

The Antirheumatic Drug Leflunomide Inhibits Osteoclastogenesis by Interfering With Receptor Activator of NF- κ B Ligand–Stimulated Induction of Nuclear Factor of Activated T Cells c1

Makoto Urushibara,¹ Hiroshi Takayanagi,² Takako Koga,² Sunhwa Kim,³ Miho Isobe,² Yasuyuki Morishita,³ Takumi Nakagawa,⁴ Monika Loeffler,⁵ Tatsuhiko Kodama,⁶ Hisashi Kurosawa,⁴ and Tadatsugu Taniguchi³

Objective. Suppression of bone destruction is required as part of an effective therapeutic strategy for autoimmune arthritis. Although numerous antirheumatic drugs are in clinical use, little is known about whether they inhibit bone destruction by acting on activated T cells or other cell types, such as bone-resorbing osteoclasts. This study was undertaken to determine whether leflunomide has a direct action on

the osteoclast lineage and to gain insights into the molecular basis for the bone-protective effect of leflunomide.

Methods. The direct effect of leflunomide on osteoclast differentiation was investigated using an in vitro culture system of bone marrow monocyte/macrophages stimulated with receptor activator of NF- κ B ligand (RANKL) and macrophage colony-stimulating factor. The molecular mechanism of the inhibition was analyzed by genome-wide screening. The T cell-independent effect of leflunomide was examined in *rag-2*^{-/-} mice.

Results. Leflunomide blocked de novo pyrimidine synthesis and RANKL-induced calcium signaling in osteoclast precursor cells in vitro; hence, the induction of nuclear factor of activated T cells c1 (NF-ATc1) was strongly inhibited. The inhibition of this pathway is central to the action of leflunomide, since the inhibition was overcome by ectopic expression of NF-ATc1 in the precursor cells. Leflunomide suppressed endotoxin-induced inflammatory bone destruction even in *rag-2*^{-/-} mice.

Conclusion. Leflunomide has a direct inhibitory effect on RANKL-mediated osteoclast differentiation by inhibiting the induction of NF-ATc1, the master switch regulator for osteoclast differentiation. Our study suggests that the direct inhibitory action of leflunomide on osteoclast differentiation constitutes an important aspect in the amelioration of bone destruction, and that the RANKL-dependent NF-ATc1 induction pathway is a promising target for pharmacologic intervention in arthritic bone destruction.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of syno-

Supported in part by a grant for Advanced Research on Cancer from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a grant from PRESTO, Japan Science and Technology Corporation (JST), a grant from the Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Japan Society for the Promotion of Science, Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan, and a grant from the Japan Orthopaedics and Traumatology Foundation.

¹Makoto Urushibara, MD: Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ²Hiroshi Takayanagi, MD, PhD, Takako Koga, MSc, Miho Isobe, BSc: Graduate School of Medicine, University of Tokyo, Tokyo, Japan, PRESTO, Japan Science and Technology Corporation, Saitama, Japan, and Graduate School, Tokyo Medical and Dental University and COE Program, Tokyo, Japan; ³Sunhwa Kim, MSc, Yasuyuki Morishita, BSc, Tadatsugu Taniguchi, PhD: Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ⁴Takumi Nakagawa, MD, PhD, Hisashi Kurosawa, MD, PhD: Juntendo University School of Medicine, Tokyo, Japan; ⁵Monika Loeffler, PhD: Molecular Enzymology Group, Institute for Physiological Chemistry, Philipps-University, Marburg, Germany; ⁶Tatsuhiko Kodama, MD, PhD: Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan.

Drs. Urushibara and Takayanagi contributed equally to this work.

Address correspondence and reprint requests to Tadatsugu Taniguchi, PhD, Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: tada@m.u-tokyo.ac.jp.

Submitted for publication June 4, 2003; accepted in revised form November 20, 2003.

vial joints, resulting in crippling bone destruction (1). Combined use of disease-modifying antirheumatic drugs (DMARDs) has greatly contributed to the improvement of patients' symptoms, such as pain and swelling, but most of the antirheumatic drugs were originally developed to suppress immune reactions. Consequently, many patients experience severe bone destruction and undergo joint replacement surgery in spite of long-term use of immunosuppressive drugs. Thus, it is widely accepted that antirheumatic drugs should have the capacity to suppress bone destruction (2,3).

Several lines of evidence indicate that bone destruction in arthritis is mainly mediated by bone-resorbing osteoclasts, and enhanced expression of receptor activator of NF- κ B ligand (RANKL) is responsible for the aberrant activation of osteoclastogenesis in inflammatory lesions. First, RANKL is highly expressed by synovial fibroblasts and T cells in arthritic joints, and the soluble decoy receptor for RANKL, osteoprotegerin (OPG), inhibits osteoclastogenesis both in vitro and in vivo (4–7). Second, bone destruction in arthritis was greatly reduced in *RANKL*^{-/-} mice or *Fos*^{-/-} mice, both of which lack osteoclasts (8,9). Finally, it is notable that antiosteoclast therapy successfully ameliorated bone damage in several models of inflammatory bone destruction (4,10,11). Thus, inhibition of RANKL-mediated osteoclastogenesis may be one of the ideal means to control bone destruction in arthritis (12).

In arthritis, T cells are involved in several pathways that lead to bone damage (3). Aberrant activation of the immune system in arthritic synovium induces macrophage-derived proinflammatory cytokines, such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1), which strongly induce RANKL on synovial fibroblasts (5,13,14). To induce bone-associated pathogenic conditions, T cells are involved in the initiation and exacerbation of this synovitis. The importance of this pathway is underscored by the observation that the neutralization of these cytokines has significant therapeutic effects on bone destruction (2,3). In addition, activated T cells expressing RANKL may contribute directly to osteoclastogenesis (4). Consequently, the level of RANKL induced by both synovial fibroblasts and T cells becomes too high to be counterbalanced by inhibitors such as interferon- γ (IFN γ) or OPG (4,13). Although some antirheumatic drugs have bone-protective effects (15,16), this complex mechanism of bone damage makes it difficult to understand the molecular basis of these protective effects.

Leflunomide is a DMARD of the isoxazole class, and it inhibits de novo pyrimidine biosynthesis by acting

on dihydroorotate dehydrogenase (DHODH) (15,17). It has a protective effect against bone damage in animal models and in humans (17–19), and it has recently been introduced for the treatment of RA (15,17). It was previously reported that leflunomide suppresses proliferation or activation of T cells (20), and it has also been introduced as an immunosuppressive drug in the transplantation of allografts (21). However, it is currently unknown if leflunomide acts directly on the lineage of bone-resorbing osteoclasts.

NF-ATc1, also referred to as NF-AT2 or NF-ATc, is a member of the NF-AT (nuclear factor of activated T cells) family of transcription factors, originally discovered in the context of the activation of the immune system (22). Members of the NF-AT family are also involved in the function and development of diverse cells in other biologic systems, including cardiovascular and musculoskeletal systems in vertebrates, where they are under the control of a Ca²⁺-regulated phosphatase, calcineurin (23). Importantly, we have recently discovered that NF-ATc1 is strongly induced by RANKL and plays a central role in RANKL-induced osteoclast differentiation (24). Thus, NF-ATc1 may be a good therapeutic target in osteoclast-mediated osteopenic diseases, including RA.

In this study, we show that leflunomide acts directly on the osteoclast precursor cells of monocyte/macrophage lineage. Evidence is provided to show that leflunomide selectively inhibits RANKL-induced differentiation of osteoclasts by interfering with de novo pyrimidine synthesis and the Ca²⁺-NF-ATc1 pathway. We further demonstrate that leflunomide has a bone-protective effect on an endotoxin-induced bone destruction model in mice deficient in the *rag-2* gene, which indicates that the bone-protective effect of leflunomide is, at least in part, independent of T cells. Our results reveal a new aspect of this antirheumatic drug and provide evidence of an interesting interrelationship between Ca²⁺ signaling and de novo pyrimidine biosynthesis.

MATERIALS AND METHODS

In vitro differentiation of osteoclasts. The osteoclast formation system has been described previously (13,25). Briefly, nonadherent bone marrow cells derived from C57BL/6 mice were seeded (5×10^4 cells per well in a 24-well plate, $1.5\text{--}2.0 \times 10^7$ cells in a 10-cm dish) and cultured in α -minimum essential medium (α -MEM; Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) containing 10 ng/ml macrophage colony-stimulating factor (M-CSF; Genzyme, Cambridge, MA). Bone marrow monocyte/

macrophages (BMMs) were maintained with this concentration of M-CSF in all experiments. After 2 days, adherent cells were used as BMMs after washing out the nonadherent cells including lymphocytes. BMMs were further cultured in the presence of 100 ng/ml soluble RANKL (PeproTech, Rocky Hill, NJ) and 10 ng/ml M-CSF to generate osteoclasts. To evaluate the effect of leflunomide, an active metabolite of leflunomide (A77 1726, a gift from Aventis Pharmaceuticals, Bridgewater, NJ) was added at the same time as RANKL unless otherwise described. Three days later, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated (>3 nuclei) cells (TRAP+ MNCs) were counted. RAW 264.7 cells (2.5×10^4 cells per well in a 24-well plate) were stimulated with RANKL (100 ng/ml) in the presence or absence of leflunomide. After 5 days, TRAP+ MNCs were counted. TRAP+ MNCs were characterized by examining the bone-resorbing activity on dentine slices and the expression of calcitonin receptors, as previously described (13). The experiments in this study were approved by the institutional committee on animal experiments.

In vitro differentiation of osteoblasts. Osteoblasts were isolated from the calvaria of newborn (3–5 days old) mice by enzymatic digestion in 0.1% collagenase and 0.2% dispase, and were cultured in α -MEM with 10% FBS. After 2 days, cells were reseeded (5×10^4 cells per well in a 24-well dish), and cultured in α -MEM with 10% FBS containing 50 μ g/ml ascorbic acid, 10 mM sodium β -glycerophosphate, and 10 nM dexamethasone, in the presence or absence of leflunomide (30 μ M), as previously described (25).

Extraction and high-performance liquid chromatography (HPLC) assay of ribonucleotides. The extraction and assay of ribonucleotides have been previously described (26). Briefly, BMMs were stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of leflunomide (50 μ M). After 24 hours, cells were deproteinized with 10% trichloroacetic acid (TCA). The cell extracts were then centrifuged for 1 minute at 12,000g, and the TCA in the supernatants was removed by back extraction with water-saturated diethyl ether to pH 5. The extracts were examined by HPLC assay.

Activation of MAP kinases, Akt, and NF- κ B. BMMs, preincubated in the presence or absence of leflunomide (30 μ M) for 24 hours, were stimulated with RANKL and analyzed by immunoblotting using antiphosphorylated MAP kinase, anti-MAP kinase, antiphosphorylated Akt, or anti-Akt antibodies (New England Biolabs, Beverly, MA), as previously described (13,25). For an electrophoretic mobility shift assay, BMMs were preincubated in the presence or absence of leflunomide (30 μ M) for 24 hours. After RANKL stimulation, a cell extract was prepared and analyzed using an oligonucleotide probe containing the NF- κ B binding site of the IFN γ promoter, as previously described (13,25). Supershift was performed with an anti-RelA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Gene analysis. Genome-wide screening was performed using essentially the same system as previously described (24). Briefly, BMMs were stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of leflunomide (100 μ M). After 24 hours, total RNA was extracted from BMMs using a Sepasol RNA extraction kit (Nacalai Tesque, Kyoto, Japan). Total RNA (15 μ g) was utilized for complementary DNA synthesis by reverse transcription, followed by synthesis of biotinylated complementary RNA (cRNA) by in vitro transcription. After cRNA fragmentation, hybridization

with mouse U74Av2 GeneChip arrays (Affymetrix, Santa Clara, CA) displaying probes for 12,000 mouse genes/expressed sequence tags was performed according to the manufacturer's protocol. Chips were washed, stained with streptavidin-phycoerythrin, and analyzed using the Affymetrix GeneChip scanner and accompanying gene expression software.

Immunoblot analysis and immunofluorescence staining. To evaluate expression of downstream molecules of RANKL signaling during osteoclastogenesis, BMMs were stimulated with RANKL/M-CSF in the presence or absence of leflunomide (30 μ M). Whole-cell lysate of BMMs was examined every 24 hours after the addition of RANKL by immunoblot analysis using specific antibodies for NF-ATc1 (7A6), tumor necrosis factor receptor-associated factor 6 (TRAF6) (H-274), and c-Fos (H-125) (Santa Cruz Biotechnology). For immunostaining, cells were cultured with RANKL/M-CSF for 3 days in the presence or absence of leflunomide (30 μ M), fixed in 4% paraformaldehyde for 20 minutes, and treated with 0.2% Triton X-100 for 5 minutes. Cells were sequentially incubated in 5% bovine serum albumin/phosphate buffered saline (PBS) for 30 minutes, 2 μ g/ml anti-NF-ATc1 antibody and 4 μ g/ml anti-c-Fos (or anti-TRAF6) antibody in PBS-Tween for 60 minutes, and then 4 μ g/ml Alexa Fluor 488 or 8 μ g/ml Alexa Fluor 546-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 60 minutes. For immunostaining of DHODH, BMMs or mature osteoclasts were incubated with 500 nM MitoTracker Orange (Molecular Probes) for 20 minutes and fixed as described above. Cells were sequentially incubated with rabbit anti-DHODH polyclonal IgG (27,28), and then with 4 μ g/ml Alexa Fluor 488-labeled secondary antibody.

Retroviral gene transfer. The retroviral vectors pMX-NF-ATc1-IRES-EGFP (pMX-NF-ATc1), pMX-c-fos-IRES-EGFP (pMX-c-fos), and pMX-TRAF6-IRES-EGFP (pMX-TRAF6) have been previously described (13,24,25). Retrovirus packaging was performed by cotransfection of these pMX vectors and pPAMpsi2 (a gift from C. L. Sawyers) into 293T cells. Two days after inoculation, BMMs were stimulated with RANKL/M-CSF plus leflunomide (30 μ M). Osteoclastogenesis was evaluated 3 days after RANKL addition by TRAP staining and bone resorption assay, as previously described (13).

Ca²⁺ measurement. BMMs were incubated with RANKL/M-CSF for 48 hours in the presence or absence of leflunomide (30 μ M). Cells were loaded with calcium indicators (fluo-4 AM; Molecular Probes) and analyzed by confocal microscopy (C1; Nikon, Tokyo, Japan), as previously described (24). The increase in the ratio of fluorescence intensity from the basal level was divided by the maximum increase in the ratio obtained by adding 10 μ M ionomycin, and was expressed as the percentage maximum ratio increase.

Endotoxin-induced bone resorption. *Rag-2*^{-/-} mice (Taconic, Germantown, NY) have been previously described (29). Mice, 7 weeks old (n = 10), were administered a local calvarial injection of lipopolysaccharide (LPS; Sigma) at 25 mg/kg body weight, as previously described (25,30). Mice were treated with a single injection of leflunomide (10 mg/kg body weight) or saline at the site of the LPS injection. After 5 days, calvarial tissues were embedded and frozen in 5% carboxymethylcellulose (CMC) sodium, and serial sections were stained for TRAP (with hematoxylin) and NF-ATc1, as previ-

ously described (24). Parameters such as the osteoclast number per millimeter of trabecular bone surface and the number of NF-ATc1-positive cells were determined.

NF-ATc1 expression in RA tissues. With informed consent, tissues were obtained from the bone-synovium interface of RA patients who underwent knee joint replacement surgery because of severe bone destruction ($n = 4$). The samples were embedded and frozen in CMC sodium and analyzed as described above.

Statistical analysis. All data are expressed as the mean \pm SEM ($n = 6$). All experiments were repeated more than 3 times throughout the study. Statistical analysis was performed using Student's *t*-test.

RESULTS

Inhibition of RANKL-induced osteoclastogenesis by restriction of pyrimidine synthesis. To determine whether leflunomide acted directly on osteoclast differentiation (and, if so, how), we first examined the effect of leflunomide on *in vitro* osteoclast formation from BMMs stimulated with RANKL in the presence of M-CSF. As shown in Figure 1a, leflunomide strongly inhibited the formation of TRAP⁺ MNCs in a dose-dependent manner (1–100 μ M). The survival and proliferation of BMMs were not significantly affected by a low concentration of leflunomide, but higher concentrations of leflunomide had a suppressive effect on the proliferation of BMMs (Figure 1b), which was much less than that on osteoclast differentiation. In addition, a similar inhibitory effect was observed in RANKL-induced osteoclastogenesis of RAW 264.7 cells, which are independent of M-CSF, suggesting that leflunomide inhibits osteoclast differentiation by interfering with RANKL signaling (Figure 1c).

In the *de novo* pyrimidine synthesis pathway, DHODH is a critical enzyme that converts dihydroorotate into orotic acid, which is essential for the synthesis of UMP, which is phosphorylated to UTP (26). The inhibitory effect of leflunomide (concentrations of 30 μ M or less) on osteoclast differentiation was completely reversed by the addition of an excessive amount of uridine, suggesting that the blockade of *de novo* uridine synthesis is responsible for this inhibition (Figures 1a and d). The inhibition was only partially reversed at 100 μ M of leflunomide, suggesting a nonpyrimidine-related effect at higher concentrations. We also examined the stage at which the osteoclast formation process is inhibited by the presence of leflunomide during RANKL stimulation. The formation of TRAP⁺ MNCs was almost completely inhibited when leflunomide was added at an early stage, i.e., between 0 and 24 hours after RANKL stimulation, whereas such inhibition was not

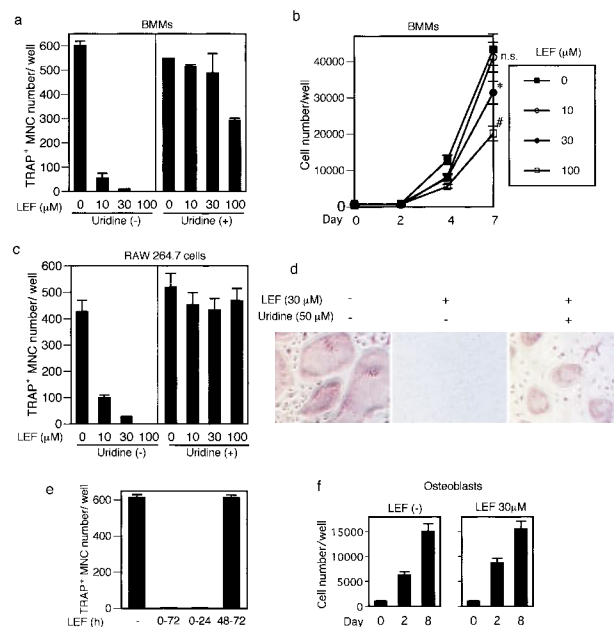


Figure 1. Effect of leflunomide (LEF) on receptor activator of NF- κ B ligand (RANKL)-induced osteoclastogenesis. **a**, LEF inhibited the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (TRAP⁺ MNCs) from bone marrow monocyte/macrophages (BMMs) stimulated with RANKL in the presence of macrophage colony-stimulating factor (M-CSF). This inhibition was reversed by the addition of uridine (50 μ M). **b**, Effect of LEF on the proliferation and survival of BMMs stimulated with M-CSF. * = $P < 0.05$; # = $P < 0.01$, versus proliferation with no LEF added. n.s. = not significant. **c**, Effect of LEF on the formation of TRAP⁺ MNCs from RAW 264.7 cells stimulated with RANKL. This inhibition was reversed by the addition of uridine (50 μ M). **d**, Photomicrograph of *in vitro* osteoclast differentiation from BMMs (TRAP staining). The inhibitory effect of LEF was reversed by uridine. **e**, Differentiation stage-dependence of the effect of LEF. The addition of LEF (30 μ M) at the early stage (0–24 hours) completely inhibited osteoclastogenesis, but addition at the late stage (48–72 hours) had no effect. **f**, Effect of LEF on the proliferation and differentiation of osteoblasts. LEF had no significant effect on the increase in the number of cultured osteoblasts. Values are the mean \pm SEM.

observed when the drug was added 48 hours after RANKL stimulation (Figure 1e). These results indicated that leflunomide suppresses cellular events that are activated by RANKL at an early stage of osteoclast differentiation.

On the other hand, when we examined the effect of leflunomide on the proliferation (Figure 1f) and differentiation (data not shown) of osteoblasts, in which RANKL signaling is not involved, little, if any, inhibitory effect was observed. These results showed that leflunomide selectively acts on the RANKL-dependent osteoclast differentiation process.

The importance of pyrimidine synthesis in

RANKL signaling suggests that DHODH, which is a critical enzyme for de novo pyrimidine synthesis and the known target of leflunomide, is induced by RANKL in osteoclast precursor cells. Using GeneChip analysis, we determined the level of messenger RNA (mRNA) for DHODH in BMMs stimulated with RANKL. As shown in Figure 2a, the level of DHODH mRNA was increased by RANKL. Furthermore, immunostaining was performed using an anti-DHODH antibody (28) and DHODH was found to be abundantly expressed by mature osteoclasts as well as by BMMs. Consistent with previous reports on other cell types (27), expression of DHODH was mainly observed in mitochondria (Figure 2b). Furthermore, using HPLC analysis, the concentration of pyrimidine ribonucleotides, including UMP and CMP, was evaluated in RANKL-stimulated BMMs. The concentration of all pyrimidines was decreased by the addition of leflunomide (Figure 2c), indicating that pyrimidine biosynthesis is actually suppressed by leflunomide in RANKL-stimulated BMMs, as seen in T cells (26). Collectively, these results suggest that de novo pyrimidine synthesis is important for RANKL-induced osteoclast differentiation, and leflunomide inhibits this process by targeting DHODH in osteoclast lineage.

Effect of leflunomide on downstream signaling pathways activated by RANKL. These results prompted us to investigate the mechanism by which leflunomide interferes with the intracellular signaling pathways activated by RANKL. Briefly, RANKL signals the cell by binding with its receptor, RANK, resulting in the activation of TRAF family proteins such as TRAF6, which activates the NF- κ B, MAP kinase, and Akt pathways (6,13,31). RANKL also induces c-Fos, which may function in the context of the activator protein 1 transcription factor complex (25,32,33). RANKL selectively induces the *NF-ATc1* gene in a TRAF6- and c-Fos-dependent manner (24). RANKL also evokes Ca^{2+} signaling, which leads to calcineurin-mediated activation of NF-ATc1, and this activation is necessary and sufficient for osteoclast differentiation (24).

To explore the molecular basis of leflunomide inhibition of osteoclastogenesis, we examined its effect on RANKL-induced activation of these downstream signaling molecules. As shown in Figure 3a, an inhibitory effect was not observed in the RANKL-induced activation of MAP kinases, including ERK, p38, and JNK, in leflunomide-treated BMMs. In addition, leflunomide had little, if any, effect on RANKL-induced activation of NF- κ B (Figure 3b) and Akt (Figure 3c), suggesting that these molecules may not be the targets of leflunomide action on RANKL signaling.

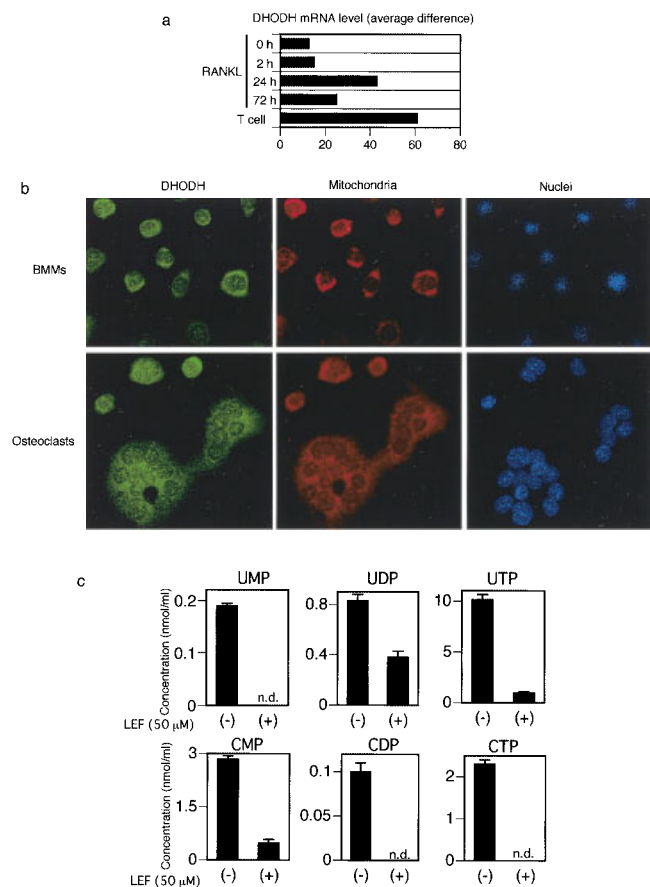


Figure 2. Expression of dihydroorotate dehydrogenase (DHODH) in the osteoclast lineage and its inhibition by LEF. **a**, Expression of DHODH mRNA in RANKL-stimulated BMMs. Expression of mRNA was detectable in unstimulated BMMs, and it was enhanced by RANKL stimulation. The induction level was comparable with that of splenic T cells. GeneChip analysis was repeated 3 times and yielded similar results; a representative set of data is shown. **b**, Immunocytochemical localization of the DHODH protein in osteoclast precursor cells and osteoclasts. DHODH was abundantly expressed in mature osteoclasts as well as in BMMs. DHODH expression was mainly observed in the mitochondria as visualized by MitoTracker Orange. Nuclei were stained with Hoechst 33342. **c**, High-performance liquid chromatography analysis of the intracellular concentration of pyrimidine nucleotides in RANKL-stimulated BMMs. In the presence of LEF, the concentration of pyrimidines was greatly down-regulated. Values are the mean and SEM. n.d. = not detectable (see Figure 1 for other definitions).

Identification of NF-ATc1 as a specific target of leflunomide action. To gain insights into the molecular targets of leflunomide, we performed a genome-wide screening of RANKL-inducible genes in BMMs stimulated with RANKL in the presence or absence of leflunomide. We utilized GeneChip arrays and analyzed the mRNA expression of transcription factors and effector molecules, which are involved in RANKL signaling

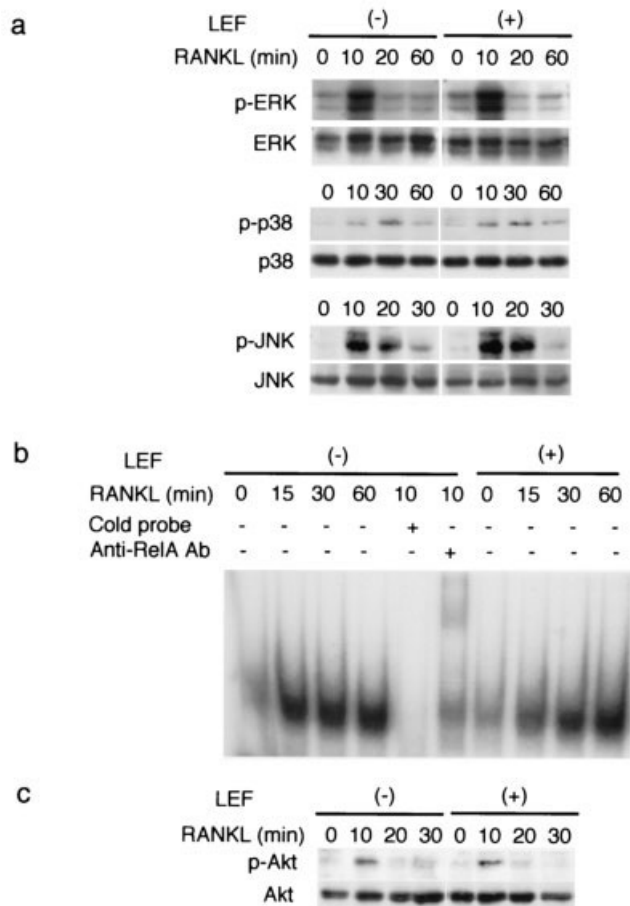


Figure 3. Effect of LEF on RANKL-activated signaling pathways. **a**, LEF had no significant effect on RANKL-induced activation of p38, ERK, or JNK in BMMs. The BMMs were incubated with LEF for 24 hours and stimulated with RANKL (100 ng/ml) in the presence of M-CSF (10 ng/ml). Whole-cell lysate was recovered after the indicated periods. **b**, Effect of LEF on RANKL-induced activation of NF- κ B in BMMs. LEF had little, if any, effect on the formation of NF- κ B complex. The band of NF- κ B complex was shifted by the addition of anti-RelA antibody (Ab). **c**, Activation of Akt by RANKL was not affected by LEF. p-ERK = phosphorylated ERK; p-p38 = phosphorylated p38; p-JNK = phosphorylated JNK; p-Akt = phosphorylated Akt (see Figure 1 for other definitions).

(Figure 4). Interestingly, a selective suppressive effect of leflunomide was observed on the transcription factor NF-ATc1. As shown in Figure 4, the expression of *NF-ATc1* mRNA was increased >20-fold by RANKL, but the addition of leflunomide reduced this expression markedly (>80% inhibition), whereas expressions of other genes were not significantly affected under this experimental condition.

As expected from the above result, leflunomide strongly inhibited the RANKL induction of NF-ATc1 protein (Figure 5a). In addition, the induction levels of

TRAF6 and c-Fos also decreased, particularly at the late stage (3 days after RANKL stimulation), but they were still observed. Consistently, NF-ATc1 expression in

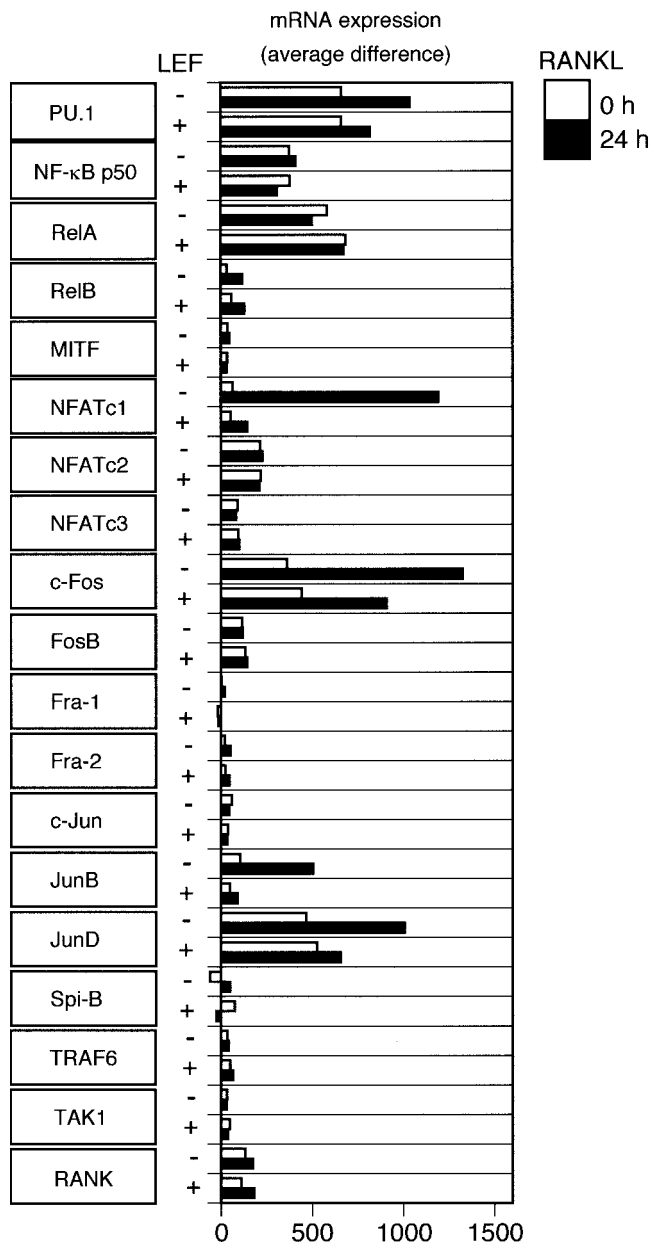


Figure 4. Genome-wide screening of targets of LEF action in RANKL-inducible genes: mRNA expression level (average difference) of genes encoding transcription factors and molecules involved in RANKL signaling. BMMs were stimulated with RANKL for 24 hours in the presence or absence of LEF, and mRNA expression was analyzed by GeneChip analysis. GeneChip analysis was repeated several times and yielded similar results; a representative set of data is shown. MITF = microphthalmia transcription factor; NF-ATc1 = nuclear factor of activated T cells c1; TRAF6 = tumor necrosis factor receptor-associated factor 6; TAK-1 = transforming growth factor β -activated kinase 1 (see Figure 1 for other definitions).

BMMs stimulated with RANKL in the presence of leflunomide was barely detected by immunostaining, but the expression of c-Fos or TRAF6 was still observed, albeit at lower levels, under the same conditions (Figure 5b).

Effect of leflunomide on the RANKL-stimulated NF-ATc1 induction pathway. The above results collectively suggest that restriction of uridine synthesis, which would generally be required for RNA synthesis, selectively suppresses the NF-ATc1 induction pathway stimulated with RANKL. It has been shown that RANKL-induced Ca^{2+} oscillation is essential for the autoamplification of *NF-ATc1* gene expression that is critical the sustained NF-ATc1-dependent transcriptional (24). We therefore examined the effect of leflunomide on Ca^{2+} signaling in BMMs stimulated with RANKL. Interestingly, as shown in Figure 5c, Ca^{2+} oscillation induced by RANKL was inhibited by leflunomide, suggesting that the inhibition of RANKL-induced Ca^{2+} signaling accounts for the inhibition of NF-ATc1 expression by leflunomide.

If the inhibition of RANKL-induced Ca^{2+} signaling and NF-ATc1 induction is the critical target of leflunomide, one would expect that the leflunomide-mediated inhibition of osteoclast differentiation would be overcome by ectopic NF-ATc1 expression. To address this issue, we examined the effect of ectopic expression of NF-ATc1, TRAF6, or c-Fos on the suppressive action of leflunomide by retrovirus-mediated gene transfer (24). Interestingly, ectopic expression of NF-ATc1, but not c-Fos or TRAF6, resulted in efficient osteoclast formation even in the presence of leflunomide (Figure 5d). These results lend further support for the notion that leflunomide exerts a selective action on the RANKL-induced Ca^{2+} /NF-ATc1 pathway.

T cell-independent effect of leflunomide on inflammatory bone destruction. Leflunomide exerts its immunosuppressive effect by acting on T cells, but it is difficult to segregate T cell-dependent immunosuppressive effects from T cell-independent effects in the in vivo mechanism of leflunomide action. To determine whether leflunomide exerts its protective effect on bone through a T cell-independent mechanism, we developed a model of inflammatory bone destruction that is induced in the absence of T cells. LPS was injected into the calvaria of mice deficient in the *rag-2* gene (*rag-2*^{-/-} mice), which lack both T and B cells (29). It has been shown that injection of a low dose of LPS does not induce bone destruction in T cell-deficient nude mice (34), but we successfully induced bone destruction in *rag-2*^{-/-} mice using a high dose of LPS, indicating

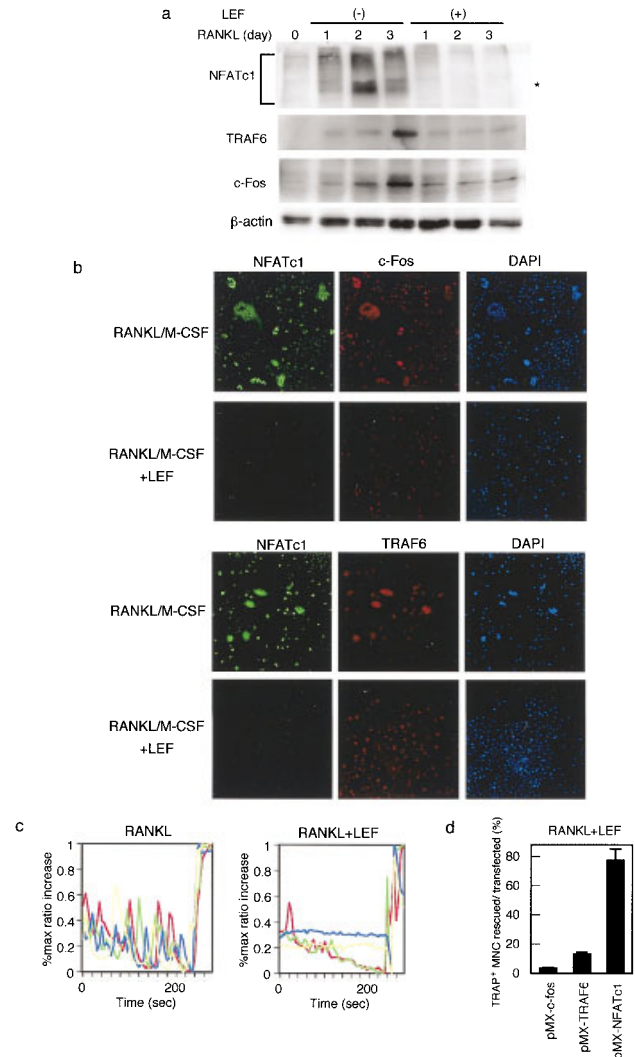


Figure 5. Inhibition of RANKL signaling by LEF through down-regulation of nuclear factor of activated T cells c1 (NF-ATc1) pathway. **a**, Effect of LEF on the expression of essential mediators of RANKL signaling. NF-ATc1 was markedly induced by RANKL and became dephosphorylated (asterisk) in the course of differentiation. LEF had a strong inhibitory effect on NF-ATc1 expression. **b**, Expression of c-Fos, tumor necrosis factor receptor-associated factor 6 (TRAF6), and NF-ATc1 in BMMs stimulated with RANKL in the presence or absence of LEF. **c**, Effect of LEF on Ca^{2+} signaling in BMMs induced by RANKL. $[Ca^{2+}]_i$ change in single cells was detected by loading Ca^{2+} indicators. Each color indicates a different cell in the same field. **d**, Effect of retroviral overexpression of c-Fos, TRAF6, and NF-ATc1 on the inhibitory effect of LEF. Values are the mean and SEM. DAPI = 4',6-diamidino-2-phenylindole (see Figure 1 for other definitions).

that lymphocytes are dispensable in this model system (Figure 6a).

Interestingly, when these mice received locally administered leflunomide, bone destruction and exces-

sive osteoclast formation were significantly ameliorated (Figures 6a and b). It was notable that a considerable number of TRAP+ MNCs in inflammatory lesions stained positively for NF-ATc1 (Figure 6a), and this was substantially reduced following treatment with leflunomide (Figure 6b). In contrast, expression of other members of the NF-AT family, such as NF-ATc2, was barely detected (results not shown). Thus, leflunomide has an antiosteoclastogenic effect in vivo, which is not targeted at T cells. Although we cannot rule out the possibility that leflunomide also acts on other cell types, our results were consistent with the above in vitro results, showing the direct inhibitory effect of leflunomide on osteoclast precursor cells by interfering with NF-ATc1 expression. Consistently, leflunomide had only a marginal effect on inflammatory reactions in *rag-2*^{-/-} mice (Figure 6c and data not shown). In contrast, when wild-type mice were treated with leflunomide in an LPS-induced bone destruction model, we observed a suppressive effect on

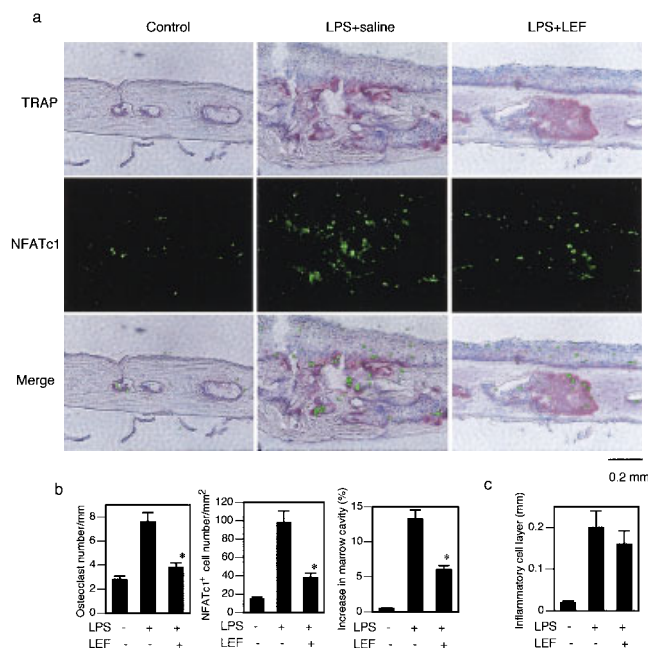


Figure 6. Effect of LEF on a T cell-independent model of inflammatory bone destruction in *rag-2*^{-/-} mice. **a**, Lipopolysaccharide (LPS) induced enhanced osteoclast formation and inflammation-related bone resorption in *rag-2*^{-/-} mice. Note that bone destruction was accompanied by an increase in the number of TRAP-positive and nuclear factor of activated T cells c1 (NF-ATc1)-positive cells. LEF ameliorated this bone resorption significantly. **b**, Effect of LEF on bone resorption parameters, such as osteoclast number, NF-ATc1-positive cell number, and increase in area of marrow cavity. * = $P < 0.05$ versus saline-only treatment. **c**, Effect of LEF on formation of an inflammatory cell layer. Values are the mean and SEM. See Figure 1 for other definitions.

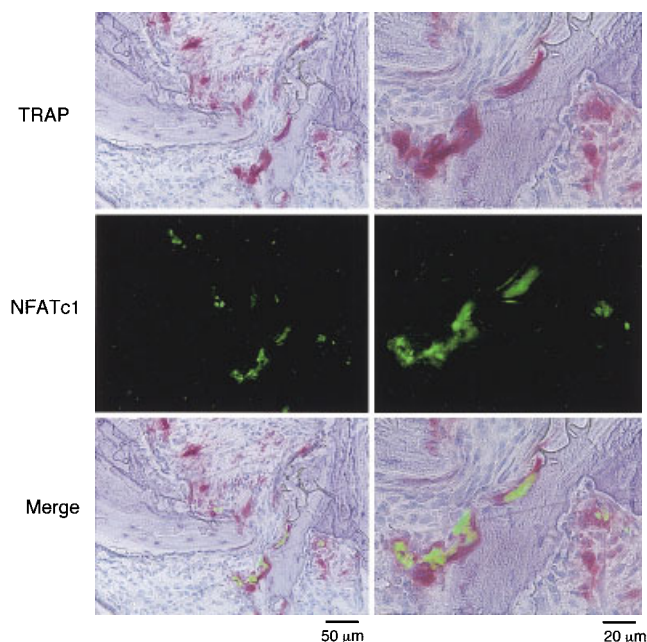


Figure 7. Expression of nuclear factor of activated T cells c1 (NF-ATc1) in osteoclasts in rheumatoid arthritis (RA). TRAP+ MNCs at the bone-synovium interface in RA patients were positive for NF-ATc1. Similar results were obtained in all samples. The right panel shows a magnified view of bone-resorbing osteoclasts, which expressed a high level of NF-ATc1 in arthritic joints. See Figure 1 for other definitions.

inflammatory reactions and a more powerful suppressive effect on osteoclast formation than that in *rag-2*^{-/-} mice (results not shown), suggesting that the bone-protective effect of leflunomide in vivo is partially due to its suppressive effect on T cells.

It is important to evaluate whether NF-ATc1 expression is up-regulated in RA tissues. In this regard, it is interesting that selective and strong expression of NF-ATc1 was observed in multinucleated osteoclasts at the bone-synovium interface in patients with RA (Figure 7).

DISCUSSION

Bone destruction in RA is initially triggered by the activation of T cells, which eventually enhances the expression of RANKL in synoviocytes, as well as in T cells themselves (4,5,13). Inflammatory cytokines also contribute to this pathogenesis by increasing the expression and activity of RANKL (6,13). Thus, there are several strategies by which to suppress bone destruction: suppression of T cell activation, inhibition of osteoclast formation/function, or blockade of inflammatory cytokines. Leflunomide has an effective therapeutic action

on animal models of autoimmune arthritis, and randomized controlled studies in human RA revealed that leflunomide is one of the antirheumatic drugs that reduces the progression of bone damage (17–19). Leflunomide has been thought to ameliorate the course of arthritis by its inhibitory effect on T cells, but we found that it has a marked suppressive effect on RANKL-activated intracellular signaling and osteoclast differentiation. Using a bone destruction model in the absence of T cells, we showed that the effect of leflunomide is also targeted at osteoclastogenesis directly, extending beyond its suppressive effect on T cells. Given the central role of RANKL in arthritic bone destruction, antirheumatic drugs that inhibit RANKL signaling, such as leflunomide, will contribute to the maintenance of joint structure through the direct inhibition of bone-resorbing osteoclasts.

Is osteoclastogenesis in RA dependent on T cells or not? As described above, T cells play important roles as an initial trigger and as a constant stimulator of bone destruction, mainly by inducing inflammatory cytokines (e.g., $\text{TNF}\alpha$, IL-1) and RANKL on synovial fibroblasts. Although RANKL is expressed on T cells (4), T cells also produce the inhibitors of RANKL, such as $\text{IFN}\gamma$ (13). Thus, the relative contribution of RANKL in these cell types remains elusive. But LPS-induced bone destruction or collagen-induced arthritis can be induced in mice lacking T cells (35). Therefore, T cell-mediated reactions are not essential for osteoclastogenesis in some models of inflammatory bone destruction. Although T cells contribute greatly to the pathogenesis of bone destruction in RA, the enhanced expression of RANKL on synoviocytes induced by synovial inflammation may be a critical molecular basis for osteoclastogenesis in arthritis. Activation of T cells should be one of the initiators of this inflammation, but RANKL on T cells may not be essential for osteoclastogenesis in inflammatory lesions. Further studies are necessary to determine in more detail how T cells contribute to osteoclastogenesis in RA.

A genome-wide screening of RANKL-inducible genes using GeneChip made it possible for us to identify the transcription factor NF-ATc1 as a selective target of leflunomide action. We found that induction of the *JunB* gene is also affected by leflunomide. However, it is reported that Jun family members play redundant roles in hematopoietic cells (36), suggesting that decreased expression of JunB cannot explain the strong suppressive effect of leflunomide on osteoclastogenesis. This is further supported by the observation that the inhibitory effect of leflunomide on osteoclastogenesis is specifically

reversed by ectopic expression of NF-ATc1. Since expression of both TRAF6 and c-Fos proteins was reduced by the addition of leflunomide at a late stage, we cannot rule out the possibility that the action of leflunomide is partly mediated through its effect on other molecules such as TRAF6 and c-Fos.

Our results raise an interesting issue of how blocking de novo pyrimidine biosynthesis selectively affects the RANKL-induced Ca^{2+} signaling pathway. In fact, purine and pyrimidine nucleotides are generally important for cellular functions. In addition, they play important roles as extracellular signaling molecules (37). For example, it has been shown that UTP and ATP evoke Ca^{2+} signaling through binding with nucleotide receptors such as P2X and P2Y (37–39). It is interesting that these receptors are expressed by cells of the osteoclast lineage (40,41), and we also detected expression of P2X7 and P2X4 in bone marrow-derived osteoclast precursor cells during osteoclastogenesis (Takayanagi H: unpublished observations). Since the mechanism by which RANKL stimulation results in Ca^{2+} oscillation is currently unknown (23), further studies will be required to determine the detailed mechanism by which the restriction of de novo pyrimidine synthesis is linked to RANKL-induced Ca^{2+} oscillation.

We demonstrated that NF-ATc1 expression is actually enhanced in inflammatory tissues adjacent to sites of bone destruction and is down-regulated by leflunomide treatment in animal models. To focus on the effect of leflunomide on bone destruction, but not on the immune system, we used the LPS-induced bone destruction model, which mimics the destructive phase of arthritis, instead of using models such as collagen-induced arthritis. We also showed that NF-ATc1 is strongly expressed in multinucleated osteoclasts at the bone-synovium interface in patients with RA, indicating the possibility that up-regulation of NF-ATc1 expression may be a feature shared among various bone-destructive or metabolic bone diseases. Although the correlation between NF-AT expression and disease severity is an issue that remains to be examined, colocalization of NF-ATc1 with TRAP suggests that NF-AT expression may also be related to severe RA with abundant TRAP+ MNCs, which actively resorb bone. Considering the specific effect of leflunomide on NF-ATc1 induction and the abundance of NF-ATc1 in osteoclasts, leflunomide should be a good therapeutic agent for suppressing osteoclastogenesis in arthritis. Suppression of the NF-ATc1 pathway would be an advantageous strategy against bone destruction in arthritis characterized by enhanced osteoclastogenesis. It is also beneficial for

suppressing steroid-induced osteoporosis that is often observed in RA patients.

We have previously reported that calcineurin inhibitors, such as FK-506 and cyclosporin A, have strong inhibitory effects on osteoclastogenesis (24). It is notable that FK-506 is also in clinical trials for the treatment of RA, with promising results (42). Methotrexate is one of the most widely used antirheumatic drugs that have a bone-protective effect. Methotrexate also has a direct suppressive effect on osteoclastogenesis (Urushibara M, Takayanagi H: unpublished observations), suggesting that bone-protective antirheumatic drugs may have a direct effect on osteoclast lineage in common. Our preliminary data also show that leflunomide and FK-506 have synergistic inhibitory effects on osteoclastogenesis (Urushibara M, Takayanagi H: unpublished observations). Therefore, a combination of antiosteoclastogenic drugs may be a promising approach to augmenting efficacy and also may reduce the possibility of side effects in the treatment of bone destruction in RA.

ACKNOWLEDGMENTS

We thank Aventis Pharmaceuticals for providing the leflunomide metabolite, and T. Yokochi and A. Suematsu for invaluable suggestions and technical help. We also thank K. Sato, M. Asagiri, M. Isshiki, A. Takaoka, K. Honda, S. Kano, C. Nakajima, S. Tanaka, H. Oda, K. Nakamura, and I. Kawai for their assistance and discussions.

REFERENCES

- Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. *Cell* 1996;85:307-10.
- Feldmann M, Maini RN. Anti-TNF α therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 2001;19:163-96.
- Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* 2001;344:907-16.
- Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;402:304-9.
- Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor κ B ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2000;43:259-69.
- Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* 2002;20:795-823.
- Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum* 2000;43:250-8.
- Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, et al. Osteoclasts are essential for TNF- α -mediated joint destruction. *J Clin Invest* 2002;110:1419-27.
- Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. *Am J Pathol* 2001;159:1689-99.
- Takayanagi H, Juji T, Miyazaki T, Iizuka H, Takahashi T, Isshiki M, et al. Suppression of arthritic bone destruction by adenovirus-mediated csk gene transfer to synoviocytes and osteoclasts. *J Clin Invest* 1999;104:137-46.
- Zhao H, Shuto T, Hirata G, Iwamoto Y. Aminobisphosphonate (YM175) inhibits bone destruction in rat adjuvant arthritis. *J Orthop Sci* 2000;5:397-403.
- Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. *Science* 2000;289:1508-14.
- Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN- γ . *Nature* 2000;408:600-5.
- Takayanagi H, Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, et al. A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis. *Biochem Biophys Res Commun* 1997;240:279-86.
- Kremer JM. Rational use of new and existing disease-modifying agents in rheumatoid arthritis. *Ann Intern Med* 2001;134:695-706.
- Jones G, Halbert J, Crotty M, Shanahan EM, Batterham M, Ahern M. The effect of treatment on radiological progression in rheumatoid arthritis: a systematic review of randomized placebo-controlled trials. *Rheumatology (Oxford)* 2003;42:6-13.
- Prakash A, Jarvis B. Leflunomide: a review of its use in active rheumatoid arthritis. *Drugs* 1999;58:1137-64.
- Sharp JT, Strand V, Leung H, Hurley F, Loew-Friedrich I, and the Leflunomide Rheumatoid Arthritis Investigators Group. Treatment with leflunomide slows radiographic progression of rheumatoid arthritis: results from three randomized controlled trials of leflunomide in patients with active rheumatoid arthritis. *Arthritis Rheum* 2000;43:495-505.
- Cohen S, Cannon GW, Schiff M, Weaver A, Fox R, Olsen N, et al, and the Utilization of Leflunomide in the Treatment of Rheumatoid Arthritis Trial Investigator Group. Two-year, blinded, randomized, controlled trial of treatment of active rheumatoid arthritis with leflunomide compared with methotrexate. *Arthritis Rheum* 2001;44:1984-92.
- Breedveld FC, Dayer JM. Leflunomide: mode of action in the treatment of rheumatoid arthritis. *Ann Rheum Dis* 2000;59:841-9.
- Williams JW, Mital D, Chong A, Kottayil A, Millis M, Longstreth J, et al. Experiences with leflunomide in solid organ transplantation. *Transplantation* 2002;73:358-66.
- Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 1997;15:707-47.
- Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000;1:11-21.
- Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 2002;3:889-901.
- Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, et al. RANKL maintains bone homeostasis through c-Fos-dependent induction of IFN- β . *Nature* 2002;416:744-9.
- Ruckemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B, et al. Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. *J Biol Chem* 1998;273:21682-91.
- Dietz C, Hinsch E, Löffler M. Immunocytochemical detection of mitochondrial dihydroorotate dehydrogenase in human spermatozoa. *Int J Androl* 2000;23:294-9.
- Löffler M, Grein K, Knecht W, Klein A, Bergjohann U. Dihydro-

- orotate dehydrogenase: profile of a novel target for antiproliferative and immunosuppressive drugs. *Adv Exp Med Biol* 1998;431:507–13.
29. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 1992;68:855–67.
 30. Chiang CY, Kyritsis G, Graves DT, Amar S. Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. *Infect Immun* 1999;67:4231–6.
 31. Wong BR, Besser D, Kim N, Arron JR, Vologodskaya M, Hanafusa H, et al. TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* 1999;4:1041–9.
 32. Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ, Wagner EF. Fos11 is a transcriptional target of c-Fos during osteoclast differentiation. *Nat Genet* 2000;24:184–7.
 33. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2002;2:389–406.
 34. Ukai T, Hara Y, Kato I. Effects of T cell adoptive transfer into nude mice on alveolar bone resorption induced by endotoxin. *J Periodontol* 1996;31:414–22.
 35. Plows D, Kontogeorgos G, Kollias G. Mice lacking mature T and B lymphocytes develop arthritic lesions after immunization with type II collagen. *J Immunol* 1999;162:1018–23.
 36. Jochum W, Passegue E, Wagner EF. AP-1 in mouse development and tumorigenesis. *Oncogene* 2001;20:2401–12.
 37. Burnstock G. The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* 1997;36:1127–39.
 38. Molliver DC, Cook SP, Carlsten JA, Wright DE, McCleskey EW. ATP and UTP excite sensory neurons and induce CREB phosphorylation through the metabotropic receptor, P2Y2. *Eur J Neurosci* 2002;16:1850–60.
 39. Kawamura M, Terasaka O, Ebisawa T, Kondo I, Masaki E, Ahmed A, et al. Integrity of actin-network is involved in uridine 5'-triphosphate evoked store-operated Ca^{2+} entry in bovine adrenocortical fasciculata cells. *J Pharmacol Sci* 2003;91:23–33.
 40. Naemsch LN, Weidema AF, Sims SM, Underhill TM, Dixon SJ. P2X₄ purinoceptors mediate an ATP-activated, non-selective cation current in rabbit osteoclasts. *J Cell Sci* 1999;112:4425–35.
 41. Weidema AF, Dixon SJ, Sims SM. Activation of P2Y but not P2X₄ nucleotide receptors causes elevation of $[Ca^{2+}]_i$ in mammalian osteoclasts. *Am J Physiol Cell Physiol* 2001;280:C1531–9.
 42. Furst DE, Saag K, Fleischmann MR, Sherrer Y, Block JA, Schnitzer T, et al. Efficacy of tacrolimus in rheumatoid arthritis patients who have been treated unsuccessfully with methotrexate: a six-month, double-blind, randomized, dose-ranging study. *Arthritis Rheum* 2002;46:2020–8.