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Adenosine A_{2A} Receptor Ligation Inhibits Osteoclast Formation

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Adenosine is generated in increased concentrations at sites of injury/hypoxia and mediates a variety of physiological and pharmacological effects via G protein–coupled receptors (A_1, A_{2A}, A_{2B}, and A_3). Because all adenosine receptors are expressed on osteoclasts, we determined the role of A_{2A} receptor in the regulation of osteoclast differentiation. Differentiation and bone resorption were studied as the macrophage colony-stimulating factor-1–receptor activator of NF-κB ligand formation of multinucleated tartrate-resistant acid phosphatase (TRAP)–positive cells from primary murine bone marrow–derived precursors. A_{2A} receptor and osteoclast marker expression levels were studied by RT-PCR. Cytokine secretion was assayed by enzyme-linked immunosorbent assay. *In vivo* examination of A_{2A} knockout (KO)/control bones was determined by TRAP staining, micro–computed tomography, and electron microscopy. The A_{2A} receptor agonist, CGS21680, inhibited osteoclast differentiation and function (half maximal inhibitory concentration, 50 nmol/L), increased the percentage of immature osteoclast precursors, and decreased IL-1β and tumor necrosis factor-α secretion, an effect that was reversed by the A_{2A} antagonist, ZM241385. Cathepsin K and osteopontin mRNA expression increased in control and ZM241385-pretreated osteoclasts, and this was blocked by CGS21680. Micro–computed tomography of A_{2A}KO mouse femurs showed a significantly decreased bone volume/trabecular bone volume ratio, decreased trabecular number, and increased trabecular space. A_{2A}KO femurs showed an increased TRAP-positive osteoclast. Electron microscopy in A_{2A}KO femurs showed marked osteoclast membrane folding and increased bone resorption. Thus, adenosine, acting via the A_{2A} receptor, inhibits macrophage colony-stimulating factor-1–receptor activator of NF-κB ligand–stimulated osteoclast differentiation and may regulate bone turnover under conditions in which adenosine levels are elevated. ***(Am J Pathol 2012, 180:775–786; DOI: 10.1016/j.ajpath.2011.10.017)***

Communication between osteoclasts and osteoblasts is essential for bone modeling and remodeling. In bone remodeling, bone formation and resorption are at equilibrium. The principal mechanism for bone remodeling, between bone resorption and formation, lies in the sequential nature of the process by which osteoblasts refill resorption lacunae with an equivalent amount of osteoid.[1,2] When this balance is disturbed in favor of bone resorption, the result is pathological bone destruction, as observed in osteoporosis[3] or inflammatory diseases, such as rheumatoid arthritis.[4]

Osteoclasts degrade bone to initiate normal bone remodeling and mediate bone loss in pathological conditions by increasing their resorptive activity. Osteoclasts are multinucleated giant cells, derived from myeloid precursors belonging to the monocyte/macrophage family,[5,6] that secret hydrochloric acid and proteases, such as cathepsin K and tartrate-resistant acid phosphatase

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Disclosures: B.N.C. holds patents on the use of the following: i) adenosine A_{2A} receptor agonists to promote wound healing and the use of A_{2A} receptor antagonists to inhibit fibrosis, ii) adenosine A_1 receptor antagonists to treat osteoporosis and other diseases of bone, iii) adenosine A_1 and A_{2B} receptor antagonists to treat fatty liver, and iv) adenosine A_{2A} receptor agonists to prevent prosthesis loosening; has received consulting fees within the past 2 years from Bristol-Myers Squibb, Novartis, CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, and Medivector; holds stock in CanFite Biopharmaceuticals; and has received research funding from King Pharmaceuticals, OSI Pharmaceuticals, URL Pharmaceuticals, Inc., and Gilead Pharmaceuticals.

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(TRAP), into an extracellular lysosomal compartment, destroying and resorbing both the mineral and matrix components of bone simultaneously.[7] The initial event associated with osteoclast commitment requires interaction with the hematopoietic growth factor, macrophage colony-stimulating factor (M-CSF)-1, which acts via its receptor. Further commitment, differentiation, and activation of osteoclasts are mediated by a complex network of regulatory factors, including systemic hormones, locally produced cytokines, and cell-cell and cell-matrix interactions that are required for transition of the osteoclast precursor (OCP) into a multinucleated and fully activated osteoclast.[8,9] Among these factors, receptor activator of NF-κB ligand (RANKL) is critical for the stimulation of osteoclast differentiation and activation.[10–14] RANKL binds to its receptor, RANK, on the surface of OCPs, activating signaling through NF-κB, c-Fos, phospholipase Cγ, and nuclear factor of activated T cells c1 (NFATc1), to induce differentiation of OCPs into osteoclasts.[9]

Adenosine, the metabolic product of adenine nucleotide dephosphorylation, is generated intracellularly and extracellularly from the catabolism of adenine nucleotides in response to stress, such as hypoxia and inflammatory injury. Ectonucleotidases, of which the apyrase CD39 and the 5′-nucleotidase CD73 are prominent examples, are present on the extracellular surface of many tissues and are crucially involved in numerous important functions, primarily via generation of adenosine.[15] These enzymes rapidly and effectively shift signaling by releasing adenine nucleotides and their products to signaling through adenosine receptors. Extracellular adenosine regulates a variety of physiological processes via interaction with specific cell surface receptors. Adenosine receptors are members of the large superfamily of G protein–coupled receptors. Four subtypes are recognized: A_1, A_{2A}, A_{2B}, and A_3 receptors, each of which has a unique pharmacological profile and is present in virtually every tissue and cell type.[16] The adenosine A_1 and A_2 receptors were initially subdivided on the basis of their effects on adenylyl cyclase activity, inhibition and stimulation, respectively,[17,18] via coupling to G_i and G_s proteins, respectively; the A_{2B} receptor is coupled to G_q, whereas the A_3 receptor is G_i coupled. Recently, evidence was presented that the A_{2A} receptor may be coupled to different G proteins in different areas and a variety of downstream signaling pathways, including cAMP-dependent, phospholipase C–dependent, and stimulation or inhibition of extracellular signal regulated kinase 1/2 via the cAMP-ras-MEK1 pathway have been implicated in signaling at this receptor.[19–23]

The study of the role of adenosine and adenosine receptors in the regulation of cells involved in bone metabolism and turnover has only recently begun. We have previously reported that both adenosine A_1 and A_{2A} receptors play an important role in promoting human monocyte fusion into giant cells *in vitro*.[24] Moreover, we have found that deletion or blockade of adenosine A_1Rs leads to increased bone density and prevents ovariectomy-induced bone loss without affecting bone formation,[25] and we demonstrated that adenosine A_1 receptor activation is required for appropriate formation and function of osteoclasts *in vitro*.[26]

Because we have previously reported that A_{2A} receptor occupancy inhibits fusion of stimulated human monocytes to form giant cells *in vitro*, in this study, we determined whether there was a similar effect of A_{2A} receptor occupancy on osteoclast formation and function both *in vitro* and *in vivo* and potentially identified novel approaches for the prevention of bone loss.

Materials and Methods

Reagents

Recombinant mouse RANKL and M-CSF were obtained from R&D Systems (Minneapolis, MN). CGS21680 and ZM241385 were obtained from Tocris (Ellisville, MO). α minimal essential medium (α-MEM; Gibco, Invitrogen, Carlsbad, CA) was used for all incubations, supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Paraformaldehyde (PFA), sodium acetate, glacial acetic acid, naphthol AS MX phosphate disodium salt, Fast Red Violet LB, and Fluoroshield with DAPI were from Sigma-Aldrich (St. Louis, MO), and sodium tartrate was from Fisher Scientific (Pittsburgh, PA). The BD BioCoat Osteologic Bone Cell Culture System was from BD Biosciences (San Jose, CA). Silver nitrate and sodium carbonate were from Sigma-Aldrich, and sodium carbonate was obtained from Fisher Scientific. Alexa Fluor 555–phalloidin was obtained from Invitrogen.

Animals

Adenosine A_{2A} receptor knockout (A_{2A}KO) mice were a gift from Dr. Jiang Fan Chen (Boston University School of Medicine, Boston, MA).[27] Female A_{2A}KO mice were bred onto a C57BL/6 background ($\geq$10 backcrosses) in the New York University School of Medicine Animal Facility. Mice described as wild type (WT) were all maintained on the C57BL/6 background by the breeder (Taconic Laboratories, Albany, NY). Genotyping was performed by PCR, as previously reported.[28–30] The WT C57BL/6 mice were used as a control. All protocols were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Osteoclast Differentiation from Bone Marrow Cells

Bone marrow cells were isolated from 6- to 8-week-old female C57BL/6 and A_{2A}KO mice (n = 6 each). Femurs and tibiae were aseptically removed and dissected free of adherent soft tissues. The bone ends were cut, and the marrow cavity was flushed out with α-MEM from one end of the bone using a sterile 21-gauge needle. The bone marrow was carefully dispersed by pipetting and incubated overnight in α-MEM containing 10% fetal bovine serum to obtain a single-cell suspension. Nonadherent cells were collected and seeded at an appropriate den-

sity (200,000 cells) for assays in both 48-well plates and the BD BioCoat Osteologic Bone Cell Culture System in α-MEM with 10% fetal bovine serum and 30 ng/mL M-CSF for 2 days. On day 3 (day 0 of differentiation), 30 ng/mL RANKL was added to the culture, together with CGS21680 (10 μmol/L to 1 nmol/L), alone or in the presence of ZM241385 (10 μmol/L to 1 nmol/L). Cultures were fed every third day by replacing half of the culture medium with an equal quantity of fresh medium and reagents. To determine whether the effect of CGS21680 on osteoclast formation varied with amount of exposure time, the A_{2A} receptor agonist was added at 10 μmol/L on consecutive days, starting on day 3 and ending 7 days later. To correlate the effects of A_{2A} agonist with cytokine expression in osteoclast differentiation, TRAP staining was performed in M-CSF–RANKL–derived osteoclasts plated and differentiated over 7 days in the presence of IL-1β, 10 ng/mL, or tumor necrosis factor (TNF)-α, 50 ng/mL, together with CGS21680 alone or with ZM241385.

Characterization of Osteoclasts in Culture

After incubation for 7 days at 37°C in a humidified atmosphere of 5% CO_2, wells were prepared for TRAP staining, to counteract osteoclast differentiation, or for Von Kossa staining, to study osteoclast function (n = 6 for each assay). Briefly, for TRAP staining, cells were washed with prewarmed PBS, fixed in 4% PFA for 10 minutes, and stained for acid phosphatase in the presence of 0.3 mol/L sodium tartrate, using naphthol AS-BI phosphate as a substrate. To determine osteoclast function, we cultured osteoclasts, as previously described, on the BD BioCoat Osteologic Bone Cell Culture System, a submicron synthetic mineralized calcium phosphate thin film coated onto culture vessels, as an alternative method to dentin slices, for direct assessment of osteoclast activity *in vitro*,[31,32] following the manufacturer's recommendations, and stained the plates with Von Kossa stain. The number of TRAP-positive multinucleated giant cells containing three or more nuclei per cell was scored.[33] The Von Kossa–stained images were analyzed with Matlab compiler software (Natick, MA). The Matlab compiler software used to analyze Von Kossa staining to quantify *in vitro* bone resorption takes an approach that is identical to that used by such commercial software packages as SigmaPlot. A program was developed using basic image analysis methods, such as edge, erode, and other imaging functions, to help extract the regions of interest and quantify the osteoclastic resorption. The edge function permitted transformation of color images into binary images, making it easier to identify and analyze the resorbed areas. After selecting the image and the region of interest, a basic threshold was applied to the images to obtain the optimum binary quality for analysis. The total area, which was equivalent to the total number of pixels in the selected image, was calculated via a simple counter. Then, the number of white pixels, which represents the resorbed areas, was also counted via a counter, and a percentage of the resorbed area was calculated. To further validate the software measurement of the area of resorption, we quantitated areas of known size and calculated the variance. The software performed these measurements in a reproducible fashion, and the results obtained accurately reflected the relative sizes of the areas measured.

Quantitative Real-Time RT-PCR

To validate the effect of A_{2A} receptors in osteoclast differentiation, we measured the activation of the two osteoclast differentiation markers, cathepsin K and NFATc1, together with osteopontin, an extracellular structural protein that initiates the development of osteoclast ruffled borders, and A_{2A} receptor by quantitative RT-PCR. WT osteoclasts derived from bone marrow were collected during the 7 days of differentiation, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Invitrogen), following the manufacturer's protocol, including sample homogenization with QIAshredder columns (Qiagen). To avoid genomic DNA contamination, we performed the on-column DNA digestion step. For first-strand cDNA synthesis, 20 μL of total RNA was retrotranscribed using the MuLV Reverse Transcriptase PCR kit (Applied Biosystems, Foster City, CA) at 2.5 U/μL, including the following reagents in the same reaction: RNase inhibitor, 1 U/μL; random hexamers, 2.5 U/μL; $MgCl_2$, 5 mmol/L; PCR buffer II, one times; and deoxyribonucleotide triphosphates, 1 mmol/L (all from Applied Biosystems). Relative quantification of gene expression was performed using real-time RT-PCR on a Stratagene Mx3005P (Agilent Technologies, La Jolla, CA) with Brilliant SYBR Green Kit QPCR Master Mix (Stratagene, Agilent Technologies), according to the manufacturer's protocol. The following primers were used in real-time PCR amplification: A_{2A} receptor, 5′-AGCCAGGGGTTACATCTGTG-3′ (forward) and 5′-TACAGACAGCCTCGACATGTG-3′ (reverse); cathepsin K, 5′-GCTGAACTCAGGACCTCTGG-3′ (forward) and 5′-GAAAAGGGAGGCATGAATGA-3′ (reverse); NFATc1, 5′-TCATCCTGTCCAACACCAAA-3′ (forward) and 5′-TCACCCTGGTGTTCTTCCTC-3′ (reverse); osteopontin, 5′-TCTGATGAGACCGTCACTGC-3′ (forward) and 5′-TCTCCTGGCTCTCTTTGGAA-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5′-CTACACTGAGGACCAGGTTGTCT-3′ (forward) and 5′-GGTCTGGGATGGAAATTGTG-3′ (reverse). The Pfaffl method[34] was used for relative quantification of A_{2A} receptor, cathepsin K, and NFATc1.

Morphological Characterization of Cultured Osteoclasts

Osteoclasts were generated from bone marrow cells extracted from the femurs and tibiae, as previously described. To differentiate osteoclasts, bone marrow cells were replated at 7500 cells/mL in fibronectin-coated glass coverslips in α-MEM containing 30 ng/mL M-CSF and 30 ng/mL RANKL, in the presence or absence of the adenosine A_{2A} agonist, CGS21680. After 7 days in culture, cells were fixed with 4% PFA in PBS, blocked with PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 30 minutes, stained fluorescently with Al-

exa Fluor 555–phalloidin for 30 minutes, and counterstained with DAPI (Fluoroshield with DAPI mounting media), as previously described.[35] To evaluate osteoclast morphological characteristics, the averages of 400 osteoclasts were examined in each sample using confocal microscopy (Leica SP5 confocal system, Buffalo Grove, IL).

Cytokine ELISAs

M-CSF–RANKL–derived osteoclasts were plated and differentiated over 7 days, as previously described. Cell supernatant was collected every day, centrifuged to remove any debris, and analyzed by Quantikine ELISA kits (R&D Systems) to determine the secretion of IL-1β and TNF-α. The manufacturer's protocol was strictly followed, and samples were run in duplicate with media-only controls and a standard curve correlation coefficient $\geq$0.98.

Bone Histological Features

The femurs from seven C57BL/6 (WT) and seven A_{2A}KO mice were excised, cleaned of soft tissue, placed into 10% formaldehyde for 24 to 48 hours, and decalcified in EDTA. Paraffin-embedded histological sections were stained, using H&E and immunohistologic techniques, for type I procollagen or for TRAP activity. We measured the bone volume (BV) in a standard zone, situated at least 0.5 mm from the growth plate, excluding the primary spongiosa and trabeculae connected to the cortical bone, and enumerated the osteoclasts and trabecular area in the same zone as that used for assessing BV (original magnification, $\times$10), using BioQuant software (Nashville, TN).[36] For measuring the osteoid, we used either Von Kossa staining (using calcified sections in methacrylate) or Goldner trichrome staining (also on calcified sections).

Electron Microscopic Examination of Osteoclasts

The femurs from five mice were fixed in 2.5% PFA plus 0.5% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer (pH 7.4) for 12 hours at room temperature. After rinsing three times for 20 minutes in the same buffer, the material was postfixed for 1 hour in 1% osmium tetroxide (in 0.1 mol/L sodium cacodylate buffer), dehydrated in a graded ethyl alcohol series, and embedded in Epon (EMbed 812; Electron Microscopy Sciences, Hatfield, PA). Thin sections (80- to 90-nm thick) of calcified bone were collected in distilled water containing one drop of bromothymol blue (pH $\geq$8.0), to prevent mineral dissolution from the thin sections. Sections were stained with lead citrate and alcoholic uranyl acetate and examined using a Philips CM-12 electron microscope (Phillips, Mahwah, NJ).

Micro–X-Ray CT Analysis of Bone Mass

For measurements of the bone volume/trabecular volume (BV/TV), the femurs of seven WT and seven A_{2A}KO mice were measured by micro–X-ray computed tomography (CT), as previously described,[25,37] using an MS-8 scanner (GE Healthcare, London, UK) at 18-μm isotropic resolution. The scans were calibrated by air, water, and a mineral standard material phantom and the Parker's algorithm for digital reconstruction.[25,38] Parameters were calculated using software supplied with the instrument.

Measurement of BMD

We assessed the bone mineral density (BMD; g/cm^2) of the whole skeletons of 4-month-old mice, using a PIXImus bone densitometer (Lunar, Madison, WI). The instrument was calibrated before each scanning session, using a phantom with known BMD, according to the manufacturer's guidelines. There were seven WT and seven A_{2A}KO mice anesthetized by i.p. injection of ketamine (100 μg/g of body weight) and xylazine (10 μg/g of body weight) and then placed in the prone position on the specimen tray to allow scanning of the entire skeleton.

Statistical Analysis

Statistical significance for differences between groups was determined by using analysis of variance or the Student's *t*-test. All statistics were calculated using GraphPad software (GraphPad, San Diego, CA).

Results

Effect of A_{2A} Receptor Activation in Osteoclast Differentiation and Function in Vitro

After 7 days of osteoclast differentiation, TRAP and Von Kossa staining were performed to counteract the role of A_{2A} receptor activation in the differentiation process and in bone remodeling function *in vitro*. When we analyzed the osteoclast number in WT cell cultures, we observed a dose-dependent inhibition of osteoclastogenesis when cultures were treated with CGS21680 alone (Figure 1A; half maximal inhibitory concentration, 50 nmol/L; maximal decrease of 38% $\pm$ 8% inhibition; $P < 0.05$; $n = 6$). Interestingly, the A_{2A} receptor agonist–mediated inhibition was more than completely reversed by pretreatment with the selective A_{2A} antagonist ZM241385 in the presence of 10 μmol/L CGS21680 (Figure 1A). Moreover, there was dose-dependent activation of osteoclastogenesis by the A_{2A} receptor antagonist (146% $\pm$ 8% of control in the presence of ZM241385, 10 μmol/L; $P < 0.05$; $n = 6$), suggesting that endogenous adenosine inhibits osteoclast formation in an autocrine fashion. When cells from A_{2A}KO mice were studied, neither the agonist nor the antagonist, at any concentration, affected the number of osteoclasts that formed (Figure 1B).

The effect of adenosine A_{2A} receptor stimulation/antagonism on bone resorption paralleled the effects on osteoclast formation. CGS21680 treatment diminished osteoclast resorption in a dose-dependent fashion (half maximal inhibitory concentration, 0.5 μmol/L; maximal resorption of 31% $\pm$ 7%, where control resorption was

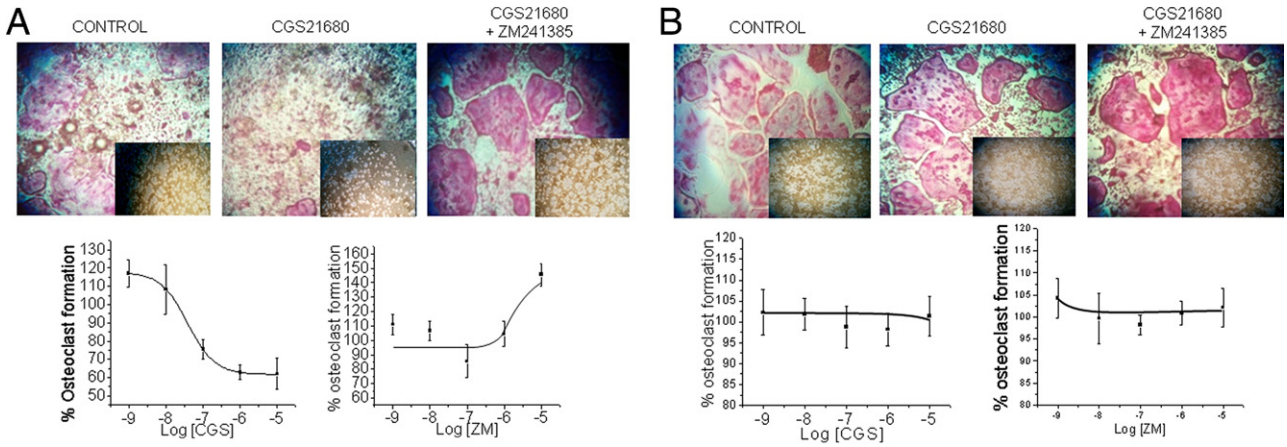

Figure 1. Effect of A_{2A} receptor on osteoclast formation of mouse bone marrow cells and bone resorption. **A:** WT mouse osteoclast primary culture cells were fixed and stained for TRAP and Von Kossa after being cultured for 7 days, in 48- or 16-well culture plates, in the presence of CGS21680 (10 μmol/L to 1 nmol/L) alone or CGS21680 + ZM241385 (10 μmol/L to 1 nmol/L for the ZM241385 and 10 μmol/L for CGS21680). TRAP-positive cells containing three or more nuclei were counted as osteoclasts (red staining). Resorbed areas (**inset** on each TRAP staining image) appear in clear, with a contrasting brown background. The results were expressed as the means of four cultures. **B:** TRAP (red staining cells) and Von Kossa (**inset** on each TRAP staining image) staining analysis in A_{2A} receptor KO mouse osteoclast primary culture cells and percentage of control in the presence of CGS21680 (10 μmol/L to 1 nmol/L) alone or pretreated with ZM241385 (10 μmol/L to 1 nmol/L for the ZM241385 and 10 μmol/L for CGS21680). The results were expressed as the means of four cultures. ZM241385 was added to cultures 30 minutes before CGS21680.

53% ± 3% of total osteoid; Figure 1A). The opposite effect was observed when cultures were pretreated with ZM241385 in the presence of CGS21680, 10 μmol/L, and resorption increased in a dose-dependent manner (Figure 1A). As with osteoclast formation, neither CGS21680 nor ZM241385 treatment affected osteoid resorption by cells from A_{2A}KO mice (Figure 1B), confirming the results obtained with TRAP staining.

To determine the stage of osteoclast formation and function affected by A_{2A} receptor stimulation, we began treatment of cultures with CGS21680 at various time points after the start of the cultures. We found that CGS21680 inhibited osteoclast differentiation (Figure 2A) and function (Figure 2B) when added to the culture at the beginning of differentiation (days 0 to 4) but had little or no effect when added after that time (Figure 2, A and B).

Expression of Osteoclast Differentiation Markers in the Presence of CGS21680

To confirm A_{2A} receptor–mediated inhibition of osteoclast differentiation, M-CSF–RANKL–derived precursors were collected during the 7 days of osteoclast differentiation and RNA was extracted. We first analyzed the expression of the A_{2A} receptor during M-CSF–RANKL–stimulated osteoclast formation. As shown in Figure 3A, the expression of A_{2A} receptor changed over time, increasing in control cells (up to 2.2-fold on days 2 and 4 of differentiation, $P < 0.005$), an effect that was abrogated in A_{2A} receptor–activated cells. Pretreatment with ZM241385 also reversed the effect of CGS21680 treatment on A_{2A} receptor message expression. When we analyzed the change in mRNA expression for cathepsin K, we observed that, both in control and ZM241385-pretreated osteoclasts, cathepsin K was up-regulated during osteoclast differentiation (up to 80-fold on day 7 for control cells and 50-fold on day 7 of differentiation for ZM241385, $P < 0.005$), but CGS21680 reduced the increase (Figure 3B). NFATc1 mRNA expression was up-regulated during osteoclast differentiation (up to sixfold on day 6 of differentiation, $P < 0.005$), and neither A_{2A} receptor activation nor blockade affected the M-CSF–RANKL–induced increase in expression (Figure 3C). Finally, mRNA expression for osteopontin was also up-regulated in the M-CSF–RANKL–stimulated osteoclast alone or pretreated with ZM241385, but CGS21680 inhibited the stimulated increase (Figure 3D). The observation that M-CSF–RANKL–induced up-regulation of NFATc1 was unaffected by the A_{2A} agonist or its antagonist supports the hypothesis that A_{2A} receptor activation selectively regulates cellular function and does not act as a general transcriptional inhibitor or cellular toxin.

Morphological Characterization of Osteoclast Cultures

As we have previously reported,[26] osteoclasts cultured on glass exhibit three distinct morphological features (Figure 4A). The least mature osteoclasts represent an early stage in osteoclast differentiation; are generally small, with fewer than five centrally located nuclei surrounded by a ring of F-actin; and are usually absent of podosomes. Maturing osteoclasts are variable in size, dendritic shaped, and contain more than five nuclei distributed throughout the cytoplasm, with podosomes located in patches at the edge of each pseudopod, and represent fusion intermediates because they are often connected with other maturing cells through cytoplasmic bridges. Finally, mature osteoclasts are large, with numerous nuclei located at the periphery near the peripheral podosome belt. We observed that the adenosine A_{2A} agonist, CGS21680, increased (Figure 4, B and C) the percentage of least-differentiated osteoclasts (from 10.5% ± 1.0% to 72.3% ± 1.10% for CGS21680, $P <$

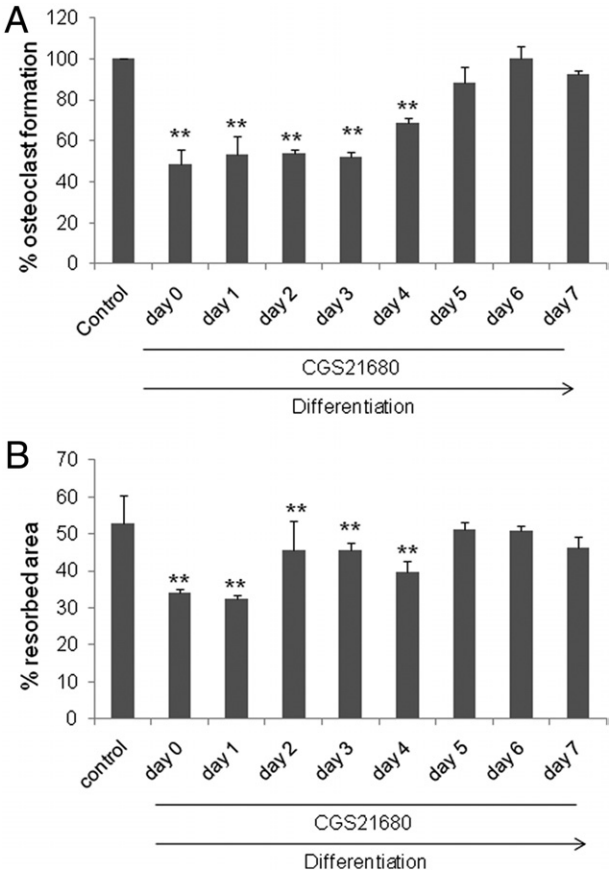

Figure 2. Day response effect of CGS21680 on A_{2A} receptor effect on osteoclast differentiation and function. **A:** WT mouse osteoclast primary culture cells stained with TRAP to counteract osteoclast differentiation being cultured for 7 days in the presence of CGS21680, 10 μmol/L, on different days. **B:** WT mouse osteoclast primary culture cells to study osteoclast function by Von Kossa staining being cultured for 7 days in the presence of CGS21680, 10 μmol/L, on different days. $^{**}P < 0.01$.

0.005). The A_{2A} receptor–mediated increase in least-differentiated osteoclasts was reversed by the A_{2A} antagonist, ZM241385 (to 17.3% ± 2.3%, $P < 0.005$). There were similar percentages of maturing cells (24.6% ± 1.2% for control, 23.3% ± 1.8% for CGS21680, and 22.6% ± 3.4% for CGS21680 + ZM241385) and an A_{2A} receptor–mediated decrease in mature osteoclasts (64.9% ± 2.3% for control, 4.5% ± 2.0% for CGS21680, and 60.2% ± 1.7% for CGS21680 + ZM241385; $P < 0.005$). These data further confirm that the activation of A_{2A} receptors plays an important regulatory role in osteoclast fusion and differentiation.

Cytokine Secretion during Osteoclast Differentiation

CGS21680 treatment markedly decreased concentrations of IL-1β in culture supernatants during osteoclast differentiation (Figure 5A, $P < 0.005$), whereas pretreatment with ZM241385 produced a marked increase in IL-1β secretion starting on day 2 of differentiation and throughout differentiation ($P < 0.005$). In control and ZM241385 pretreated cells, there was an increase in TNF-α secretion over time, and treatment with CGS21680 diminished levels of this cytokine during differentiation (Figure 5B, $P < 0.005$). When we analyzed the levels of IL-1β and TNF-α in A_{2A}KO cell culture supernatants, we observed an increase in cytokine levels compared with WT cell cultures. There was a detectable increase in IL-1β levels by day 1 of differentiation that remained stable during the 7 days of osteoclast differentiation and was unaffected by any of the treatments (Figure 5C, $P < 0.005$), whereas the secretion of TNF-α was not significantly changed in culture supernatants during osteoclast differentiation (Figure 5D). To determine whether the changes in TNF-α and IL-1β played a role in A_{2A} receptor–mediated regulation of osteoclast formation, we determined the effect of A_{2A} receptor stimulation on osteoclast formation in the presence of these two cytokines. Interestingly, both IL-1β and TNF-α completely abrogated the capacity of CGS21680 to inhibit osteoclast formation *in vitro* (2.5% ± 2.9% inhibition in the presence of IL-1β and 5.1% ± 4.4% inhibition in the presence of TNF-α; $P = 0.15$; Figure 5, E and F). These observations suggest that adenosine A_{2A} receptor–mediated inhibition of IL-1β and TNF-α might play a role in the adenosine A_{2A} receptor-mediated inhibition of osteoclast formation.

Micro–X-Ray CT, Dual X-Ray Absorptiometry, and Histomorphometric Analysis of Bone in A_{2A}KO Mice

To correlate the *in vitro* A_{2A} receptor–mediated effects on osteoclast differentiation and function with the *in vivo* effects, we studied the skeletons of A_{2A}KO mice compared with those of WT mice. As previously described, A_{2A}KO mice were similar in external appearance, body weight, and organ weight to WT controls (data not shown). Micro–X-ray CT analysis of femurs from WT and A_{2A}KO mice showed a significantly decreased BV/total volume ratio and trabecular number in A_{2A}KO mice ($P < 0.05$ and $P < 0.001$, respectively) and an increased trabecular space ($P < 0.001$) (Figure 6A and Table 1). Cortical bone, total area, and the outer perimeter in A_{2A} receptor KO mice were similar to the WT mice (Table 1).

To better characterize the bone phenotype of A_{2A}KO mice, we examined the long bones of the mice histomorphometrically. Whole body dual X-ray absorptiometry scanning confirmed the decreased bone mineral content in the A_{2A}KO mice (Figure 6A). We also observed a significant decrease in both BMD and bone mineral content (Table 1, $P < 0.01$). Interestingly, TRAP staining (Figure 6B) showed an increased number of TRAP-positive osteoclasts in the femoral metaphyses of A_{2A}KO mice when compared with WT mice (six fields each from femurs of two different mice each) and a decrease in histomorphometrically determined BV/trabecular BV (Figure 6C, Table 1, $P < 0.01$), reflecting the bone loss.

Transmission electron microscopy of bone showed an apparent increase in osteoclast membrane folding and bone resorption in the femurs from A_{2A}KO mice, compared with WT mice, consistent with our demonstration of

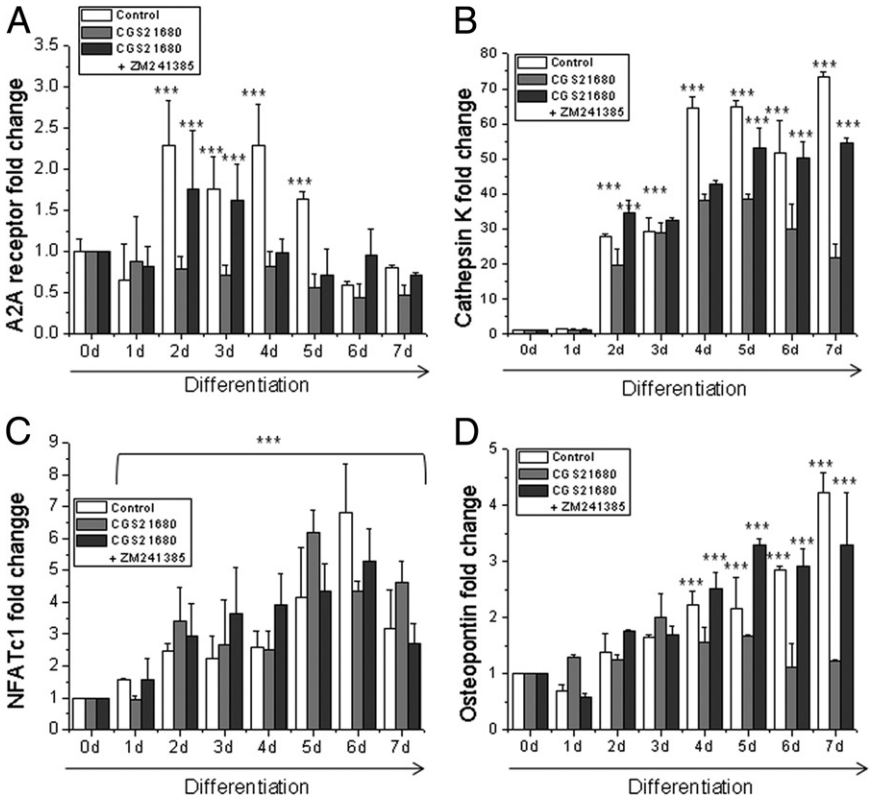

Figure 3. Expression of A_{2A} receptor and osteoclast differentiation markers mRNA. **A:** Fold change in A_{2A} receptor mRNA in M-CSF–RANKL OCPs during the 7 days of osteoclast differentiation in the presence of CGS21680 alone or with ZM241385. **B:** Fold change from control in cathepsin K mRNA in M-CSF–RANKL–treated OCPs during the 7 days of osteoclast differentiation in the presence of CGS21680 alone or with ZM241385. **C:** Fold change in NFATc1 mRNA in M-CSF–RANKL–treated OCPs during the 7 days of osteoclast differentiation in the presence of CGS21680 alone or with ZM241385. **D:** Fold change in osteopontin mRNA in M-CSF–RANKL–treated OCPs during the 7 days of osteoclast differentiation in the presence of the A_{2A} agonist alone or with ZM241385. $^{***}P < 0.005$.

enhanced bone resorption in the ZM241385-treated osteoclasts (data not shown).

Discussion

Previous results[25,26] from our laboratory demonstrated that osteoclasts express all four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3). Because all four adenosine receptor subtypes are expressed on osteoclasts, we have examined the role of distinct adenosine receptors to determine their roles in regulating bone physiological and pathological characteristics. Our laboratory previously reported that deletion or blockade of adenosine A_1 receptors diminished osteoclast formation and function and osteoclast-mediated bone loss *in vitro*. Adenosine A_1 deletion or blockade led to osteopetrosis in KO mice and inhibition of post–ovariectomy-induced bone loss, a model for post-menopausal osteoporosis.[25,26] These findings underlined the potential therapeutic importance of adenosine receptors in regulating bone physiological and pathological characteristics. Herein, we demonstrate that adenosine A_{2A} receptors also regulate osteoclast formation and function *in vitro* and that deletion of these receptors leads to enhanced osteoclast formation and function both *in vitro* and *in vivo*, with a resulting decline in BMD.

Our *in vitro* studies reveal that adenosine A_{2A} receptor stimulation diminished the number of differentiated TRAP-positive cells, bone resorption by these cells, and expression of osteoclast differentiation markers, such as cathepsin K and osteopontin. This effect is more notable when the agonist was added at early stages of culture. This observation correlates with the down-regulation in A_{2A} receptor mRNA that occurred during differentiation. During differentiation, A_{2A} receptor levels undergo no increase after CGS21680 treatment, whereas in RANKL alone or ZM241385-stimulated cells, the A_{2A} receptor is overexpressed, a phenomenon consistent with the known effects of TNF-α stimulation and stimulation of NF-κB activation on adenosine A_{2A} receptor expression.[39–42] This, together with *in vitro* differentiation and function assays performed in A_{2A}KO cells, suggests that the A_{2A} receptor has to be either activated or blocked from the beginning of cell differentiation to exert its effect on osteoclast formation.

Although the KO animals studied were universal knockouts and not specific for osteoclasts or OCPs, it is likely that the primary effect on bone metabolism observed herein is primarily the result of specific loss of adenosine receptors on osteoclasts and their precursors. The studies of *in vitro* differentiation of osteoclasts studied herein start with a relatively pure population of myeloid cells containing precursors that will differentiate into osteoclasts in the presence of M-CSF and RANKL. As shown herein, an adenosine A_{2A}-selective receptor agonist suppresses osteoclast formation in cells from WT mice (an effect completely reversed at pharmacologically relevant concentrations of selective antagonists) but not in the OCPs derived from A_{2A} receptor KO mice. The increase in osteoclasts observed *in vivo* in the KO mice was clearly consistent with this *in vitro* effect, although it is possible that indirect effects of adenosine receptor dele-

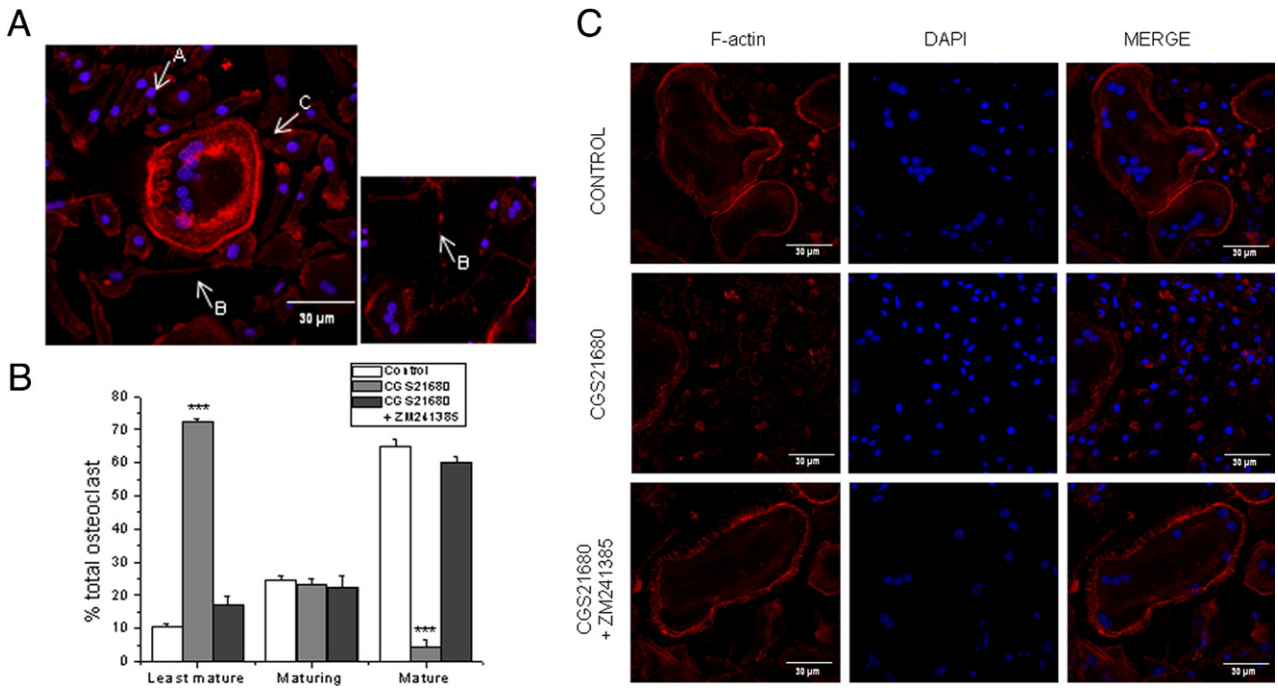

Figure 4. Morphological characterization of osteoclast cultures. **A:** Morphological characteristics of the least mature (A), maturing (B), and mature (C) osteoclasts cultured on glass. **B:** Quantitative evaluation of the number of least mature, maturing, and mature osteoclasts in osteoclast cultures treated with CGS2160 alone or in the presence of ZM241385 compared with control cultures. **C:** F-actin was detected by Alexa 555–phalloidin staining in osteoclast cultures treated with CGS2160 alone or in the presence of ZM241385 compared with control cultures. Original magnification for all parts, ×63. $^{***}P < 0.005$.

tion on other cells that regulate osteoclast formation could have been responsible for the increase in osteoclasts observed. Even if adenosine receptors on other cell types (eg, osteoblasts or inflammatory cells) play a role in the A_{2A} receptor–mediated regulation of osteoclast formation *in vivo*, the direct effects of adenosine A_{2A} receptor engagement on osteoclast differentiation remain critical to the observed changes in osteoclast number and bone resorption. The use of selective cell-specific knockouts could have more definitely demonstrated that the observed changes were only the result of the effects on OCPs.

The order of affinity of adenosine receptors for adenosine is $A_1 > A_{2A} \gg A_{2B} = A_3$; thus, as previously observed by Yang et al,[43] endogenous adenosine levels are more potent stimuli for A_1 and A_{2A} receptors than A_{2B} or A_3 receptors. The potency of adenosine as an agonist for adenosine receptors also depends on the density of the receptors.[44] Under physiological conditions, adenosine levels in most tissues are low but are sufficient to partially activate A_1, A_{2A}, and A_3 receptors, which are abundantly expressed and highly sensitive. Based on the capacity of the A_{2A} antagonist to increase osteoclast formation and function, it is likely that A_{2A} receptors are activated by endogenous levels of adenosine (as are A_1 receptors). Because A_1 and A_{2A} receptors have mutually antagonistic actions, it is likely that blockade of A_{2A} receptors permits full activation and signaling of A_1 receptors by endogenous adenosine. Similarly, blockade of A_1 receptors may uncover endogenous activation of A_{2A} receptors.

Pellegatti and colleagues[45] recently reported that the purinergic axis plays a crucial role in osteoclast formation and confirms previous evidence advocating a key role for either ATP or adenosine receptors in multinucleated giant cell formation. Although a message for adenosine receptors was present in peripheral blood OCPs, an adenosine A_1 receptor message was present at low levels. Surprisingly, Pellegatti and colleagues found that ATP, which activates P_2X_7 receptors, blocks osteoclast fusion and that catabolism of adenine nucleotides promotes osteoclast formation from precursors in peripheral blood, an effect they ascribed to A_{2A} receptor activation by its selective agonist, CGS21680. However, in their work, blockade or stimulation of adenosine receptors regulates cellular fusion only when the P_2X_7 receptor is blocked or adenine nucleotides are catabolized. This work stands in contrast to prior work by Merrill and coworkers,[24] in which adenosine A_1 receptor stimulation promotes multinucleated giant cell formation and A_{2A} receptor stimulation inhibits fusion of peripheral blood monocytes. The difference between their findings in human peripheral blood OCPs and the findings reported herein may be due to a species-dependent difference in response to A_{2A} receptor stimulation. Another explanation may be that OCPs in peripheral blood differ from precursors in the marrow with respect to P_2X_7 or adenosine receptor expression or function.

During osteoclast maturation, proinflammatory cytokines cause an imbalance in bone metabolism favoring bone resorption.[46] Indeed, even in the absence of RANKL-TNF–related activation-induced cytokine, TRAF6,

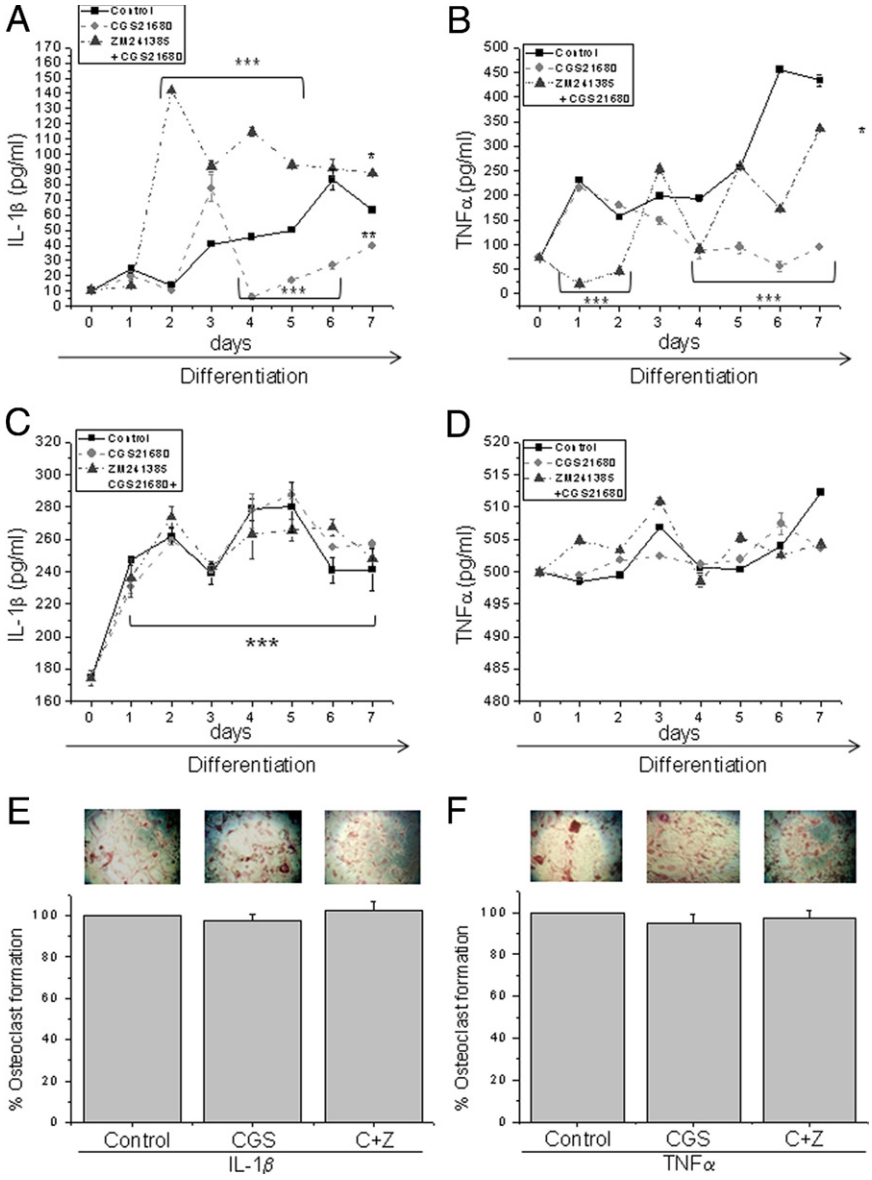

Figure 5. Cytokine secretion changes during osteoclast differentiation. **A:** IL-1β–secreted values during the 7 days of osteoclast differentiation in WT M-CSF–RANKL cultures in the presence of CGS21680 alone or with ZM241385, compared with control. **B:** TNF-α–secreted values during the 7 days of osteoclast differentiation in WT M-CSF–RANKL cultures in the presence of CGS21680 alone or with ZM241385, compared with control. **C:** IL-1β–secreted values during the 7 days of osteoclast differentiation in A_{2A}KO M-CSF–RANKL cultures in the presence of CGS21680 alone or with ZM241385, compared with control. **D:** TNF-α–secreted values during the 7 days of osteoclast differentiation in A_{2A}KO M-CSF–RANKL cultures in the presence of CGS21680 alone or with ZM241385, compared with control. **E:** M-CSF–RANKL–derived osteoclast treated with CGS21680 alone or with ZM241385 in the presence of IL-1β were fixed and stained for TRAP. **F:** M-CSF–RANKL–derived osteoclasts treated with CGS21680 alone or with ZM241385 in the presence of TNF-α were fixed and stained for TRAP. $^{***}P < 0.005$.

or RANK, TNF can stimulate osteoclast formation.[47] The well-documented inhibitory effects of adenosine A_{2A} receptor stimulation on TNF-α and IL-1β secretion probably contribute, in our work, to inhibition of osteoclast formation and bone resorption in inflammatory diseases and help explain the therapeutic effects of methotrexate, the anti-inflammatory effects of which are mediated by adenosine acting at its receptors[48] in the treatment of rheumatoid arthritis. Thus, it is likely that, under inflammatory conditions, the net effect of adenosine A_{2A} receptor stimulation will be even further inhibition of osteoclast formation.

Adenosine is a potent biological mediator that affects numerous cell types, including neuronal cells, platelets, neutrophils, and smooth muscle cells, among others. Adenosine A_{2A} receptors are coupled to G_S and signal, primarily by activation of adenylate cyclase, accumulation of cAMP, and downstream activation of either protein kinase A or exchange protein activated by cAMP. Signaling downstream from protein kinase A proceeds, in part, through phosphorylation of the cAMP response element binding transcription factor, resulting in activation leading to either interaction of cAMP response element binding with specific promoters and gene expression or competition with NF-κB or other transcription factors. Prior reports[49] had suggested that increases in cAMP inhibited osteoclast formation, but inhibition of protein kinase A had no effect on osteoclastogenesis. Similarly, Lerner et al[50] detected the presence of functional A_2 and P-site receptors, but not A_1 receptors, in mouse calvaria and osteoblast-like cells; both receptors regulate cAMP, but the authors assumed they were not intimately linked to bone. Recently, Zhang et al[51] linked the stimulation of adenylyl cyclase by A_{2A} and A_{2B} activation, which led to increased cAMP levels that, in turn, activated the canonical protein kinase A pathway and the exchange protein directly activated by cAMP[52]; in contrast, A_1 and A_3 activation diminishes cAMP.[53] In addition, adenosine receptor signaling in mast cells has also been linked to

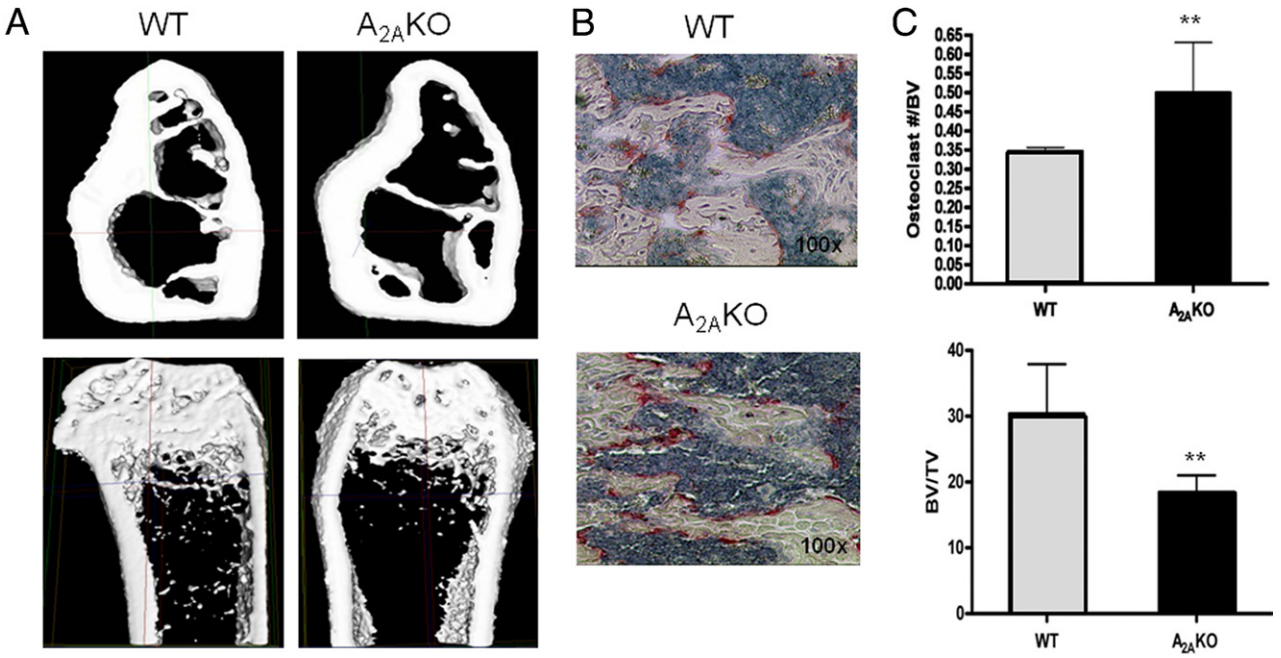

Figure 6. Decreased bone mass in adenosine A_{2A}KO mice. Histological and histomorphometric analysis of the femurs of A_{2A}KO and WT mice. **A:** Representative high-resolution microfocal CT images showing significantly greater trabecular and cortical bone density in 4-month-old A_{2A}KO mice compared with WT mice. Three-dimensional panel reconstruction of the femurs revealed increased bone mass in A_1KO mice compared with their WT littermates. **B:** Representative histological sections obtained from the femurs of WT and A_{2A}KO mice, stained for TRAP activity as a marker of osteoclasts and counterstained with hematoxylin. **C:** Osteoclast number/BV and BV/trabecular BV (TV) in WT and A_{2A}KO mice. Data are from representative sections from transmission electron microscopy of femurs from WT and A_{2A}KO mice after fixation in 2.5% PFA plus 0.5% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer, postfixed in 1% osmium tetroxide and embedded in Epon, divided into sections, and stained with lead citrate and alcoholic uranyl acetate. $^{**}P < 0.01$.

phospholipase C and calcium mobilization (A_{2B} and A_3), phosphatidylinositol 3-kinase (A_3), and protein kinase C and mitogen-activated protein kinases (A_1, A_{2A}, and A_3 receptors).[21] Future experiments will be directed at the dissection of adenosine receptor signaling in OCPs and the role of cAMP or other intermediates in the regulation of osteoclast formation.

In rheumatoid arthritis and other inflammatory diseases that affect the bone, there is inflammation of surrounding tissues with activation of osteoclasts and resorption of bone. Adenosine A_{2A} receptors have diminished inflammation for a long time by directly inhibiting the inflammatory function of macrophages, dendritic cells, and neutrophils and by stimulating anti-inflammatory functions of macrophages and T cells.[54] Moreover, methotrexate, which diminishes inflammation by increasing extracellular adenosine concentrations,[55] diminishes bone erosions in rheumatoid arthritis, although not as well as anti-TNF agents.

Because adenosine mediates the anti-inflammatory effects of methotrexate,[56] we further speculate that the capacity of methotrexate to inhibit bone erosion in patients with rheumatoid arthritis may be mediated by methotrexate-stimulated increases in adenosine concentration. In KO mouse models, the A_{2A} receptor, together with the A_3 receptor, mediated the anti-inflammatory effect of methotrexate, which is used as a treatment of arthritis.[57,58] We first determined whether methotrexate induced an increase of extracellular adenosine via intracellular adenosine production or whether it was generated extracellularly from adenine nucleotides. The pathway leading to increased extracellular adenosine by methotrexate has been mostly delineated in studies[56–58] that confirmed that low-dose methotrexate therapy increases tissue aminoimidazole carboxamide ribonucleotide (an intermediate in the generation of inosine monophosphate) levels in animal models of rheumatoid arthritis and urinary aminoimidazole carboxamide ribonucleotide levels in patients with psoriasis and rheumatoid arthritis.

Table 1. Histomorphometric Examination of Long Bones in 4-Month-Old WT and A_{2A}KO Mice

Variable	WT mice	A_{2A}KO mice
BV/tissue volume ratio	22.61 ± 2.154	17.49 ± 0.98*
Trabecular no. (mm^{-1})	5.8 ± 0.07	4.4 ± 0.15**
Trabecular separation (μm)	0.13 ± 0.004	0.19 ± 0.006**
Cortical area (mm^2)	0.68 ± 0.04	0.6 ± 0.016*
Total area (mm^2)	1.5 ± 0.03	1.3 ± 0.02*
BMD (HA/cm^3)	0.053 ± 0.0025	0.049 ± 0.0016***
BMC (g)	0.48 ± 0.051	0.43 ± 0.028***
Outer perimeter (mm)	4.6 ± 0.04	4.4 ± 0.04***
TMC (g)	1.9 ± 0.12	1.7 ± 0.05*
TRAP-positive osteoclasts (no./lpf)	35 ± 1	50 ± 13***

Data are expressed as the mean ± SEM of three independent animals and related to control.

$^*P < 0.5$, $^{**}P < 0.005$, and $^{***}P < 0.01$.

BMC, bone mineral content; HA, hydroxyapatite; lpf, low-power field; TMC, trabecular mineral content.

Finally, downstream signaling from RANK activation includes activation of the NF-κB complex, NFATc1 transcription factor, C-Jun kinase, p38, and extracellular signal regulated kinase mitogen-activated protein kinases and the phosphatidylinositol 3-kinase–Akt axis.[59,60] Each of these pathways plays a key role in osteoclast differentiation and/or function. Thus, mice lacking the p50 and p52 subunits of NF-κB or c-Fos, a member of the AP-1 family of transcription factors and a downstream target of stress-activated protein kinase/C-Jun kinase, generate no osteoclasts.[61] Ectopic expression of NFATc1 in bone marrow macrophages induces the formation of multinuclear osteoclasts in the absence of known fusion-inducing factors, such as RANKL.[62] In addition, adenosine A_{2A} receptor activation increases mitogen-activated protein kinase phosphorylation and activation and activation of AKT, Cdc42, and other pathways.[63–65] The interactions of adenosine A_{2A} receptors with these signaling pathways in the formation and function of osteoclasts and in the final mechanism of the differentiating phenotype need further investigation.

In conclusion, these results indicate that adenosine A_{2A} receptors inhibit M-CSF–RANKL–stimulated osteoclast differentiation and function and, thereby, regulate bone turnover.

Acknowledgments

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