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

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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

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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-kB 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 antioestrogen 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 (TNFα) 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 (IFNγ) 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, 1.5–2.0 × 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/
enzymatic digestion in 0.1% collagenase and 0.2% dispase, and isolated from the calvaria of newborn (3–5 days old) mice by in vitro transcription. After cRNA fragmentation, hybridization synthesis of biotinylated complementary RNA (cRNA) by in situ DNA synthesis by reverse transcription, followed by cultured in M/s. After 5 days, TRAP RANKL (100 ng/ml) in the presence or absence of leflunomide (30 μM) in the presence or absence of leflunomide (100 ng/ml) in the presence of 10% FBS containing 50 M/ M glycerophosphate, and 10 nM sodium 104 cells per well in a 24-well dish), and BMMs were preincubated in the presence or absence of leflunomide (30 μM). Cells were sequentially incubated in 5% bovine serum albumin/phosphate buffered saline (PBS) for 30 minutes, 2 μg/ml anti–NF-ATc1 antibody and 4 μg/ml anti–c-Fos (or anti-TRAF6) antibody in PBS–Tween for 60 minutes, and then 4 μg/ml Alexa Fluor 488 or 8 μg/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 subsequently incubated with rabbit anti-DHODH polyclonal IgG (27,28), and then with 4 μg/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 pMAMps2 (a gift from C. L. Sawyer) into 293T cells. Two days after inoculation, BMMs were stimulated with RANKL/M-CSF plus leflunomide (30 μM). 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 μM). 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 μM 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 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 μM). After 24 hours, total RNA was extracted from BMMs using a Sepasol RNA extraction kit (Nacalai Tesque, 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 × 104 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 × 104 cells per well in a 24-well dish), and cultured in α-MEM with 10% FBS containing 50 μg/ml ascobic acid, 10 mM sodium β-glycerophosphate, and 10 nM dexamethason, in the presence or absence of leflunomide (30 μM), 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 μM). After 24 hours, cells were deproteinized with 10% trichloroacetic acid (TCA). The cell extracts were then centrifuged for 1 minute at 12,000 g, 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-κB. BMMs, preincubated in the presence or absence of leflunomide (30 μM) 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 μM) for 24 hours. After RANKL stimulation, a cell extract was prepared and analyzed using an oligonucleotide probe containing the NF-κB binding site of the IFNγ promoter, as previously described (13,25).

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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 μM). 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, BMMs were cultured with RANKL/M-CSF for 3 days in the presence or absence of leflunomide (30 μM), 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 for 30 minutes, 2 μg/ml anti–NF-ATc1 antibody and 4 μg/ml anti–c-Fos (or anti-TRAF6) antibody in PBS–TWEEN for 60 minutes, and then 4 μg/ml Alexa Fluor 488 or 8 μg/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 μg/ml Alexa Fluor 488–labeled secondary antibody.

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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 ± SEM (n = 6). All experiments were repeated more than 3 times throughout the study. Statistical analysis was performed using Student’s t-test. The importance of pyrimidine synthesis in osteoblasts. Values are the mean ± SEM.

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**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 μM). 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 μM 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 μM 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 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-κB, 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-κB (Figure 3b) and Akt (Figure 3c), suggesting that these molecules may not be the targets of leflunomide action on RANKL signaling.

**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.
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
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 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 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).
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., TNFα, 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 IFNγ (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 Ca²⁺ oscillation 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 (Urushihara M, Takayanagi H: unpublished observations). Therefore, a combination of calcineurin inhibitors and FK-506 have synergistic inhibitory effects on osteoclastogenesis (Urushihara M, Takayanagi H: unpublished observations). Therefore, a combination of antosteoclastogenic 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.

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