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Nucleosides Accelerate Inflammatory Osteolysis, Acting as Distinct Innate Immune Activators

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Abstract

The innate immune system and its components play an important role in the pathogenesis of inflammatory bone destruction. Blockade of inflammatory cytokines does not completely arrest bone erosion, suggesting that other mediators also may be involved in osteolysis. Previously we showed that nucleosides promote osteoclastogenesis and bone-resorption activity in the presence of receptor activator for nuclear factor κB ligand (RANKL) in vitro. The studies described here further demonstrate that selected nucleosides and nucleoside analogues accelerate bone destruction in mice immunized with collagen II alone (CII) but also further enhance bone erosion in mice immunized by collagen II plus complete Freund's adjuvant (CII + CFA). Abundant osteoclasts are accumulated in destructive joints. These data indicate that nucleosides act as innate immune activators distinct from CFA, synergistically accelerating osteoclast formation and inflammatory osteolysis. The potential roles of the surface triggering receptor expressed on myeloid cells (TREM) and the intracellular inflammasome in nucleoside-enhanced osteoclastogenesis have been studied. These observations provide new insight into the pathogenesis and underlying mechanism of bone destruction in inflammatory autoimmune osteoarthritis.

Keywords

Nucleoside; Inflammatory Osteoarthritis; Osteoclast/Osteolysis; Trem; Inflammasome

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Additional Supporting Information may be found in the online version of this article.

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Introduction

Inflammation-induced osteolysis, such as occurs in rheumatoid arthritis (RA) and osteoarthritis (OA), is a common chronic joint disorder that affects approximately 1% of the adult population in the United States. However, there is no completely effective therapy because we do not fully understand the pathogenesis of bone destruction in inflammatory osteoarthritic diseases. Most studies have focused on the immune system and its components, assuming that inflammatory cytokines influence osteoclast-like cells either by regulating the expression of receptor activator for nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) or by directly affecting osteoclastogenesis and bone erosion.\(^1\) Although these mechanisms are important, blockade of inflammatory cytokines does not uniformly prevent, delay, or reduce bone destruction. Therefore, inflammatory cytokines only partially mediate the pathogenesis of bone destruction in inflammatory osteoarthritic diseases. Other mediators and mechanisms are likely involved in inflammation-induced osteolysis.\(^2\)

In recent years, research into the cause of osteoarthritis has focused on the innate immune system and its components because of its remarkable ability to generate specific immunity. The host innate immune system typically is activated by pathogen-recognition receptors (PRRs) on antigen-presenting cells (APCs), which recognize key conserved pathogenic molecules and pathogen-associated molecular patterns (PAMPs). PRRs include both surface and intracellular receptors. The Toll-like receptor (TLR) and the triggering receptor expressed on myeloid cells (TREM) are two types of surface PRRs that survey the extracellular fluids and endosomal compartments. TREM proteins, members of the innate immune receptor family, are restricted to granulocytes, monocytes, macrophages, osteoclasts, and dendritic cells and play critical roles in the development of inflammatory and immune responses in general and in the maintenance of bone homeostasis by regulating osteoclast development.\(^3\)

The intracellular PRRs include nucleotide binding and oligomerization 1 or 2 (NOD)–like receptors and other members that recognize intracellular danger-signaling molecules. A number of NOD-like receptors activate caspase-1 within the inflammasome, leading to the processing and secretion of proinflammatory cytokines interleukin 1β (IL-1β), IL-18, and IL-33. The NACHT–, LRR–, and PYD domain–containing protein 3 (Nalp3) inflammasome, one of the main cellular platforms for IL-1β production, is composed of multiple protein components, including Nalp proteins in the NOD-like receptor family, adaptor molecule apoptosis–associated specklike protein (ASC), and procaspase-1.\(^4\) Thus aberrant regulation of or mutations in the inflammasome components may cause autoinflammatory disorders characterized by elevated proinflammatory cytokines. The inflammasome also cooperates with TLRs to mediate appropriate responses to a number of endogenous danger signals such as uric acid, ATP/ADP, and nucleosides and contributes to the pathogenesis of autoimmune disease. For example, activation of the Nalp3 inflammasome by gout-associated uric acid crystals results in the production of active inflammatory cytokines such as IL-1β and IL-18 and gout arthritis.\(^4\)
In fact, these PRRs may be a specialized family of nucleic acid sensors that recognize various nucleic acid components, including RNA, DNA, nucleotide/nucleoside, and low-molecular-weight nucleic acids. Although the specific ligand for TREMs has not been identified, the natural ligand(s) would have the ability to connect these responses by virtue of the important roles of TREMs in inflammation, immunity, and bone erosion. Therefore, the ability to detect bacterial or viral infections by the host innate immune system depends in part on the recognition between pathologic nucleic acids and PRRs. For molecules that are rather poorly recognized by the host immune system, immune adjuvants such as mineral salts and oil-based emulsions are used widely to enhance this recognition.

Nucleic acid products are an important component of the pathogen-associated molecular patterns that activate the innate immune system. During inflammation, especially chronic inflammation, chromosomal DNA, nucleoside 5′-triphosphates (NTPs), and their metabolic nucleic acid products are released from apoptotic host cells or infectious pathogens that are recognized by PRRs, resulting in activation of innate immune responses. DNase II knockout (KO) mice developed chronic polyarthritis, characterized by severe inflammation, bone destruction, and elevated DNA and tumor necrosis factor α (TNF-α) in serum, supporting the role of nucleic acid products in inflammation, immune response, and bone metabolism. Notably, the concentration of nucleosides is markedly increased in synovial fluid and plasma from patients with rheumatoid arthritis and osteoarthritis. However, little is known regarding the role of nucleic acid products in arthritic bone destruction or the underlying cellular and molecular mechanisms.

Previously, we demonstrated that partial antiretroviral nucleoside reverse transcriptase inhibitors (NRTIs) such as azidothymidine (AZT) stimulate osteoclast formation in the presence of RANKL in vitro. Subsequently, we also reported that nucleosides upregulate TREM2 expression, implicating the TREM pathway in nucleoside-induced osteoclastogenesis. However, these data were obtained in the mouse RAW264.7 cell line and mouse bone marrow macrophages in vitro. In the studies described herein, we test more nucleosides and analogues and show that nucleosides not only promote osteoclastogenesis and bone resorption activity in vitro but also profoundly accelerate bone destruction and joint deformity in mice immunized with collagen II plus complete Freund’s adjuvant (CFA). However, nucleosides alone neither stimulate osteoclastogenesis in the absence of RANKL in vitro nor accelerate osteolysis in unimmunized mice in vivo. These results indicate that selected nucleosides, acting as a unique immune activator distinct from CFA, activate innate immune responses and accelerate inflammatory osteolysis. The surface TREMs and intracellular inflammasomes are two potential pathways activated by nucleosides, thereby producing inflammatory cytokines, recruiting osteoclasts, increasing osteoclast activity, and synergistically accelerating subsequent bone destruction in collagen II–induced arthritis (CIA) mice. Our studies provide new insight into the pathogenesis of bone destruction and joint deformity in various inflammatory osteoarthritic diseases.
Materials and Methods

Reagents and animals

All chemicals, including superpurified (>99%) nucleosides for cell culture, were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise indicated. Recombinant macrophage colony-stimulating factor (M-CSF) and RANKL for osteoclastogenesis were from R&D Systems (Minneapolis, MN, USA). Stock solutions of nucleosides (25 mM) were prepared in PBS for cell culture in vitro. The aliquots were frozen at −20°C for up to 6 months. The kit for A2 siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for flow cytometry and Western blot were from BD Biosciences (San Jose, CA, USA) and eBiosciences (San Diego, CA, USA). DBA1 and C57BL/6 were purchased from Harlan Industries (Livermore, CA, USA).

Primary mouse bone marrow macrophages for osteoclastogenesis and bone resorption assay (pit assay)

Bone marrow macrophages (BMMs) were obtained from mouse femurs and tibias by aseptic isolation, as described previously. Mouse bone marrow macrophages (5 × 10^4/well on 24-well plates) were plated and cultured in complete α modified essential medium (α-MEM) containing RANKL (100 ng/mL) and M-CSF (10 ng/mL) for 4 more days. Cells from each animal were cultured separately.

For bone-resorption assays, primary macrophages (1 × 10^5) were plated on sterile dentine slices and cultured in complete α-MEM containing M-CSF (10 ng/mL) plus RANKL (100 ng/mL) in the presence or absence of nucleosides (10 μM) for 10 to 14 days. The medium was changed every 3 days. Dentine slices were fixed with 0.25 M ammonium hydroxide for 30 minutes, and cells were gently removed mechanically. Dentine slices were examined by scanning electron microscope. The data were quantified by measuring the area and expressing it as a percent of the total slice area. This was determined using image-analysis software obtained from the National Institutes of Health.

Arthritis induction and nucleoside treatment

For generation of collagen II–induced arthritic (CIA) mice, 8- to 12-week-old DBA/1J mice were injected subcutaneously with 200 μg of collagen type II emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis*. One week after collagen II immunization, mice were administered intraperitoneally 200 μL of PBS (untreated) or PBS containing 25 mg/kg of nucleosides daily (~3.2 mg nucleosides/mL of PBS) for 5 days per week. Nucleoside treatment was continued for 4 weeks. Animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and Emory University.

To further characterize the individual contribution of CFA and nucleosides in the development of arthritis, DBA1 mice were immunized with a single injection of 200 μg of collagen II alone (CII), CFA alone containing 200 μg of *M. tuberculosis* (CFA), and collagen II plus CFA (CII + CFA), respectively. One week after immunization, the three groups of mice were injected daily intraperitoneally with PBS or 25 mg/kg/200 μL of...
nucleosides per mouse for 5 days per week. Nucleoside treatment was continued for 4 weeks.

**Clinical assessment, histology, and histomorphometry**

Clinical arthritis (swelling of the paws) was assessed by two independent, blinded examiners using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, moderate swelling and edema; grade 3, extreme swelling and pronounced edema; and grade 4, joint rigidity. Each limb was graded separately, and the mean grade per paw was calculated. The paws/knees were collected after euthanizing the animals at the end of experiments, fixed in 4% buffered formaldehyde, and then analyzed by micro-computed tomography (μCT). A total of 185 consecutive tomographic slices were obtained in mouse paws/knees with a slice thickness of 12.8 μm at 8-μm resolution. After scanning, 3D microstructural image data were reconstructed, and structural indices were calculated using 3D trabecular bone analysis software.

The paws/knees were decalcified, embedded in paraffin, and sectioned for histology assay. Decalcified paraffin-embedded 2-mm sections of the paws/knees were stained with hematoxylin and eosin (H&E) for assessing signs of inflammatory arthritis and osteolysis. Osteoclast formation was measured by tartrateresistant acid phosphatase (TRACP) staining assay using TRACP enzyme with the Leukocyte Acid Phosphatase Assay Kit (Sigma), as per the manufacture’s instructions. Osteoclast-like TRACP⁺ cells in each well were scored by counting the number of TRACP⁺ multinucleated cells with more than three nuclei.

Quantitative bone histomorphometric measurements were performed independently by experimental technicians in the Histomorphometry and Molecular Analysis Core Laboratory of the Center for Metabolic Bone Disease (CMBD) at the University of Alabama at Birmingham and expressed in units following the guidelines established by the ASBMR Histomorphometry Nomenclature Committee. The paw/knee slides were analyzed using Bioquant Image Analysis Software (R&M Biometrics, Nashville, TN, USA).

**Polychromatic flow cytometry (PFC)**

For determination of intracellular cytokine profiles, whole-blood specimens were isolated, and the surface markers were first stained with specific fluorescent-conjugated antibodies on ice for 30 minutes, followed by lysis of red blood cells and permeabilization of white blood cells (BD Biosciences). Intracellular cytokines then were stained with fluorescent-conjugated antibodies. Data were acquired using LSR II flow cytometry and analyzed by Flowjo analysis software (TreeStar, Ashland, OR, USA).

**Inflammasome activation and cytokine assay in mouse macrophages**

Mouse bone marrow macrophages derived from wild-type C57BL/6 mice and *Nalp3*⁻/⁻ knockout mice were plated and then incubated for 24 hours with 5 mM ATP, 0.5 mg/mL of nucleosides, adenosine (A), thymidine, (T) or azidothymidine (Z). The IL-1β level in supernatants was measured by ELISA, and the data were presented as the mean ± SEM.
Statistical analysis

All statistical evaluation and graphing were performed using Prism 5.0 for Macintosh (GraphPad, San Diego, CA, USA). The standard Student's $t$ test was used for statistical analysis of in vitro experiments. A one-way analysis of variance (ANOVA) was used to determine statistical significance between arthritic and control groups of mice with the Newman-Keuls multiple-comparison test. A value of $p < .05$ was considered the limit of statistical significance.

Results

Nucleosides enhance inflammatory osteolysis in CIA mice

We first used collagen-induced arthritic (CIA) DBA1 mice to determine whether nucleosides accelerate bone destruction under inflammatory condition. Although several animal models with inflammatory arthritis have been produced, the CIA model shares several pathologic features with rheumatoid arthritis and is used widely to study the pathologic mechanism of autoimmunity.\(^{11}\) Collagen II triggers an autoimmune response in joints, and complete Freund's adjuvant (CFA) facilitates the development of autoimmune arthritis.\(^{11}\) After 1 week of immunization with collagen II plus CFA, mice were injected intraperitoneally with thymidine (T) or nucleoside analogue azidothymidine (Z) or PBS as a control. Anti-collagem II antibodies (IgG\(_1\) and IgG\(_2\)), which are observed in CIA mice, were detected in serum after immunization. Nucleosides, thymidine, and azidothymidine did not increase antibody concentrations in all CIA groups, suggesting that nucleosides do not affect the antibody response to collagen II antigen (Fig. 1A). Nucleosides accelerated paw inflammatory swelling within 1 week that progressed over the course of treatment in CIA mice, whereas paws did not appear swollen until after 3 weeks in the PBS-treated CIA mice (Fig. 1B). The effects of nucleosides on bone and joints were assessed in CIA mice using \(\mu\)CT imaging (Fig. 1C). In contrast to slight bone erosions in PBS-treated CIA mice, nucleoside treatment markedly enhanced bone erosion and joint deformity. Trabecular bone volume (BV/TV) by \(\mu\)CT were reduced by nucleosides to approximately 50% of that of control mice. Furthermore, systemic bone mineral density (BMD) also was significantly reduced by nucleosides ($p < .05$ versus control; Fig. 1D).

Nucleosides stimulate osteoclastogenesis and bone resorption in vivo and in vitro

The effects of nucleosides on bone erosion were further evaluated by histologic examination of the paws. The results showed severe inflammation and bone destruction in nucleoside-treated mice that were characterized by tenosynovitis, villus proliferation, pannus, bone erosion, and joint destruction. The pannus was infiltrated by macrophages and neutrophils. In contrast, paws from nonimmunized or PBS-treated CIA mice revealed either normal or slight proliferation of the synovial lining layer without cell infiltration and destruction of cartilage and bone (Fig. 2A). Notably, inflammation and tissue damage enhanced by nucleosides were limited to joints but were not seen not in other important organs, including lung, pancreas, thymus, kidney, and spleen (data not shown).

Given the causal role of osteoclasts in inflammatory bone destruction, osteoclast infiltration was assessed in each group of mice by TRACP staining. Large numbers of TRACP$^+$
osteoclasts accumulated in the paws of nucleoside-treated CIA mice. In contrast, no osteoclasts were found in paws of nonimmunized mice (Fig. 2B). Histomorphometric analysis of femoral trabecular bone is summarized in Table 1. The ratio of bone volume to total volume (BV/TV%), as well as the number and thickness of trabecular bone, was significantly reduced in nucleoside-treated CIA mice. Compared with control CIA mice, the surface and number of osteoclasts were markedly increased, whereas the surface and number of osteoblasts were not changed by nucleosides (CIA versus CIA + T or CIA + Z).

This osteoclastogenic effect of nucleosides was further confirmed in cultured mouse bone marrow macrophage precursors in vitro. The tested nucleosides, adenosine, azidothymidine, and thymidine, stimulated bone marrow macrophages to differentiate into TRACP\(^+\) multinucleated osteoclasts in the presence of RANKL (Fig. 2C). Total numbers of multinucleated osteoclasts induced by RANKL plus nucleosides were increased twofold compared with RANKL alone. Furthermore, the differentiated osteoclasts retained bone-resorption activity, as determined by pit assays. The percentages of pit area produced by adenosine, thymidine, and azidothymidine treatments were 5.4%, 6.7%, and 12.0%, respectively. They were greater than the less than 2% obtained with control osteoclasts treated with RANKL alone without nucleosides (Fig. 2D).

However, nucleosides alone neither stimulate osteoclast differentiation in the absence of RANKL in vitro nor induce the development of arthritis in nonimmunized mice in vivo, suggesting that nucleosides may act as an enhancer but not an inducer of inflammatory autoimmune arthritis (Supplemental Fig. 1). All results support the idea that bone loss in nucleoside-treated CIA mice is associated with increased osteoclast activity.

**Nucleosides activate the innate immune response**

CFA is a typical immune adjuvant for enhancing inflammation and bone erosion in the CIA model. To further determine whether nucleosides by themselves are able to accelerate inflammation and osteolysis in mice, the frequency and severity of nucleoside-induced osteolysis were compared in mice that were immunized with collagen II alone, CFA alone, or collagen II plus CFA. Nucleosides accelerated osteolysis in mice immunized with collagen II alone. Furthermore, the severity of osteolysis was similar to that in PBS-treated mice immunized with collagen II plus CFA, suggesting that nucleosides stimulate inflammatory osteolysis in CIA mice as an immune activator such as CFA (Fig. 3A). In μCT analysis of the hind paws/knees, nucleosides reduced the ratio of BV/TV in mice immunized with collagen II alone or collagen II plus CFA \((p<.01\) versus PBS) but not in mice treated with CFA alone \((p>.05\) versus PBS). BV/TV% in mice immunized with collagen II plus CFA was further significantly reduced compared with mice immunized with collagen II alone (Fig. 3B). The histologic score showed that the most severe inflammation and bone destruction were detected in nucleoside-treated mice that were immunized with collagen II plus CFA \((\text{mean scores} > 2)\). In mice immunized with collagen II alone, however, nucleosides produced moderate pathologic changes that were similar to those in PBS-treated CIA mice \((\text{mean score} < 2)\). Only modest proliferation of monolayer fibroblasts in the synovium was detected without marked bone erosion. No inflammation or bone erosion was detected in any mice receiving CFA alone \((\text{mean score} = 0);\) Fig. 3C and Supplemental Fig.
The differentiation capacity of osteoclast precursors was determined ex vivo using bone marrow cells isolated from each group and revealed that abundant bone marrow cells differentiated into osteoclasts in all nucleoside-treated collagen II–immunized mice. Moreover, nucleosides and CFA had a synergistic or additive effect on osteoclast differentiation in mice immunized with collagen II plus CFA (Fig. 3D). This enhancement of inflammation and osteolysis by CFA and nucleosides in CIA mice is likely mediated through two distinct activated innate immune responses.

Although CFA contains ligands for TLRs and non-TLRs, CFA activates the innate immune response principally by triggering the TLR proteins on dendritic cells. Another family of innate immune receptors, TREM, has the potential for recognition of nucleic acid products and regulation of the host immune responses. Given that TREM2 is also a coreceptor involved in osteoclast differentiation, we determined whether nucleoside-enhanced osteolysis is mediated through the TREM pathway. We first determined the expression of TREM2 transcript in mouse RAW264.7 cells (Fig. 4A) and the level of TREM2 protein in mouse bone marrow macrophages (Fig. 4B). Nucleosides increased TREM2 and TRACP mRNA and protein in the presence of RANKL. Notably, increased levels of TREMs also were seen in spleen cells from nucleoside-treated CIA mice compared with control and PBS-treated CIA mice (Fig. 4C). Then the function of increased TREM2 in nucleoside-induced osteolysis was determined by blocking the TREM pathway using a TREM2 antibody. TREM2 antibody reduced nucleoside-induced osteoclastogenesis of mouse bone marrow macrophages to the levels induced by RANKL alone (Fig. 4D). Furthermore, nucleosides failed to stimulate osteoclast differentiation in RAW264.7 cells that were transfected with TREM2-silencing RNA (siRNA) to block the TREM pathway (Fig. 4E).

To further characterize the mechanism by which nucleosides accelerate osteoclastogenesis, the target of TREM signaling, which is responsible for increasing numbers of TRACP+ cells, was determined. Mouse bone marrow macrophages were cultured with nucleosides in the presence of M-CSF and RANKL for 24 hours, and then the levels of PLCγ and NFATc1 in total cell lysate were detected by Western blot analysis. Despite RANKL-increased phosphorylated PLCγ and cytoplasmic NFATc1, nucleosides continued to stimulate the phosphorylation of PLCγ and increase the expression of NFATc1 (Fig. 4F). All evidence supports the idea that nucleosides activate innate immune responses likely mediated by the TREM pathway that is distinct from CFA.

Role of the inflammasome pathway in nucleoside-enhanced osteoclastogenesis

In addition to the surface TLR and TREM receptors, the intracellular innate immune receptors, such as Nalp3 inflammasome, also can detect cytoplasmic nucleic acid products and activate the innate immune system. The activation of Nalp3 ultimately results in the production of several proinflammatory cytokines, including IL-1β and IL-18. It has been demonstrated that classic aluminium adjuvants activate the Nalp3 inflammasome, producing IL-1β and regulating the innate immune response. To determine whether nucleosides activate the Nalp3 inflammasome, we measured the levels of IL-1β proinflammatory cytokine in supernatants from mouse bone marrow macrophages by ELISA. Nucleosides induced release of IL-1β from wild-type mouse bone marrow macrophages. Furthermore,
the increased IL-1β in wild-type mouse bone marrow cells was inhibited by addition of a caspase-1 inhibitor (zYVAD; Fig. 5A).

To further determine the role of Nalp3 in nucleoside-enhanced osteoclastogenesis, nucleoside-induced osteoclastogenesis was compared using bone marrow macrophages from Nalp3−/− knockout mice and control wild-type C57BL/6 mice. Although IL-1β was undetectable in control bone marrow macrophages from both wild-type and Nalp3−/− knockout mice, ATP and nucleosides only stimulated bone marrow cells from wild-type mice but not from Nalp3−/− knockout mice to release IL-1β into the medium (Fig. 5B). However, macrophages from Nalp3−/− knockout mice still were able to differentiate to osteoclasts in the presence of RANKL and M-CSF (Fig. 5C). As seen in macrophages from wild-type mice, nucleosides significantly increased osteoclastogenesis of bone marrow macrophages from Nalp3−/− knockout mice (Fig. 5C), but the increase in magnitude (p < .05 versus control) was lower than that observed in macrophages from wild-type mice (p < .01 versus control). Similarly, the osteoclastogenic effect of nucleosides in bone marrow macrophages from Nalp3−/− knockout mice was less effective than that from wild-type mice (p < .05, wild-type versus knockout mice). Therefore, IL-1β produced by activating the intracellular Nalp3 inflammasome pathway is partially involved in the osteoclastogenic effect of nucleosides.

**The purinergic receptors do not mediate nucleoside-stimulated osteoclastogenesis**

Other levels of control over the release of IL-1β have been described, such as the P2X7 purinergic receptor. Furthermore, effects of extracellular nucleotides generally are mediated through the purinergic receptor exhibiting a number of functional responses. Increased bone destruction in nucleoside-treated CIA mice may be mediated in part by the purinergic receptors. To determine the role of the purinergic receptor in nucleoside-enhanced osteoclastogenesis, a set of purinergic receptor antagonists, including 8-phenyltheophylline (8-PT), 8-cyclopentylxanthine (DPCPX), and ZM241385, was used to block the purinergic pathways. Although the concentrations of these antagonists were shown to completely inhibit their respective receptors, none of the purinergic antagonists significantly inhibited nucleoside-enhanced osteoclastogenesis in vitro using mouse bone marrow macrophages (Fig. 6A). Because mouse RAW264.7 cells primarily expressed the A2b receptor (data not shown), the role of the purinergic receptor in nucleoside-stimulated osteoclastogenesis was further examined by using A2b receptor (A2bR) siRNA. Nucleosides still markedly promoted osteoclast differentiation in RAW264.7 cells transfected with A2R siRNA (Fig. 6B). Therefore, the purinergic receptors most likely do not play crucial roles in nucleoside-enhanced osteoclastogenesis.

**Nucleosides recruit osteoclasts and increase inflammatory cytokines**

Another important issue is which cell populations respond to the nucleoside-enhanced innate immune response. Osteoclast precursors and intracellular proinflammatory cytokines in whole blood were assessed by polychromatic flow cytometry (PFC, 8-color). The CD11b-gated cell population in PFC analysis consists primarily of monocytes and macrophages (Supplemental Fig. 3). The proportions of CD11b+ monocytes and macrophages were markedly increased to 20% of the total blood cells in nucleoside-treated
CIA mice compared with 10% in nonimmunized and PBS-treated CIA mice (Fig. 7A). Consistent with the increased monocyte/macrophage recruitment, nucleoside-treated CIA mice showed marked splenomegaly (Fig. 7B).

Compared with control mice, the proportion of TNF-α+ monocytes/macrophages was markedly increased in peripheral macrophages prepared from nucleoside-treated CIA mice ($p < .001$ versus control; Fig. 7C). The serum level of TNF-α from each group of mice was measured by ELISA (Fig. 7D); collagen II immunization remarkably increased TNF-α in serum compared with control mice. Nucleosides further significantly upregulated TNF-α production ($p < .05$ versus control). Nucleosides had no significant effect on the IL-4- and interferon γ (IFN-γ)–producing monocyte/macrophages from CIA mice ($p = \text{NS}$ versus control). IL-2-producing macrophages were not detected. Importantly, neither the proportion of T-lymphocytes nor their intracellular cytokines were altered by nucleosides in all groups of mice (Fig. 7E). Therefore, activated T-lymphocytes are likely an important source of bone erosion through regulating RANKL expression but not via production of inflammatory cytokines. Nucleosides specifically promoted proliferation and cytokine production in monocytes/macrophages but not in T-lymphocytes, further supporting the concept that TREM receptors on monocytes/macrophages are the site for nucleoside interaction.

**Discussion**

Most of the previous investigations into the pathogenesis of inflammatory osteolysis attributed it to inflammatory cytokine networks. Our results in vivo and in vitro demonstrate for the first time that nucleosides, acting as unique immune activators distinct from CFA, are involved in inflammatory osteolysis in CIA mice. Nucleosides activate the innate immune system likely via the surface TREM receptors and the intracellular Nalp3 inflammasome to enhance inflammatory osteolysis. The activation of the innate immune system by nucleosides recruits more osteoclast precursors to the joint space and increases the level of TREM receptors, enhancing their differentiation capacity. Furthermore, nucleosides also contribute to the processing and production of proinflammatory cytokines that trigger the subsequent adaptive immune responses. In contrast with increased osteoclast number in nucleoside-treated CIA mice, osteoblast formation is not significantly affected by nucleosides, suggesting that nucleoside-enhanced osteolysis is mediated solely by enhanced osteoclastogenesis. Together these pathways synergistically accelerate osteoclastogenesis and inflammatory bone destruction in inflammatory osteoarthritis.

Nucleosides are produced constitutively by cells under physiologic conditions. The concentration of extracellular nucleosides is thought to be below 1 μM, whereas nucleoside levels in inflammation, ischemia/hypoxia, and trauma/hemorrhage are increased to as high as 100 μM. Increased nucleosides generally are thought to play anti-inflammatory roles because they inhibit immunity and facilitate protection from microorganisms or infectious agents. However, under chronic inflammation and autoimmune diseases, tissue damage releases chromosomal DNA and dephosphorylates ATP and other NTPs, forming a large number of extracellular nucleosides. Accumulated nucleosides could, directly or indirectly, further accelerate tissue damage, such as occurs in systemic lupus erythematosus.
(SLE), Crohn disease, chronic obstructive pulmonary disease (COPD), and chronic polyarthritis in DNase II knockout mice.\(^7\)

Our results support this concept and begin to elucidate the underlying molecular mechanisms by which accumulated nucleosides, acting as innate immune activators, activate the innate immune responses. It has been reported that there is an increase in purine metabolism in the rheumatoid arthritis joint and that hypoxanthine is a potential marker of synovitis.\(^8\) However, whether the incidence and severity of inflammatory osteolysis in patients with osteoarthritis are correlated with the systemic and/or intraarticular local concentration of nucleosides has not been elucidated. Furthermore, our data are only obtained using selected nucleosides that represent common metabolic nucleosides released from a variety of cells in response to metabolic and inflammatory stress and pharmaceutical nucleoside analogues used in AIDS therapy. In fact, the concentrations of nucleosides and their metabolic components in synovial fluid or plasma have been measured in very few patients. Therefore, it is necessary to measure nucleosides in patients with osteoarthritis, which potentially could predict the development and prognosis of bone destruction as valuable biomarkers.

Nucleosides could be recognized by the TLR receptor, TREM receptor, inflammasome, and purinergic receptor. However, the immune activator effects of nucleic acids generally are mediated through the TLR pathway. The TLRs have been implicated in promotion of osteoclast formation and inflammatory osteolysis. At least four TLRs (TLR3, -7, -8, and -9) are able to recognize and respond to extracellular nucleic acids.\(^22\) Notably, human TLR7 and TLR9 recognize self nucleic acids that hamper glycocorticoid activity in lupus.\(^22\) It has been reported that activation of the TLR pathway in inflammation upregulates RANK expression, thereby increasing the sensitivity of osteoclast precursor to RANKL.\(^23\) Thus it can be hypothesized that the osteoclastogenic effects of nucleosides are also partially mediated through activation of the TLR pathway.

A critical role for TREM2 in osteoclast formation is suggested by the impaired bone-resorptive activity in TREM2-deficient patients.\(^24\) Our results show that TREM2 expression in osteoclast precursors is upregulated by nucleosides, thereby inducing osteoclast precursors with high TREM expression to differentiate at lower concentrations of RANKL.\(^25\) To further define the role of TREM2 in the osteoclastogenic effect of nucleosides, we focused on the phosphorylation of PLC\(\gamma\) and translocation of NFATc1 that are known to be potent signals in the TREM2 activation.\(^26–28\) Despite the fact that these two signals are also activated by the RANK pathway,\(^29\) nucleosides further increased the levels of phosphorylated PLC\(\gamma\) and cytoplasmic NFATc1 activated by RANKL, suggesting the role of another pathway beyond RANK (Fig. 4). Thus we cannot exclude the possibility that the TREM2 pathway may be involved in nucleoside-activated innate immune responses. However, whether these two receptors, RANK and TREM2, cross-talk in osteoclastogenesis will be further defined in the future.

Whether TREM, like TLR, specifically recognizes nucleic acids/nucleosides has not yet been determined. For most of the TLRs, TLRs bind and become activated by many different ligands, including the large-molecular-weight nucleic acid components of pathogens as well
as small-molecule synthetic nucleoside analogues. Moreover, no specific receptor-binding assay for TLRs has been reported. Ligand recognition specificity generally is defined by using a technique of gene knockout.\(^{(30)}\) Owing to the failure of osteoclastogenesis of bone marrow cells from \textit{wither TREM} or DAP12\(^{-/-}\) knockout mice, this technique is not available for TREM. Considering the similar adaptors and functional features of TREM and TLR, it is not surprising that nucleosides are potential ligands for the TREM receptors. Previous studies have demonstrated that \textit{Escherichia coli} and several other species of bacteria bind specifically to TREM2-transfected cells, suggesting that small anionic metabolic molecules from self antigens and pathogens might be candidate ligands for TREM.\(^{(31)}\) In fact, it has been reported that TREM, like many other innate immune receptors, may have the ability to bind multiple ligands.\(^{(23)}\) Future studies using binding assays for nucleosides and the TREM2 receptor and TREM-2A/IgG\(_1\)-Fc fusion protein or TREM2-expressing BWZ cells could provide direct evidence.

The Nalp3 inflammasome is an intracellular innate immune receptor that recognizes endogenous uric acid, ATP/ADP, and nucleosides. The Nalp3 inflammasome recognizes gout-associated uric acid crystals, resulting in the production of active proinflammatory cytokines.\(^{(17)}\) Our results show that nucleosides stimulated the production and secretion of IL-1\(\beta\) from wild-type mouse bone marrow macrophages but not from \textit{Nalp3}\(^{-/-}\) knockout mouse bone marrow cells (Fig. 5). The production and release of IL-1\(\beta\) not only trigger the subsequent adaptive immune responses, amplifying inflammatory cytokine production,\(^{(17)}\) but also stimulate RANKL-mediated osteoclastogenesis.\(^{(32)}\) Despite the fact that bone marrow cells from \textit{Nalp3}\(^{-/-}\) knockout mice retain nucleoside-enhanced osteoclastogenesis in vitro, the low level of IL-1\(\beta\) reduced the osteoclastogenic efficacy of nucleosides, suggesting that the increase in proinflammatory cytokine IL-1\(\beta\) by nucleosides likely mediates at least in part the nucleoside-enhanced osteoclastogenesis.

Recent reports have shown that \textit{Nalp3}\(^{-/-}\) and \textit{caspase-1}\(^{-/-}\) knockout mice also had severe collagen- and antigen-induced arthritic bone destruction that was similar to that of wild-type mice. These knockout mice were susceptible to arthritic osteolysis induced by collagen or antigen immunization. In contrast, only \textit{ASC}\(^{-/-}\) knockout mice showed reduced severity of arthritic osteolysis.\(^{(33,34)}\) It is still not clear what role various components of the inflammasome complex play in mechanism underlying activation of innate immunity and inflammatory osteolysis. ASC may modulate the immune response in arthritis through its effect on cell-mediated immune responses but not via inflammasome formation.\(^{(33,34)}\) Thus the effect of the Nalp3 inflammasome on nucleoside-enhanced osteoclastogenesis needs further investigation in various inflammasome knockout mice in vivo.

In addition to the nucleoside-induced increased osteoclastogenesis via stimulating TREM expression and activating the processing and secretion of IL-1\(\beta\), another important observation is the recruitment of the CD11b\(^{+}\) cell population in peripheral blood in nucleoside-treated CIA mice, which is a critical product of immune adjuvant stimulator (Fig. 7). Human CD11b is expressed primarily on monocytes and macrophages, especially osteoclast precursors in bone marrow. It has been reported that functional osteoclasts are derived from CD11b\(^{+}\) cells in human peripheral blood mononuclear cells (PBMCs).\(^{(35)}\) It also has been reported that administration of aluminum in mice triggered the recruitment of
neutrophils, monocytes, and dendritic cells (DCs) and enhanced both cellular and humoral immunity.\(^{36}\) The expansion of monocytes/macrophages has been reported to correlate with the progression of inflammatory arthritis.\(^{37}\) The increase of CD11b\(^+\) monocytes/macrophages in nucleoside-treated CIA mice facilitates monocyte/macrophage-driven inflammation and constitutes a substantial source from which osteoclast precursors are recruited to the joint. In addition, nucleosides specifically promoted monocyte/macrophage recruitment and their intra-cellular cytokine production but not T-lymphocytes, providing indirect evidence that the nucleoside‐induced effects are mediated through TREM on monocytes/macrophages. In nucleoside‐treated CIA mice, activated T‐lymphocytes may only provide RANKL for recruiting RANKL‐primed osteoclast precursors but do not produce inflammatory cytokines.\(^{21}\)

Recently, a high prevalence of osteopenia, osteoporosis, and osteoarthritis has been reported in HIV-infected patients receiving highly active antiretroviral therapy (HAART). A new clinical study showed that bone mineral density (BMD) declined gradually every year in hip and spine of HIV-infected patients with continuous antiretroviral therapy.\(^{38}\) However, controversial negative evidence also has been showing that not all individuals receiving HAART exhibit osteopenia and osteoporosis.\(^{39}\) Neither the pathogenesis of bone loss nor the reason for the conflicting results in HIV-infected patients on HAART have been elucidated. Therefore, most therapeutic management schemes are focused only on reducing the risks of complications. Our studies provide a likely new mechanism of underlying HAART‐induced osteopenia, osteoporosis, and osteoarthritis in HIV/AIDS patients. Antiretroviral nucleoside compounds directly and indirectly stimulate osteoclast formation, increasing the prevalence and severity of osteopenia and osteoporosis in HIV-infected patients. However, this stimulatory effect requires the presence of RANKL most likely released from activated T‐lymphocytes. The functions of T‐lymphocytes in HIV-infected patients are likely associated with the prevalence of these bone‐joint disorders. It has been observed that osteoarthritis occurs frequently when the CD4 cells are increased by HAART therapy, suggesting that improvement of HIV disease and restoring of CD4 T‐lymphocytes during the setting of immune reconstitution may allow HIV‐infected individuals to produce more RANKL and to be more susceptible to the onset of osteoarthritis.\(^{40,41}\) It has already been reported that macrophage depletion diminished whereas T‐cell depletion accelerated inflammatory osteolysis in mice, suggesting that monocytes/macrophages, and not T cells, are likely responsible for the osteoclastogenic effect of nucleosides.\(^{38}\) However, the function of lymphocytes in nucleoside‐enhanced osteolysis must be further confirmed using lymphocyte‐deficient CIA mice.\(^{42}\)

In conclusion, our studies provide novel insight into the pathogenesis of bone destruction in inflammatory joint diseases, in which nucleosides likely activate both the innate and adaptive immune systems via the TREM and Nalp3 pathways (Fig. 7F). Owing to chronic inflammation, large amounts of host cell apoptosis and bacterial or viral pathogens cause the local accumulation of nucleosides in joints that are recognized by the TREM receptors on macrophages and the intracellular Nalp3 inflammasome, activating the innate immune system. Accumulated nucleosides either recruit abundant macrophages or upregulate expression of the TREM receptors on macrophages, thereby increasing RANKL‐mediated osteoclastogenesis. Simultaneously, the recognition of nucleosides by the intracellular Nalp3
inflammasome produces proinflammatory cytokines IL-1β and IL-18. IL-1 exerts direct and indirect effects on the formation of osteoclasts.\(^\text{43}\) T cells primed by IL-1β not only activate adaptive immune responses but also provide RANKL, thereby generating abundant RANKL-primed osteoclast precursors. These pathways synergistically accelerate inflammatory bone destruction. Blockade by small-molecule inhibitors of the TREM receptor may provide a new generation of antiarthritis/anti-bone-destruction drugs. In order to further confirm the role of nucleosides in inflammatory osteolysis, other small-molecule nucleosides/nucleoside analogues and osteoarthritis animal models such as antigen-induced arthritis (AIA) and specific knockout mice, including \(\text{TREM2}^-\), \(\text{Nalp3}^-\), or \(\text{ASC}^-\) knockdown mice should be used in the future.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### References


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Fig. 1. Nucleosides accelerate inflammation and osteolysis in CIA mice. Arthritis was induced in DBA-1 mice by immunization with collagen II and complete Freund's adjuvant, as described previously. (11) One week after immunization (day 7), mice were injected intraperitoneally with PBS (CIA + PBS, n = 6) or with thymidine (CIA + T, n = 8) or azidothymidine (CIA + Z, n = 8) (25 mg/kg/200 μL PBS/mouse) daily for 5 days per week. Nucleosides were administrated for 4 weeks. Nonimmunized DBA1 mice were used as controls (Control, n = 3). Mice were euthanized on day 35, and the following tests were performed. (A) The level of bovine collagen type II antibodies in mouse serum samples was determined quantitatively by ELISA. The results are expressed as the mean ± SEM. (B) The severity of inflammatory arthritis was assessed by scoring the degree of paw swelling. For each mouse, the mean score per paw (maximum value = 4) was calculated. Data are the mean swelling score/paw ± SEM for each group. (C) The hind limbs were scanned horizontally by μCT. The parameters of total volume (TV), bone volume (BV), and ratio of BV to TV (BV/TV) are shown, and the figures are a representative image of each experimental group of mice. (D) Noncephalic whole-body bone mineral density (BMD) of mice was measured using dual-energy X-ray absorptiometry (DXA). The results are expressed as the mean ±SEM. The p values are shown compared with control.
Fig. 2.
Nucleosides stimulate osteoclastogenesis and bone resorption in vivo and in vitro. (A) Decalcified paraffin-embedded 2-μm sections of the paws were stained with hematoxylin and eosin for assessing signs of inflammation and bone destruction (arrows). Representative micrographs are shown. (B) TRACP staining was performed to detect TRACP⁺ osteoclasts. Representative TRACP staining of paw in collagen II–immunized mice treated with nucleosides is shown. TRACP⁺ osteoclasts are purple. (C) Mouse bone marrow macrophages were incubated without and with nucleosides, azidothymidine (Z), adenosine (A), or thymidine (T) at 10 μM in the absence (medium) and presence of RANKL (RANKL, 100 ng/mL) plus M-CSF (10 ng/mL) for 4 more days. TRACP staining was performed, and TRACP⁺ osteoclasts with up to 3 nuclei were counted per well. The values are presented as the mean TRACP⁺ osteoclasts per well±SE. Representative TRACP⁺ osteoclast cells are shown from four individual experiments. *p < .05 and **p < .01 for RANKL plus nucleosides versus RANKL alone. (D) Mouse bone marrow macrophages were plated on sterile dentine slices and cultured in complete α-MEM containing RANKL(100 ng/mL) alone (R) or RANKL plus adenosine (R + A), azidothymidine (R + Z), and thymidine (R + T) (10 μM nucleosides) in the presence of M-CSF (10ng/mL) for 10 days. Fresh medium was changed in every 3 to 4 days. Dentine slices were examined by scanning electron microscopy. Representative pits caused by osteoclasts are marked with arrows. The data were quantified by measuring the percentage of the pit area in whole dentine slices.
Fig. 3. Nucleosides accelerate osteolysis in mice immunized with collagen II in the presence and absence of CFA. (A) DBA1 mice were immunized with a single injection of collagen II alone (CII), CFA alone (CFA), and collagen II plus CFA (CII + CFA), as described previously. After 1 week of immunization, mice were injected intraperitoneally with PBS (top panel) or thymidine (T, middle panel), or azidothymidine (Z, bottle panel) (25 mg/kg/200 μL/mouse) daily 5 days per week. After euthanization, osteolysis was detected by μCT. Images are representative examples of the hind paw of mice from the different groups. Arrows indicate bone erosion. (B) The ratio of BV to TV (BV/TV) is calculated in each group of mice. The results are expressed as the mean ± SEM (n = 3/group). The p values for the relations are shown. (C) Semiquantification of histologic features was scored in each group of mice, as described previously. (D) Mouse bone marrow macrophages were isolated from mouse tibia of each group and plated on a 24-well plate at a concentration of 5 × 10⁴ cells. Cells were cultured in complete a-MEM containing RANKL and M-CSF for 4 more days. TRACP staining was performed, and TRACP⁺ osteoclasts with up to 3 nuclei were counted per well. The values are presented as the mean TRACP⁺ osteoclasts per well ± SEM.
Fig. 4.
Nucleosides activate the innate immune system by activating the TREM receptors. (A) Mouse RAW264.7 cells were treated without (C) or with RANKL (100 ng/mL) alone (R) or with RANKL plus nucleosides adenosine (R + A), thymidine (R + T), or azidothymidine (R + Z) (10μM) for 2 days. RT-PCR for Tracp, Trem2, and Gapdh genes was performed. (B) Mouse bone marrow macrophages were cultured with RANKL alone (100 ng/mL, R), RANKL plus 10 μM nucleosides adenosine (R + A), azidothymidine (R + Z), or thymidine (R + Z) in the presence of M-CSF (10ng/mL) for 24 hours. Cells were cultured with M-CSF alone as control (C). The expression levels of TRACP, TREM2, and actin were determined by Western blot in cell lysates. (C) Total RNA was prepared from spleens of nonimmunized control mice or collagen II–immunized mice with PBS or nucleosides (Tand Z). The levels of Tracp, trem1, Trem2, and Gapdh or 18S genes were analyzed by RT-PCR. (D) Mouse bone marrow cells were cultured without or with anti-TREM antibody (5 μg) in the absence or presence of RANKL (100 ng/mL) and/or nucleosides A, Z, and T (10 μM) for 4 days. TRACP staining was performed, and the number of TRACP+ multinuclear osteoclasts per well was measured. The values are presented as the mean ± SEM in three independent experiments. The p values are shown. (E) RAW264.7 cells were transfected with Trem2 siRNA and negative siRNA as a control per the manufacture's instructions (Santa Cruz Biotechnology). After 36 hours of transfection, cells were treated with or without nucleosides azidothymidine (Z) and thymidine (T) (10 μM) in the presence (R) or absence (C) of RANKL (100 ng/mL) for 4 days. The expression of Trem2 in transfected cells was determined by RT-PCR (upper gels). TRACP+ osteoclasts were counted. The results are expressed as the mean ± SEM of TRACP+ osteoclasts per well. (F) Mouse bone marrow macrophages were cultured with the nucleosides adenosine (A), thymidine (T), or azidothymidine (Z) for 8 hours in the presence of M-CSF and RANKL. Whole-cell lysates were subjected to Western blot analysis for phosphorylated PLCγ (p-PLCγ), total PLCγ, and NFATc-1 proteins. Actin is shown as loading control.
Fig. 5. The role of the Nalp3 pathway in nucleoside-enhanced osteoclastogenesis. (A) Bone marrow cells from C57BL/6 mice (white columns) were plated and then cultured with ATP, nucleosides [adenosine (A), thymidine (T), and azidothymidine (Z)] for 24 hours in the absence or presence of caspase-1-specific inhibitor (zYVAD-fmk; black columns). Supernatants were harvested and assayed for mature IL-1β by ELISA. (B) Mouse bone marrow cells from C57BL/6 mice (white columns) and Nalp3−/− knockout mice (black columns) were treated with nucleosides for 24 hours. Supernatants were harvested and assayed for mature IL-1β by ELISA. (C) Bone marrow cells from C57BL/6 mice (white columns) and Nalp3−/− knockout mice (black columns) were cultured for 4 more days in the presence of M-CSF (10ng/mL) and RANKL (100 ng/mL). TRACP staining was performed, and TRACP+ osteoclasts with up to 3 nuclei were counted per well. The values are presented as the mean ± SEM. The p values are shown compared between macrophages from wild-type and knockout control mice in each indicated treatment. *p < .05 and **p < .01 indicate comparisons with non-nucleoside-treated macrophages from control wild-type and knockout mice.
Fig. 6.
The role of purinergic receptors in nucleoside-treated mouse bone marrow macrophages. (A) Mouse bone marrow macrophages were incubated for 4 days with PBS (C), azidothymidine (Z), adenosine (A), and thymidine (T) at 10μM in the absence or presence of RANKL (100 ng/mL, R) and purinergic receptor inhibitor (10 μM 8-PT, 50 μM DPCPX, or 10 μM ZM241385), as indicated. TRACP\(^+\) osteoclasts were counted. (B) RAW264.7 cells were transfected with A2bR siRNA and negative siRNA as control per the manufacture’s instructions (Santa Cruz Biotechnology). Cells were treated with or without nucleosides in the presence or absence of RANKL at 36 hours after transfection. The level of A2bR was measured by RT-PCR (top panels). TRACP\(^+\) osteoclasts were counted. The results are expressed as the mean ± SEM of TRACP\(^+\) osteoclasts per well.
Fig. 7.
Analysis of cell populations that are involved in inflammation and bone destruction in peripheral blood cells derived from nucleosides-treated CIA mice. Heparinized blood was withdrawn (day 35) from nonimmunized control mice and CIA mice treated with nucleosides or PBS. Whole blood cells were stained with different fluorescence-conjugated antibodies specific for cell surface markers CD11b, CD4, CD8, and B220. Monocytes/macrophages and lymphocytes were discriminated based on their characteristic forward and side light-scatter profiles. Intracellular cytokines were analyzed by polychromic flow cytometry in peripheral blood samples from each group of mice. Following surface staining and lysis of red blood cells (BD Bioscience), cells were permeabilized and stained with fluorescence-conjugated antibodies to detect intracellular cytokines TNF-α, IFN-γ, IL4, and IL2. Analysis of flow cytometry data was performed using FlowJo software. The results are expressed as the mean ± SE, and the p values are shown compared with control. (A) The percentage of CD11b+ monocytes/macrophages within the pool of myeloids was determined for mice in different treatment groups. The p values are shown to compare with control mice. (B) Spleen-to-body-weight ratios in the different groups of mice were analyzed. The representative spleens from control and CIA + Z groups are shown. (C) The percentage of peripheral blood CD11b+ monocytes/macrophages expressing TNF-α, IL-4, IFN-γ, or IL-2 was determined for individual mice in different treatment groups. The results are expressed as the mean ± SEM. The standard Student's t test shows the statistical significance of cytokine-producing monocytes/macrophages in the nucleoside-treated CIA mice. (D) The concentrations of TNF-α in mouse serum from each group of mice were measured by sandwich ELISA according to the manufacturer's protocol (R&D Systems). Both intra- and interassays coefficients of variation were less than 8% for all assays. The results are
expressed as the mean ± SEM. (E) The percentages of CD4 and CD8T-lymphocytes in the total blood cells were determined in the different groups of mice. The results are expressed as the mean ± SEM. (F) Schematic illustration of the mechanisms of nucleoside-accelerated osteolysis.
### Table 1

**Histomorphometry of Femoral Trabecular Bone**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CIA</th>
<th>CIA+T</th>
<th>CIA+Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>10.75 ± 0.01</td>
<td>10.68 ± 0.58</td>
<td>8.14 ± 0.68 (p&lt;.05)</td>
<td>6.83 ± 0.50 (p&lt;.01)</td>
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<tr>
<td>Trabecular number (/mm)</td>
<td>3.97 ± 0.34</td>
<td>3.62 ± 0.19</td>
<td>2.18 ± 0.19 (p&lt;.05)</td>
<td>2.13 ± 0.24 (p&lt;.01)</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>38.35 ± 2.21</td>
<td>37.19 ± 1.15</td>
<td>31.91 ± 1.34 (p&lt;.03)</td>
<td>28.69 ± 0.91 (p&lt;.01)</td>
</tr>
<tr>
<td>OcS/BS</td>
<td>5.03 ± 0.36</td>
<td>5.37 ± 0.09</td>
<td>6.99 ± 0.49 (p&lt;.02)</td>
<td>6.52 ± 0.39 (p&lt;.01)</td>
</tr>
<tr>
<td>ObS/BS</td>
<td>16.75 ± 3.21</td>
<td>15.57 ± 1.89</td>
<td>16.21 ± 2.03 (NS)</td>
<td>17.21 ± 2.75 (NS)</td>
</tr>
<tr>
<td>N.Oc/BS</td>
<td>1.38 ± 0.16</td>
<td>1.48 ± 0.17</td>
<td>2.42 ± 0.39 (p&lt;.01)</td>
<td>2.45 ± 0.16 (p&lt;.01)</td>
</tr>
<tr>
<td>N.Ob/BS</td>
<td>7.13 ± 1.07</td>
<td>6.88 ± 1.15</td>
<td>7.23 ± 1.31 (NS)</td>
<td>7.91 ± 1.93 (NS)</td>
</tr>
</tbody>
</table>

NS = not significant; BV=bone volume; TV=total volume; OcS = osteoclast surface; ObS = osteoblast surface; BS = bone surface; N.Oc = osteoclast number; N.Ob = osteoblast number.

Results are given as the mean ± SE. There were from 3 to 8 mice in each group (see Fig. 1).