were decreased by IL-4 and IL-13 treatment (Fig. 2A). Similarly, in bones stimulated by D3 (10 nmol/liter), it was found that IL-4 and IL-13 decreased the D3 enhanced expression of TRAP and CTR mRNA (Fig. 2B).

Semiquantitative RT-PCR further demonstrated that the basal, unstimulated mRNA expression of RANK was clearly decreased by both IL-4 and IL-13, with no effect on RANKL mRNA expression and a small increase in OPG mRNA (Fig. 2A). In bones stimulated by D3 (10 nmol/liter), RANKL mRNA was slightly increased, an effect decreased by IL-4 and IL-13 (Fig. 2B). As expected, the mRNA of RANKL was up-regulated by D3, and this effect was clearly decreased by IL-4 and IL-13 (Fig. 2B). In addition, the expected down-regulation of OPG mRNA by D3 was reversed by addition of IL-4 and IL-13 (Fig. 2B).

The inhibitory effects of IL-4 and IL-13 on D3-stimulated RANK and RANKL mRNA in mouse calvarial bones were confirmed using quantitative real-time RT-PCR analyses (Fig. 2, C and D). Likewise, the decrease of OPG mRNA caused by D3, and the reversals caused by IL-4 and IL-13 were confirmed using quantitative real-time RT-PCR analyses (Fig. 2E).

Effects of IL-4 and IL-13 on RANKL and OPG Protein Expression in Mouse Calvariae—The amount of RANK protein in D3 (10 nmol/liter) stimulated mouse calvarial bones was increased more than 10-fold after 48 h of stimulation (Fig. 3A). The enhancement of RANK protein caused by D3 was significantly decreased by IL-4 (50 ng/ml) and IL-13 (50 ng/ml).

IL-4 (50 ng/ml) and IL-13 (50 ng/ml) treatment for 48 h significantly enhanced OPG protein levels in calvarial bones (Fig. 3B). The amount of OPG protein in mouse calvarial bones was significantly decreased by treatment with D3 (10 nmol/liter) for 48 h, an effect that was reversed by addition of IL-4 (50 ng/ml) and IL-13 (50 ng/ml); Fig. 3B).

Effects of IL-4 and IL-13 on RANKL and OPG mRNA in Mouse Calvarial Osteoblasts—Quantitative real-time RT-PCR analyses demonstrated that stimulation of mouse calvarial osteoblasts for 48 h with D3 (10 nmol/liter) resulted in the expected increase in RANKL mRNA and the expected decrease in OPG mRNA (Fig. 4, A and B). IL-4 (50 ng/ml) and IL-13 (50 ng/ml) significantly decreased the enhancement of RANKL mRNA by D3 (Fig. 4A) and significantly reversed the down-regulation of OPG mRNA by D3 (Fig. 4B).

Similar data were also observed in osteoblast cultures treated with D3 and cytokines for 8 h (data not shown).

6 U. H. Lerner and H. H. Conaway, unpublished data.
Protein levels of RANKL in calvarial osteoblasts were too low to be detected by the ELISA method used, both in unstimulated control cells and in cells stimulated by D3. However, OPG was more highly expressed and stimulation by D3 for 48 h resulted in decreased amounts of OPG protein, an effect that was reversed both by IL-4 (50 ng/ml) and IL-13 (50 ng/ml; Fig. 4C).

Effects of IL-4 and IL-13 on Osteoclastogenesis and Gene Expression in Mouse Spleen Cells—The development of TRAP^+ multinucleated osteoclasts in mouse spleen cell cultures induced by co-treatment with M-CSF (25 ng/ml) and RANKL (100 ng/ml) was abolished by addition of IL-4 (50 ng/ml) and IL-13 (50 ng/ml; Fig. 5A). In addition, no TRAP^+ mononuclear cells were seen in the IL-4- and IL-13-treated cultures (data not shown). In agreement with previous observations in IL-4-treated mouse bone marrow cultures (34), a substantial number of TRAP^+ multinucleated cells were seen in both the IL-4- and IL-13-treated spleen cell cultures, but no such cells were seen in the OPG-treated spleen cells (data not shown).

Semiquantitative RT-PCR indicated that stimulation of osteoclastogenesis by M-CSF/RANKL was associated with significantly increased levels of CTR, TRAP, cathepsin K, RANK, and NFAT2 mRNA (Fig. 5B). Quantitative real-time RT-PCR demonstrated that IL-4 and IL-13 significantly decreased the M-CSF/RANKL-induced up-regulation of CTR, TRAP, and cathepsin K mRNA (Fig. 5C–E). The expression of RANK mRNA in M-CSF/RANKL-treated spleen cells was significantly decreased by IL-4 and IL-13 (Fig. 5F). In addition, the increase of NFAT2 mRNA stimulated by M-CSF/RANKL was significantly decreased by IL-4 and IL-13 (Fig. 5G). In contrast, TRAF6 mRNA was unaffected by M-CSF/RANKL both in the absence and presence of IL-4 and IL-13 (data not shown).
Effects of IL-4 and IL-13 on Osteoclastogenesis and Gene Expression in Mouse Bone Marrow Macrophages—Treatment of BMM with M-CSF (100 ng/ml) and RANKL (50 ng/ml) for 4–5 days resulted in a large number of TRAP⁺ multinucleated osteoclasts, an effect substantially decreased by IL-4 (50 ng/ml) and IL-13 (50 ng/ml; Fig. 6A). In contrast to M-CSF- and RANKL-stimulated spleen cell cultures, no TRAP⁺- multinucleated cells were seen in IL-4- or IL-13-treated BMM (data not shown). Inhibition of osteoclastogenesis was associated with decreased mRNA expression of the osteoclast markers CTR and cathepsin K (Fig. 6, B and C). The increased mRNA expression of RANK induced by RANKL was also significantly decreased by IL-4 and IL-13 (Fig. 6D).
Effects of IL-4 and IL-13 on Gene Expression in RAW 264.7 Cells—The monocytic cell line RAW 264.7 can be induced to differentiate to the osteoclast lineage by treatment with RANKL (100 ng/ml). This was demonstrated by the time-dependent stimulations of mRNA expressions for CTR, TRAP, and cathepsin K (Fig. 7A). IL-4 (50 ng/ml), IL-13 (50 ng/ml), as well as OPG (300 ng/ml), decreased RANKL-stimulated enhancements of TRAP and CTR mRNA, with a more pronounced effect on CTR mRNA (Fig. 7B).

In RANKL-stimulated RAW 264.7 cells, semiquantitative RT-PCR revealed that mRNA expression of RANK was decreased by IL-4 and IL-13 (Fig. 7B). Quantitative real-time RT-PCR showed that the inhibitory effect of IL-4 on RANK mRNA was concentration-dependent (Fig. 7C).

Effects of IL-4 on RANK Protein in RAW 264.7 Cells—The expression of RANK protein was assessed by analyzing cell surface expression of RANK in RAW 264.7 cells using fluorescein isothiocyanate-labeled anti-RANK. Treatment of RAW 264.7 cells with IL-4 (50 ng/ml) for 48 h consistently decreased the binding of anti-RANK antibodies in six of six experiments. A representative experiment is shown in Fig. 7D. Analysis of data obtained in the six experiments showed that the effect was statistically significant (Fig. 7E).

Expression of IL-4 Receptor Components in Calvarial Osteoblasts, Spleen Cells, and Bone Marrow Macrophages—Mouse calvarial osteoblasts, mouse spleen cells, and mouse BMM were found to constitutively express mRNA for γc, IL-4Rα, IL-13Rα1, and IL-13Rα2 (Fig. 8, A–C).

D3 (10 nmol/liter) increased the mRNA expression of IL-4Rα in osteoblasts, but did not alter mRNA expression of γc, IL-13Rα1, or IL-13Rα2 (Fig. 8A). Expression of γc mRNA was enhanced by addition of IL-4 to osteoblasts treated with D3, but no effect was noted with IL-13. On the other hand, addition of either IL-4 or IL-13 to osteoblasts treated with D3 resulted in robust increases in IL-13Rα2 mRNA.

In spleen cells, M-CSF (25 ng/ml)/RANKL (100 ng/ml) decreased mRNA expression of IL-4Rα, IL-13Rα1, and IL-13Rα2, but did not alter mRNA expression of γc (Fig. 8B). Neither IL-4 (50 ng/ml) nor IL-13 (50 ng/ml) affected γc or countered the decrease of IL-4Rα and IL-13Rα1 mRNA produced by M-CSF plus RANKL. However, IL-13, but not IL-4, counteracted the decrease of IL-13Rα2 elicited by M-CSF plus RANKL.

In BMM, M-CSF and RANKL treatment decreased the mRNA expression of IL-4Rα and IL-13Rα2 (Fig. 8C). Very similar to the findings in spleen cells. IL-4 and IL-13 only marginally affected the decrease of IL-4Rα caused by M-CSF and RANKL, also similar to the observations in spleen cells. As such, like in spleen cells, IL-13 had a greater suppressive effect on mRNA of IL-13Rα1 than IL-4. However, in contrast to spleen cells, the decrease of IL-13Rα2 caused by M-CSF and RANKL was reversed by both IL-4 and IL-13 in BMM cells.
No Effects of IL-4 and IL-13 on Bone Resorption and mRNA Expressions of RANKL and RANK in Mouse Calvarial Bones from Stat6$^{-/-}$ Mice—D3 (10 nmol/liter) stimulated $^{45}$Ca release in cultured mouse calvarial bones from wild type mice (BALB/cJ) and from Stat6$^{-/-}$ and Stat4$^{-/-}$ mice in a BALB/c background to a similar degree (Fig. 9, A–C). Similarly, IL-1$\beta$ (300 pg/ml) stimulated $^{45}$Ca release in calvariae from wild type mice and Stat6$^{-/-}$ mice to a similar extent (Fig. 9, D and E). IL-4 (50 ng/ml) and IL-13 (50 ng/ml) did not inhibit D3-stimulated $^{45}$Ca release in bones from Stat6$^{-/-}$ mice (Fig. 9B). In contrast, significant inhibition was obtained in D3-stimulated bones from wild type (Fig. 9A) and Stat4$^{-/-}$ mice (Fig. 9C), which was in agreement with observations in bones from CsA mice (Table 2, Fig. 1A). Paralleling these observations, IL-4 and IL-13 did not inhibit $^{45}$Ca release in bones from Stat6-deficient mice stimulated by IL-1$\beta$ (Fig. 9D) and from Stat4$^{-/-}$ mice (data not shown).

Increased RANKL mRNA observed in D3 (10 nmol/liter)-stimulated calvarial bones from wild type mice was significantly decreased by IL-4 (50 ng/ml) and IL-13 (50 ng/ml; Fig. 9F). However, no inhibition of D3-stimulated RANKL mRNA was obtained by IL-4 and IL-13 treatment of bones from Stat6$^{-/-}$ mice (Fig. 9G).

The enhancement of RANK mRNA expression in D3-stimulated calvariae from wild type mice was significantly decreased by IL-4 and IL-13 (Fig. 9F). In contrast, IL-4 and IL-13 did not affect D3-induced enhancement of RANK mRNA in bone from Stat6$^{-/-}$ mice (Fig. 9F).

No Effects of IL-4 and IL-13 on Osteoclastogenesis and Gene Expression in Spleen Cells from Stat6$^{-/-}$ Mice—Treatment of spleen cells from wild type mice, Stat4$^{-/-}$ and Stat6$^{-/-}$ mice with M-CSF (100 ng/ml) and RANKL (50 ng/ml) resulted in enhanced formation of TRAP$^{+}$ osteoclasts in spleen cell cultures from all three genotypes (Fig. 10, A–C). IL-4 (50 ng/ml) abolished and IL-13 (50 ng/ml) substantially decreased M-CSF/RANKL-stimulated TRAP$^{+}$ osteoclast formation in spleen cells from wild type and Stat4$^{-/-}$ mice (Fig. 10, A and C). In contrast, no inhibitory effect of IL-4 and IL-13 could be observed in spleen cell cultures from Stat6$^{-/-}$ mice (Fig. 10C). OPG (300 pg/ml), however, inhibited osteoclast formation in M-CSF- and RANKL-stimulated spleen cells from all three genotypes.
The increased mRNA expressions for CTR and cathepsin K observed in M-CSF/RANKL-treated cells from wild type and Stat6−/− mice were significantly inhibited by IL-4 and IL-13 in spleen cell cultures from wild type (Fig. 10, D and F), but not from Stat6−/− mice (Fig. 10, E and G). Similarly, the inhibition of TRAP mRNA by IL-4 and IL-13 in wild type cells was not observed in spleen cells from Stat6−/− mice (data not shown). In spleen cell cultures from Stat6−/− mice, IL-4 and IL-13 did not affect M-CSF/RANKL induced enhancement of RANK mRNA (Fig. 10), whereas significant inhibition was observed in spleen cells from wild type mice (Fig. 10H).

DISCUSSION

Since the initial discovery that osteoclast activating factor (45) was IL-1 (46), numerous cytokines have been shown to stimulate bone resorption and be important in the physiological and pathological remodeling of bone (15, 16). Much less is known about cytokines that inhibit bone resorption. In agreement with previous observations (24), we found that IL-4 inhibits bone resorption in neonatal mouse calvariae stimulated by PTH, D3, and IL-1β. In ongoing projects, it has also been observed that IL-4 inhibits bone resorption in calvarial bones stimulated by trans-retinoic acid and dexamethasone, ligands that activate the retinoic acid and glucocorticoid hormone receptors, respectively.4 In the present study, IL-4 was additionally found to inhibit bone resorption stimulated by the cytokines TNF-α, IL-6 (in the presence of the soluble IL-6 receptor), OSM, and RANKL. The capacity of IL-4 to inhibit resorption induced by different stimulators was shared with IL-13. Furthermore, IL-4 and IL-13 were found to be equipotent.

IL-4 and IL-13 inhibit prostaglandin biosynthesis by decreasing mRNA expression of cyclooxygenase-2 in osteoblasts and it has been suggested that the cytokines inhibit IL-1-induced bone resorption by suppressing prostaglandin biosynthesis (32, 33). In agreement with this hypothesis, it was observed in initial experiments that IL-4/IL-13, as
FIGURE 7. Interleukin-4 and interleukin-13 decrease osteoclast differentiation and the mRNA and protein expression of RANK in Raw 264.7 cells. Semiquantitative RT-PCR analyses show the time-dependent mRNA expressions of CTR, TRAP, and cathepsin K in RAW 264.7 cells cultured in the absence (-) and presence (+) of RANKL (100 ng/ml) for 2, 4, and 6 days (A). In B, RAW 264.7 cells were cultured in the presence of RANKL (100 ng/ml) with and without IL-4 (50 ng/ml), IL-13 (50 ng/ml), and OPG (300 ng/ml) for 6 days with the expression of mRNA for RANK, TRAP, and CTR assessed using semiquantitative RT-PCR. In C, quantitative real-time RT-PCR analysis shows the concentration-dependent inhibitory effect of 24 h IL-4 treatment on RANK mRNA expression in RAW 264.7 cells. The inhibitory effect of IL-4 was statistically significant (p < 0.05) at and above 0.5 ng/ml. In D a representative fluorescence-activated cell sorter analysis showing the effect of IL-4 (50 ng/ml) on RANK protein expression on the cell surface of RAW 264.7 cells is demonstrated, and in E the cumulative data obtained in six independent experiments are given. The data shown in E are expressed as percent of control and the amount of cells expressing RANK in control cultures was 50.2 ± 0.6% (mean ± S.E.; n = 6). Statistically significant effects are shown by the asterisks; * p < 0.05. The expression of mRNA for the genes of interest was normalized to that of GAPDH in the semiquantitative RT-PCR analyses and to that of β-actin in the quantitative real-time RT-PCR analyses. The figures shown in A and B denote the number of RT-PCR cycles. Values in C and E are means for five and six wells, respectively, and S.E. is shown as vertical bars when larger than the height of the symbol. Cells cultured in the absence of RANKL were used as controls (Co).

FIGURE 8. The four receptor proteins known to constitute the different components of the interleukin-4 and interleukin-13 receptors can be found in mouse calvarial osteoblasts, mouse spleen cells, and bone marrow macrophages and are regulated by D3 and M-CSF/RANKL, as well as by interleukin-4 and interleukin-13. Mouse calvarial osteoblasts were cultured in the absence (control) or presence of D3 (10 nmol/liter), with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml), for 6 days with the expression of mRNA for IL-4Ra, IL-13Ra1, and IL-13Ra2 was assessed by semiquantitative RT-PCR (A). Mouse spleen cells were cultured in the presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml) (M/R), with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml), for 4 days and the expression of γC, IL-4Ra, IL-13Ra1, and IL-13Ra2 was assessed by semiquantitative RT-PCR (B). Mouse BMM were cultured in the presence of M-CSF (100 ng/ml) and RANKL (50 ng/ml) (M/R), with and without IL-4 (50 ng/ml) or IL-13 (50 ng/ml), for 4 days and the expression of γC, IL-4Ra, IL-13Ra1, and IL-13Ra2 was assessed by semiquantitative RT-PCR (C). The mRNA expression of the genes of interest was normalized to that of GAPDH. Numbers indicate the number of cycles in the RT-PCR analyses.
well as indomethacin, inhibited resorption of mouse calvariae stimulated by IL-1β, IL-6 (plus soluble IL-6 receptor), and OSM. However, whereas bone resorption stimulated by PTH, D3, TNF-α, and RANKL was unaffected by indomethacin, significant inhibition was noted in the presence of IL-4 and IL-13. Similarly, it has been observed that the stimulatory effects of trans-retinoic acid and dexamethasone are inhibited by IL-4 and IL-13 and unaffected by indomethacin. Together, these findings suggest that inhibitory effects of IL-4 and IL-13 on bone resorption must involve mechanisms that are not dependent on inhibition of cyclooxygenase-2 induction.

In contrast to the transient inhibitory effect of CT that was observed in time course experiments, inhibition of bone resorption by IL-4 was sustained in long term (5 days) cultures. However, IL-4 did not cause inhibition when the cytokine was added to bones in which resorption was prestimulated by treatment with D3. This also differed from the response to CT and suggested that the action of IL-4 does not involve the activity of terminally differentiated osteoclasts, but is because of inhibition of mononuclear osteoclast progenitor cell differentiation. This view was supported by results showing that both IL-4 and IL-13 decreased the expression of TRAP and CTR mRNA in calvarial bones. In contrast to our findings, a recent study has reported that IL-4, but not IL-13, inhibits the activity of mature osteoclasts in cell cultures (47). Aside from the possibility that differential expression of receptor components in the two systems might be occurring, it is not clear why mature osteoclasts in cell cultures and the osteoclasts found in D3 prestimulated calvarial bones might differ in their sensitivity to IL-4 and IL-13.

The finding that both IL-4 and IL-13 inhibited bone resorption stimulated by many different agonists supports the idea that the cytokines may be inhibiting a primary mechanism involved in resorption. This, together with data indicating that the cytokines can inhibit osteoclast progenitor cell differentiation, suggests that IL-4 and IL-13 may be inhibiting a mechanism commonly employed for stimulation of osteoclast differentiation. One obvious candidate for this is the RANKL-RANK-OPG system. Thus, effects of IL-4 and IL-13 on bone resorption stimulated by recombinant RANKL, mRNA expression of RANK, RANKL, and OPG, and the protein levels of RANKL and OPG in calvarial bones were evaluated.

IL-4 and IL-13 were found to inhibit RANKL-induced mineral release in calvarial bones with similar potencies and with dose-response curves that were closely aligned to those observed in D3-stimulated bones. This suggests that IL-4 and IL-13 inhibit bone resorption by targeting receptors on RANKL-sensitive cells and inhibiting either the interaction between RANK and RANKL, or mechanisms downstream of receptor activation. RANK signaling includes TRAF6-mediated phosphorylation of IκBα, which leads to the release of NF-κB dimers and their translo-
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expressions of TRAP and CTR, and formation of TRAP and CTR in RANKL-stimulated RAW 264.7 cells, IL-4 inhibited mRNA expression of TRAP, RANK, as well as the enhancement of RANK mRNA caused by D3 in calvarial bones. In addition to inhibiting mRNA expression of TRAP and CTR in RANKL-stimulated RAW 264.7 cells, IL-4 inhibited mRNA expressions of TRAP and CTR, and formation of TRAP multilocusated osteoclasts in M-CSF/RANKL-stimulated mouse spleen cells and BMM. Effects similar to the inhibitory effects of IL-4 on osteoclast differentiation in RAW 264.7 cells, mouse spleen cells, and BMM were also noted following treatment with IL-13. Furthermore, in agreement with observations in calvarial bones, IL-4 and IL-13 were found to inhibit mRNA expression of RANK in mouse spleen cells, BMM, and RAW 264.7 cells. The dosages of IL-4 required for inhibition of bone resorption in mouse calvariae and RANK mRNA expression in RAW 264.7 cells were very similar, with concentration-dependent effects observed at 0.15 ng/ml and above. In the RAW cell line it was also demonstrated that IL-4 and IL-13 receptors on osteoclast progenitor cells are linked to inhibition of RANK signaling, either at steps in the cascade of events required for osteoclast differentiation, or at a step proximal to NF-kB, JNK, and ERK.

Semiquantitative RT-PCR and quantitative real-time RT-PCR showed that IL-4 and IL-13 inhibited the basal mRNA expression of RANK, as well as the enhancement of RANK mRNA caused by D3 in calvarial bones. In addition to inhibiting mRNA expression of TRAP and CTR in RANKL-stimulated RAW 264.7 cells, IL-4 inhibited mRNA expressions of TRAP and CTR, and formation of TRAP multilocusated osteoclasts in M-CSF/RANKL-stimulated mouse spleen cells and BMM. Effects similar to the inhibitory effects of IL-4 on osteoclast differentiation in RAW 264.7 cells, mouse spleen cells, and BMM were also noted following treatment with IL-13. Furthermore, in agreement with observations in calvarial bones, IL-4 and IL-13 were found to inhibit mRNA expression of RANK in mouse spleen cells, BMM, and RAW 264.7 cells. The dosages of IL-4 required for inhibition of bone resorption in mouse calvariae and RANK mRNA expression in RAW 264.7 cells were very similar, with concentration-dependent effects observed at 0.15 ng/ml and above. In the RAW cell line it was also demonstrated that IL-4 and IL-13 receptors on osteoclast progenitor cells are linked to inhibition of RANK signaling, either at steps in the cascade of events required for osteoclast differentiation, or at a step proximal to NF-kB, JNK, and ERK.

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FIGURE 10. No effect on osteoclast formation or on the mRNA expressions of CTR, cathepsin K, and RANK by IL-4 and IL-13 in M-CSF/RANKL-stimulated spleen cell cultures from Stat6-deficient mice. In A–C, formation of multinucleated TRAP osteoclasts (TRAP OCL) in mouse spleen cultures from wt, Stat4−/−, and Stat6−/− mice stimulated by M-CSF (100 ng/ml) and RANKL (50 ng/ml) for 4 days with or without IL-4 (50 ng/ml) and IL-13 (50 ng/ml) and OPG (300 pg/ml) is shown. OPG inhibited OCL formation in M-CSF/RANKL-stimulated spleen cells from all three genotypes (A–C), whereas IL-4 abolished and IL-13 substantially inhibited the stimulatory effect on TRAP OCL formation induced by M-CSF/RANKL in wt and Stat4−/− mice (A and C), but had no effect in Stat6−/− mice (B). The data shown are expressed as percent of the number of TRAP OCLs obtained in wells stimulated by M-CSF/RANKL (100%). The number of TRAP OCLs in stimulated controls (A–C) varied from 173 to 273 cells/well. Data shown in A–C represent means for four wells and S.E. is shown as vertical bars. In D–I, quantitative real-time RT-PCR shows the effects of IL-4 (50 ng/ml) and IL-13 (50 ng/ml) treatment on mRNA expression of CTR, cathepsin K, and RANK in spleen cell cultures stimulated by M-CSF (100 ng/ml) and RANKL (50 ng/ml) (M/R) for 4 days. The increased mRNA expressions for CTR, cathepsin K, and RANK in M-CSF/RANKL-treated cells were significantly inhibited by IL-4 and IL-13 in spleen cell cultures from wt (D, F, and H), but not Stat6−/− mice (E, G, and I). The mRNA expressions of CTR, cathepsin K, and RANK were normalized to that of β-actin. The data shown are expressed as percent of the expression obtained in cells stimulated by M-CSF and RANKL (100%). Data shown in D–I represent means for four wells and S.E. is shown as vertical bars. Statistically significant effects are shown by the asterisks; *, p < 0.05.
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decreased RANK expression. However, it seems the inhibitory effect of IL-4 on RANK expression in osteoclast progenitor cells may be cell specific, for RANK mRNA expression in human peripheral blood T-lymphocytes activated by either phytohemagglutinin or anti-CD3 has been reported to be enhanced by IL-4 (50).

Mechanisms proposed for inhibition of osteoclastogenesis by IL-4 include a direct effect of IL-4 on osteoclast progenitor cells and an indirect effect mediated by T-cells (37). The observation that inhibition of RANK expression by IL-4 and IL-13 was obtained both in M-CSF/RANKL-stimulated BMM, shown to be devoid of T-cells, and in RANKL-stimulated RAW 264.7 cells, indicate that decreased RANK mRNA was not an effect mediated by IL-4 or IL-13 stimulation of T-cells, but rather because of a direct action of the cytokines on osteoclast progenitor cells.

Inhibition of osteoclastogenesis in M-CSF/RANKL-stimulated spleen cells and BMM by IL-4 and IL-13 was associated with decreased mRNA expression of the master transcription factor for osteoclastogenesis, NFAT2. NFAT2 is activated by calcineurin-regulated dephosphorylation (2). Calcineurin activation is regulated by intracellular calcium, which has been linked to activation of two immunoreceptor tyrosine-based activation motifs harboring adaptor proteins, FcRy and DAP12. Two of these molecules heterodimerize with different ligand recognizing receptors such as DAP12-associated receptors, osteoclast-associated receptor, and paired immunoglobulin-like receptor-A. The current data showing decreased expression of NFAT2 mRNA caused by IL-4 and IL-13 suggest that not only was RANK signaling inhibited, but that FcRy/DAP12 signaling could also be inhibited (51). In agreement with this, preliminary data have indicated that IL-4 and IL-13 inhibit the mRNA expression of osteoclast-associated receptor in bone marrow macrophages.5

Osteoclastogenesis and bone resorption are dependent on both stromal cells/osteoblasts expressing RANKL/OPG and osteoclast progenitor cells expressing RANK (52). In neonatal mouse calvariae, bone resorption stimulated by D3 is associated with increased RANKL mRNA and protein, and decreased OPG mRNA and protein (20). Assessments of RANKL and OPG expression, using semiquantitative RT-PCR and quantitative real-time RT-PCR for mRNA expression and ELISA for protein analyses, showed that IL-4 and IL-13 inhibited the D3-stimulated enhancement of RANKL and reversed the D3-induced decrease of OPG in calvariae. Similar observations were made in osteoblasts isolated from the calvarial bones, in which IL-4 and IL-13 were found to decrease D3 stimulation of RANKL mRNA expression. In addition, IL-4 and IL-13 counteracted the D3-induced down-regulation of OPG mRNA and OPG protein in the isolated calvarial osteoclasts. These observations point to the presence of IL-4 and IL-13 receptors in osteoclasts and suggest that the two cytokines inhibit osteoclast differentiation, not only by interacting with osteoclast progenitor cells, but also by interfering with the osteoclastogenesis inducing capacity of stromal cells/osteoblasts. In agreement with the present observations, IL-4 has recently been shown to decrease RANKL expression and increase OPG expression in human fibroblast-like synoviocytes from patients with rheumatoid arthritis. Furthermore, osteoclast formation in D3-stimulated co-cultures of synoviocytes and human peripheral blood mononuclear cells was inhibited by IL-4 (53). Local overexpression of IL-4 in mice with collagen-induced arthritis has also been reported to cause a decrease in mRNA expression of RANKL in synovial extracts (28). However, unlike the current study, no change in OPG mRNA was noted in the mouse arthritis model.

Because control of gene expression by receptors for IL-4 and IL-13 has been shown to be dependent on homodimerization of the transcription factor STAT6 (21), we sought to determine whether the effects of these two cytokines on bone resorption, osteoclast formation, and expression of RANKL and RANK were also STAT6 dependent. Using mouse calvarial bones from STAT6-deficient mice, we found that the inhibitory effects of IL-4 and IL-13 on bone resorption, stimulated by either D3 or IL-1β were lost. Also, the inhibitory effects of IL-4 and IL-13 on the enhancement of RANKL and RANK expression by D3 were abolished in calvarial bones from STAT6-deficient mice, but not in bones from STAT4-deficient mice. Likewise, the inhibitory effects of IL-4 and IL-13 on osteoclast formation, mRNA expression of the osteoclastic genes CTR, TRAP, and cathepsin K, and on the mRNA expression of RANK were absent in M-CSF/RANKL-stimulated spleen cells from STAT6-deficient mice, but still present in cells from STAT4-deficient mice. These observations indicate that STAT6 plays a crucial role in both osteoblasts and osteoclast progenitor cells to mediate the inhibitory effects of IL-4 and IL-13 on osteoclast formation and bone resorption.

Following treatment with IL-4 and IL-13, the mRNA expression profiles of RANKL, OPG, and RANK suggested that receptors for IL-4 and IL-13 existed in both osteoblasts and osteoclast progenitor cells. In subsequent experiments, mRNA expression of the components (IL-4Ra, γc, IL-13Rα, and IL-13Rα2) known to constitute the IL-4 and IL-13 receptor subtypes was observed in osteoblasts, spleen cells, and BMM. However, because there was expression of mRNA for all of the components, these results do not distinguish if the effects of the cytokines on RANKL, OPG, and RANK were mediated by IL-4 receptors Type 1 or 2 or by the IL-13 receptor. Because IL-13Rα2 was expressed, it is also possible that binding to this putative decoy receptor may have affected the response of IL-13.

In spleen cells and BMM, M-CSF/RANKL had no effect on mRNA expression of the γc receptor component, but decreased mRNA expression of IL-4Ra and IL-13Ra2. This suggests that increased osteoclast differentiation might normally be characterized by decreased sensitivity to the IL-4 and IL-13 receptors. Although addition of IL-4 and IL-13 to spleen cell and BMM cultures treated with M-CSF/RANKL counteracted the decrease in IL-13Rα2 mRNA, the similar inhibitory effectiveness of IL-4 and IL-13 in spleen cells argues against a decoy function of IL-13Rα2 that might significantly depress responsiveness to IL-13.

D3 increased IL-4Ra mRNA in osteoblasts, but had no effect on mRNA expression of the γc, IL-13Rα1, or IL-13Rα2 components. It is possible that up-regulation of IL-4Ra mRNA by IL-4 increased Type 1 and 2 IL-4 receptors and enhanced the inhibitory effect of IL-4 in osteoblasts treated with D3. In addition, because both IL-4 and IL-13 caused up-regulation of IL-13Rα2 mRNA, it is possible that the responses of the Type 2 IL-4 receptor and the IL-13 receptor were decreased. However, no dramatic changes in receptor function were apparent, for IL-4 and IL-13 were equally effective in osteoblasts treated with D3 and equipotent as inhibitors of bone resorption in mouse calvariae.

In summary, data in the present study suggest that IL-4 and IL-13 can function by inhibiting mechanisms that are commonly used by agents to stimulate osteoclastic differentiation and bone resorption. One mechanism used by IL-4 and IL-13 appears to involve activation of receptors on osteoclasts, which subsequently causes a decrease in RANK formation in osteoclast progenitor cells. A second mechanism thought to play an important inhibitory role is activation of IL-4 and IL-13 receptors on osteoblasts, which leads to decreased RANKL formation and increased OPG formation. Both mechanisms are critically dependent on activation of STAT6 by IL-4 and IL-13.

5 P. Palmqvist, P. Lundberg, E. Persson, I. Lundgren, A. Lie, H. H. Conaway, and U. H. Lerner, unpublished data.
