Proteasome Inhibition Aggravates Tumor Necrosis Factor–Mediated Bone Resorption in a Mouse Model of Inflammatory Arthritis

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Objective. The proteasome inhibitor bortezomib has potent anti-myeloma and bone-protective activity. Recently, bortezomib was shown to directly inhibit osteoclastogenesis. The aim of this study was to analyze the influence and therapeutic effect of bortezomib in a mouse model of inflammatory arthritis.

Methods. Heterozygous human tumor necrosis factor α (hTNFα)–transgenic mice and their wild-type (WT) littermates were intravenously injected with 0.75 mg/kg of bortezomib or phosphate buffered saline twice weekly. The mice were assessed for clinical signs of arthritis. After 6 weeks of treatment, mice were analyzed for synovial inflammation, cartilage damage, bone erosions, and systemic bone changes. Osteoclast precursors from WT and hTNF-transgenic mice were isolated from bone marrow, treated with bortezomib, and analyzed for osteoclast differentiation, bone resorption, and expression of osteoclast-specific genes as well as apoptosis and ubiquitination.

Results. Bortezomib-treated hTNF-transgenic mice showed moderately increased inflammatory activity and dramatically enhanced bone erosions associated with a significant increase in the number of synovial osteoclasts. Interestingly, bortezomib did not alter systemic bone turnover in either hTNF-transgenic mice or WT mice. In vitro, treatment with therapeutically rele- vant concentrations of bortezomib resulted in increased differentiation of monocytes into osteoclasts and more resorption pits. Molecularly, bortezomib increased the expression of TNF receptor–associated factor 6, c-Fos, and nuclear factor of activated T cells c1 in osteoclast precursors.

Conclusion. In TNF-mediated bone destruction, bortezomib treatment increased synovial osteoclastogenesis and bone destruction. Hence, proteasome inhibition may have a direct bone-resorptive effect via stimulation of osteoclastogenesis during chronic arthri-

tis.

Rheumatoid arthritis (RA) is a chronic and progressively destructive inflammatory disease of the diarthrodial joints. Affecting ~0.5% of the population, RA causes substantial morbidity and mortality and is associated with a significant decrease in the quality of life. In terms of morphology, RA is based on inflammatory synovial tissue composed of leukocytes and resident mesenchymal cells that invades and destroys cartilage and bone (1,2). Although synovitis can be therapeuti
cally targeted at any given point of time and is considered reversible, structural damage is irreversible and is associated with loss of joint function and poor quality of life and thus is considered a major target of therapy.

Bone erosions along joints result from the forma-
tion of multinucleated osteoclasts, which resorb bone, as well as the absence of appropriate skeletal repair mechanisms (3,4). In RA, monocytes migrate into inflamed joints and differentiate to osteoclasts upon a concerted challenge by cytokines such as macrophage colony-stimulating factor (M-CSF) and RANKL, which are produced by synovial fibroblasts and T lymphocytes. In addition, proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) enhance osteoclast differentiation and activity and accelerate structural damage in RA. Despite the introduction of TNF-blocking agents, which are effective for retarding inflammatory bone erosion, many patients with RA treated with TNF blockers still experience progressive structural damage, suggesting that additional strategies are required to target osteoclasts (5,6).

The ubiquitin–proteasome system controls the turnover of most intracellular proteins, including many regulatory factors such as cyclins and inhibitors of NF-κB. The proteasome inhibitor bortezomib specifically blocks the chymotrypsin-like activity of the 26S proteasome and has been shown to effectively treat multiple myeloma and associated osteolysis (7). Bortezomib has been approved for the treatment of relapsed multiple myeloma and mantle cell lymphoma, has been used successfully in patients with treatment-refractory multiple myeloma, and mantle cell lymphoma, and has been shown to effectively treat multiple myeloma and mantle cell lymphoma, and thus far represents the only proteasome inhibitor in routine clinical use. Blockade of the proteasome primarily targets rapidly proliferating cells through perturbation of cell cycle regulation and cells that are dependent on NF-κB activity for survival. The cells that appear to be most sensitive to proteasome inhibition, however, are those that produce large amounts of secretory proteins, namely, myeloma cells as well as normal plasma cells. In the case of proteasome inhibition, defective or unfolded proteins accumulate within the endoplasmic reticulum, inducing the terminal unfolded protein response leading to apoptosis (8).

Although the treatment effect of bortezomib in multiple myeloma may be based purely on directly targeting malignant plasma cells and thus affecting the underlying disease, direct effects of bortezomib on bone-active cells have recently been reported (9,10). Hence, we hypothesized that bortezomib might exert direct effects on osteoclasts in vitro and in vivo and thus could also affect local as well as inflammatory bone loss in vivo. In this study, we investigated the effects of proteasome inhibition by bortezomib on inflammatory arthritis as well as the bone loss associated with inflammatory arthritis.

MATERIALS AND METHODS

Animals and treatment. Heterozygous human TNFα (hTNFα)–transgenic mice (strain tg197) and their wild-type (WT) littermates were intravenously injected with 0.75 mg/kg of bortezomib or phosphate buffered saline (PBS) twice weekly from week 6 to week 12. A total of 25 mice were treated in this study, and only littermates were used. Mice were clinically evaluated in a blinded manner every week from week 5 to week 12. At week 12, mice were killed by cervical dislocation. Blood was withdrawn by cardiac puncture, and histologic, cellular, and molecular analyses were carried out. All animal studies were approved by the local animal use committee.

Clinical arthritis assessment. Mice were assessed weekly for body weight and clinical signs of arthritis, as described previously (11). Paw swelling was evaluated using a semiquantitative score, where 0 = no swelling, 1 = mild swelling of the toes and ankle, 2 = moderate swelling of the toes and ankle, 3 = severe swelling of the toes and ankle, and 4 = formation of bulk. Grip strength was scored as follows: 0 = normal grip strength, −1 = mildly reduced grip strength, −2 = severely reduced strength, and −3 = no grip at all.

Assessment of joint histology. Hind paws and knee joints were fixed in 4% formalin and decalcified afterward in 14% EDTA (Sigma-Aldrich) until the bones were pliable. Paraffin sections of hind paws (2 μm) were stained with hematoxylin and eosin for the detection of synovial inflammation. For the determination of proteoglycan loss of articular cartilage, toluidine blue staining was performed. Joint sections were also stained for tartrate-resistant acid phosphatase (TRAP) to detect osteoclasts and to assess bone erosions. Synovial inflammation, bone erosions, numbers of osteoclasts, and cartilage destruction were quantified using a Nikon microscope with a digital image analysis system (OsteoMeasure; OsteoMetrics), as described previously (12).

Immunohistochemical analysis. Different cell populations were analyzed in the synovial membrane by immunohistochemistry for T cells (anti-CD3; Serotec), B cells (anti-CD45R; BD Bioscience PharMingen), and macrophages (anti-F4/80; Serotec). Briefly, hind paw sections were left untreated for detection of B cells or were pretreated with citrate buffer, pH 6, for B cells or proteinase K for T cells. Subsequently, sections were incubated with biotinylated species-specific secondary antibodies (Vector) and avidin–biotin–peroxidase complex, using 3,3′-diaminobenzine (Sigma-Aldrich) as a chromogen. Positively stained cells were counted using a Nikon microscope and the OsteoMeasure system (OsteoMetrics). All cells per high-power field (hpf) were counted, and the percentage of positively stained cells was determined. Ten representative hpf per paw were evaluated.

Bone histomorphometry. Paraffin sections were prepared from tibial bones and were stained for TRAP activity. Trabecular bone and the osteoclast number were quantified using the OsteoMeasure system. The following parameters were analyzed: fraction of bone volume of the total sample volume (bone volume/total volume [BV/TV]), trabecular thickness (TbTh), trabecular number (TbN), trabecular separation (TbSp), and osteoclast number/bone perimeter (OcN/BPm).
**Ex vivo osteoclastogenesis.** Bone marrow cells obtained from hTNF-transgenic mice were isolated, cultured in α-minimum essential medium (Gibco) containing 10% fetal calf serum, 30 ng/ml M-CSF, and 30 ng/ml RANKL (both from R&D Systems) and incubated with different concentrations (5 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, 0.001 μg/ml, or 0.0001 μg/ml) of bortezomib or were left untreated. Osteoclasts were then fixed and stained for TRAP activity using a commercially available leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer’s instructions. Mature osteoclasts were identified as large, purple-stained cells containing >2 nuclei.

Furthermore, we analyzed whether bortezomib has apoptotic effects on osteoclasts. We performed osteoclast assays with control (no bortezomib) and 2 different bortezomib concentrations, one stimulatory and the other inhibitory (1 μg/ml and 0.0001 μg/ml, respectively), and counted TRAP-positive cells at 4 different time points (2 hours, 4 hours, 6 hours, and 8 hours) after the addition of bortezomib. We stained cell nuclei with 4',6-diamidino-2-phenylindole to count apoptotic cell nuclei. Apoptotic cell bodies are defined as sharp-bordered, compact nuclei, which outshine intact nuclei under fluorescent light.

**In vitro bone resorption assay.** For detection of bone resorption by osteoclasts, a similar protocol was applied, but cells were cultured on a BioCoat Osteologic Bone Cell Culture System (BD Bioscience) for 3 days, as previously described, allowing visualization of resorption pits. The areas of the resorption pits were counted using the OsteoMeasure system, as described above.

**Serum enzyme-linked immunosorbent assay (ELISA).** Blood was obtained by heart puncture of untreated and bortezomib-treated hTNF-transgenic mice. Serum levels of RANKL, osteoprotegerin (OPG) (R&D Systems), and type I collagen cleavage products (Immunodiagnostic Systems) were analyzed by ELISA, according to the manufacturer’s instructions.

**RNA isolation from knee joints.** Knee joints were put into Precellys tubes (Peqlab) containing 2.8-mm metal balls and 1 ml of peqGOLD TriFast reagent (Peqlab) and were homogenized. RNA isolation was performed using a standard protocol with chloroform and alcohol precipitation. Purity was measured with a standard photometer (Eppendorf). One microgram of RNA was reverse transcribed using murine leukemia virus reverse transcriptase and random hexamer primers. Quantitative polymerase chain reaction (PCR) was performed according to the manufacturer’s protocol on an ABI Prism 7300 Sequence Detection System (Applied Biosystems).

**Quantitative RNA analysis of osteoclasts.** RNA was isolated from osteoclasts using TRIzol reagent (Invitrogen) and was reverse transcribed with murine leukemia virus reverse transcriptase, using the GeneAmp RNA PCR Kit (Applied Biosystems) with oligo(dT) primers. To measure the quantity of primer-specific RNA in osteoclasts, real-time PCR was performed using LightCycler technology (Applied Biosystems) and the FastStart SYBR Green 1 Kit for amplification and detection (10-minute denaturing step; 55 cycles of 5 seconds at 95°C, 15 seconds at 65°C, and 15 seconds at 72°C; melting point analysis in 0.1°C steps; final cooling step). Expression of the target molecule was normalized to the expression of β-actin.

The following primers were used: for c-Fos, forward 5’-CGGGTTTC-AACGCCGACTAC-3’, reverse 5’-CAGGT-CTGGGCTGTGGAGA-3’; for TRAP, forward 5’-ACAGCC-CCACC-TCCACCCT-3’, reverse 5’-TCAGGGCTCTGGGT-TCCTTGGG-3’; for Iκb, forward 5’-TGAAGGACGAGGATT-ACGAGC-3’, reverse 5’-TCCTGGGATGATTGCCAAGTG-3’; for β-actin, forward 5’-TGTGATGTTGGGAAT-GGGT-CAG-3’, reverse 5’-TGTGATGTTGGGAATGGGT-CAG-3’, reverse 5’-TGTGATGTTGGGAATGGGT-CAG-3’, reverse 5’-GAATCCTGAGA-CCTCCATGAAAACGC-3’, reverse 5’-CCATGAGCCTT-CCATCATA-GCTGG-3’.

**Western blotting.** The phenol fraction of TRIzol was kept to isolate the osteoclast proteins with or without bortezomib treatment. To assess the amount of ubiquitination, osteoclasts were stimulated with bortezomib and MG132, another proteasome inhibitor, at the given concentrations for 30 minutes. Protein was boiled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. After blocking the membrane with binding with 10% Tris buffered saline with 0.1% Tween 20 and 5% nonfat dry milk, anti–TNF receptor—associated factor 6 (anti–TRAF-6) (Santa Cruz Biotechnology), anti–c-Fos, anti-RANK, and anti–nuclear factor of activated T cells c1 (anti–NF-ATc1) (all from Santa Cruz Biotechnology). Anti-ubiquitin and anti-actin were used as primary antibodies, followed by the addition of horseradish peroxidase–conjugated secondary antibody and visualization with a chemiluminescence detection system (Pierce).

**Serum cytokine measurement.** Blood was drawn by heart puncture, and serum samples were collected after centrifugation at 15,000 g for 2 minutes at 4°C (BD Microtainer SST). Serum levels of TNFα, IL-4, IL-10, IL-17, interferon-γ (IFNγ), and granulocyte–macrophage CSF (GM-CSF) were measured using Luminex technology.

**Cytofluorometric analyses.** For immunophenotyping, cells were isolated from the spleens and lymph nodes, stained with antibodies against CD4, CD8, CD25, CD45R (B220), TNF, IL-10, IL-17, and granulocyte–macrophage CSF (GM-CSF) (BD Pharmingen), and analyzed by flow cytometry on a FACS Calibur (Becton Dickinson). Data analysis was performed using CellQuest software (Becton Dickinson).

**Statistical analysis.** Data are presented as the mean ± SEM. One-way factorial analysis of variance with the Bonferroni–Dunn test or the Mann-Whitney U test was used for group comparisons. P values less than 0.05 were considered significant.

**RESULTS**

**Effect of bortezomib on TNF-mediated arthritis.** To determine the effects of proteasome inhibition on TNF-mediated arthritis, we treated hTNF-transgenic mice with either vehicle (PBS) or bortezomib (0.75 mg/kg weekly). The dose of bortezomib for blockade of the proteasome in vivo was selected based on previous protocols (13). Weekly assessments of body weight throughout the observation period revealed no significant differences between vehicle and bortezomib treat-
ment (Figure 1A). Interestingly, compared to vehicle-treated mice, bortezomib-treated mice had a slightly more severe clinical course of disease, as evidenced by more pronounced paw swelling and loss of grip strength, although the difference did not reach statistical significance (Figures 1B and C).

The clinical findings were confirmed by quantitative histologic analysis. Bortezomib-treated hTNF-transgenic mice showed a modest increase in the area of inflammation compared with vehicle-treated mice (mean ± SEM 1.04 ± 0.14 versus 0.52 ± 0.1 mm²; \( P < 0.05 \)) (Figure 2A). This was accompanied by more severe cartilage proteoglycan loss (mean ± SEM 36.49 ± 3.28% and 21.78 ± 4.29%, respectively; \( P < 0.05 \)) (Figure 2B). Interestingly, the inflammatory infiltrate was not only quantitatively but also qualitatively altered in bortezomib-treated mice; immunohistochemical analysis revealed an altered composition of immune cells infiltrating the synovium (Figure 3). T cells were slightly more abundant in bortezomib-treated mice (mean ± SEM 18.35 ± 1.76% versus 13.39 ± 3.17% in untreated hTNF-transgenic mice; \( P \) not significant [NS]), whereas B cells (1.00 ± 0.58% versus 5.67 ± 0.88%; \( P < 0.05 \)) and macrophages (27.98 ± 5.53% versus 59.04 ± 9.13%; \( P < 0.01 \)) were less prevalent in bortezomib-treated mice than in untreated mice (Figure 3).

We also assessed the effects of bortezomib on immune cells in the spleen. Apart from a slight increase in the numbers of CD8+ T cells in the spleens of bortezomib-treated hTNF-transgenic mice, no major differences between treated and untreated mice in the number of mature B cells, macrophages, regulatory T cells, and CD4+ T cells could be detected (data not shown). The serum concentrations of TNFα, GM-CSF, IFNγ, IL-17, IL-4, and IL-10 were not altered by bortezomib treatment (Figure 2C).

**Effect of bortezomib on local bone destruction in hTNF-transgenic mice.** Next, we assessed the specific effects of bortezomib on TNF-mediated bone destruction. Although vehicle-treated hTNF-transgenic mice displayed signs of periarticular bone erosions (mean ± SEM area 0.13 ± 0.02 mm²) at 12 weeks of age, treatment with bortezomib strongly aggravated rather than reduced TNF-mediated bone destruction (0.42 ± 0.09 mm²; \( P < 0.05 \)) (Figure 4A). This finding was associated with a significant increase in synovial osteoclast numbers in treated mice compared with untreated mice (mean ± SEM 94 ± 19 versus 33 ± 5; \( P < 0.05 \)). Thus, bortezomib treatment worsened the TNF-mediated arthritic bone destruction associated with increased synovial osteoclastogenesis.

**Effect of bortezomib on RANKL expression.** We also analyzed the expression of RANKL and OPG, systemically and in the inflamed knee joints of untreated and bortezomib-treated hTNF-transgenic mice. Assessment of the serum levels of OPG revealed no significant difference between untreated and bortezomib-treated hTNF-transgenic mice (mean ± SEM 7.263 ± 630 and 6,950 ± 388 pg/ml, respectively). However, we observed a significant (\( P < 0.05 \)) increase in serum levels of RANKL in bortezomib-treated hTNF-transgenic mice (46.0 ± 8.8 pg/ml) compared to untreated hTNF-transgenic mice (24.4 ± 3.9 pg/ml) (Figure 4B). The expression of serum type I collagen cleavage products,
Figure 2. Histologic analysis of synovial inflammation and cartilage destruction. A, Hematoxylin and eosin–stained sections of hind paws from untreated and bortezomib-treated human tumor necrosis factor–transgenic (hTNFtg) mice, showing synovial inflammation (arrows). B, Proteoglycan loss as determined using toluidine blue–stained sections. Proteoglycan-depleted areas are unstained as compared to proteoglycan-rich dark blue–stained areas (arrows). C, Serum levels of cytokines after 6 weeks in untreated and bortezomib-treated hTNF-transgenic mice, as assessed by Luminex technology. Values are the mean ± SEM. Original magnification × 25 in A; × 100 in B. * = P < 0.05 versus untreated mice. GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; IL-17 = interleukin-17.
Figure 3. Quantitative evaluation of immune cells in inflamed synovium and in spleen. Immunohistochemical analysis of T cells (CD3+), macrophages (F4/80+), and B cells (CD45R+) in the inflamed synovium of untreated human tumor necrosis factor–transgenic (hTNFtg) mice and hTNF-transgenic mice treated with bortezomib was performed. Positively labeled cells appear brown. Representative images are shown. All cells per high-power field (hpf) were counted, and the percentage of positively stained cells was determined. Ten representative hpf per paw were evaluated. Values are the mean ± SEM. Original magnification ×100. * = P < 0.05 versus untreated mice.

Figure 4. Effects of bortezomib on local bone loss in tumor necrosis factor (TNF)–mediated arthritis. A, Arthritic bone erosions in untreated and bortezomib-treated human TNF–transgenic (hTNFtg) mice were quantified in tartrate-resistant acid phosphatase (TRAP)–stained sections. Osteoclasts appear as purple-stained multinucleated cells. Representative sections are shown. Original magnification ×25 in top row; ×100 in bottom row. B, Serum levels of RANKL and osteoprotegerin (OPG) in untreated and bortezomib-treated hTNF-transgenic mice. C, Relative (rel.) synovial expression of RANKL and OPG mRNA in the inflamed joints of untreated and bortezomib-treated hTNF-transgenic mice. Values are the mean ± SEM. * = P < 0.05 versus untreated mice.
however, was not changed following bortezomib treatment, indicating no enhanced resorption of systemic bone (mean ± SEM 25.98 ± 2.0 versus 22.8 ± 2.1 ng/ml in untreated hTNF-transgenic mice).

Next, we performed quantitative PCR analysis to detect synovial RANKL and OPG messenger RNA (mRNA) levels. As observed with serum analysis, synovial RANKL expression was increased in bortezomib-treated versus untreated hTNF-transgenic mice (mean ± SEM relative mRNA expression 3.03 ± 1.0 versus 0.91 ± 0.1; *P < 0.05) (Figure 4C). We did not detect a difference in synovial OPG expression between untreated and treated mice (mean ± SEM 0.36 ± 0.17 versus 0.19 ± 0.04; P NS) (Figure 4C). This net increase in synovial RANKL expression could contribute to increased synovial osteoclastogenesis and subsequent joint damage.

**No effect of bortezomib on systemic bone architecture.** To address whether proteasome inhibition affects systemic bone architecture, we performed a histomorphometric analysis of trabecular bone. Human TNF–transgenic mice developed severe osteopenia when compared to their WT littermates (mean ± SEM BV/TV 8.47 ± 2.21% versus 18.89 ± 3.23%; *P < 0.05) (additional information is available from the corresponding author). Bortezomib treatment, however, did not significantly change trabecular bone mass in either WT (mean ± SEM BV/TV 9.7 ± 1.7%; P NS versus untreated WT) or hTNF-transgenic mice (mean BV/TV 9.7 ± 1.7%; P NS versus untreated hTNF-transgenic). In addition to osteopenic bone mass, hTNF-transgenic mice showed severe alterations in bone microarchitecture. Trabeculae were thinner (mean reduction in thickness 27.5% versus WT; *P < 0.05), reduced in numbers (2.23 ± 0.44 versus 3.16 ± 0.43/mm in WT mice; *P < 0.05), and more distant from each other (450.5 ± 100.5 versus 261.1 ± 52.56 μm in WT mice; *P < 0.05), resulting in an overall reduced connectivity density of bone.

Figure 5. Effects of bortezomib on osteoclast differentiation and function. A, Osteoclasts were generated from murine hTNF-transgenic monocytes by stimulation with macrophage colony-stimulating factor (30 ng/ml) and RANKL (30 ng/ml) for 4 days. Monocytes were additionally treated with bortezomib at the indicated concentrations after 3 days of culture. Representative TRAP-stained osteoclasts (purple-stained multinucleated cells) are shown. B, Osteoclasts were cultured on a bone matrix, and resorption pits were analyzed after 5 days of culture. C, Apoptosis of osteoclasts was analyzed using different concentrations of bortezomib. Arrows indicate apoptotic nuclei. Values are the mean ± SEM. * = *P < 0.05 versus untreated mice. Original magnification × 200 in A and B; × 400 in C. See Figure 4 for other definitions.
hTNF-transgenic mice. When the number of osteoclasts in tibial bones was analyzed, hTNF-transgenic mice showed an accumulation of bone-resorbing osteoclasts as compared to WT mice (mean ± SEM 12.62 ± 0.66 versus 7.56 ± 1.77; *P* < 0.05). Proteasome inhibition did not alter osteoclast numbers in either WT mice (mean ± SEM OcN/BPm 10.06 ± 2.57) or hTNF-transgenic mice (mean ± SEM OcN/BPm 11.18 ± 1.05). Thus, bortezomib dramatically increased local bone erosions in the presence of synovial inflammation but did not alter systemic bone turnover.

**Bortezomib-stimulated osteoclast formation in vitro.** To further elucidate the effects of proteasome inhibition on osteoclasts, we performed in vitro osteoclast differentiation assays with bone marrow cells from hTNF-transgenic mice (Figure 5A). Interestingly, we observed a strong dose-dependent stimulatory effect of bortezomib on osteoclast formation at clinically relevant concentrations. Concentrations ranging from 0.0001 μg/ml to 0.01 μg/ml stimulated osteoclastogenesis. Bortezomib increased the fusion of osteoclast precursors, as assessed by counting the number of nuclei per osteoclast (data not shown), when added to osteoclast cultures on day 3. Higher concentrations of bortezomib had toxic effects on cells and induced cell death in vitro. To confirm our findings, we also investigated in vitro bone resorption. In accordance with the effects on osteoclast differentiation, bortezomib dose-dependently increased the resorptive capacity of osteoclasts (mean ± SEM area resorbed by osteoclasts following treatment with 0.0001 μg/ml bortezomib 37 ± 6% versus 25 ± 7% in control osteoclasts; *P* < 0.05) (Figure 5B). We also assessed osteoclast differentiation in WT monocytes following bortezomib stimulation and observed similar results (additional information is available from the corresponding author).

Furthermore, we analyzed whether bortezomib has apoptotic effects on osteoclasts. We observed that osteoclasts stimulated with 1 μg/ml of bortezomib had significantly more apoptotic cell bodies than osteoclasts that were treated with low concentrations of bortezomib or were left untreated. Thus, high bortezomib concentrations decreased osteoclast numbers by inducing cell death, whereas lower concentrations of bortezomib did not affect cell viability (Figure 5C).

**Effect of bortezomib on the expression of osteoclast-associated genes.** To further define the effects of bortezomib on osteoclasts, we analyzed the expression levels of osteoclast-associated genes. As shown in Figure 6A, bortezomib affected gene expression in osteoclasts. The gene expression of c-Fos and NF-ATc1 increased at osteoclast-stimulatory bort-
Bortezomib concentrations. We also tested the effects of proteasome inhibition by bortezomib on protein expression. First, we assessed accumulation of ubiquitinated proteins in osteoclasts by using bortezomib and MG132, another proteasome inhibitor, at different concentrations. We showed that doses of bortezomib that stimulate osteoclasts were associated with increased levels of ubiquitinated proteins. Higher concentrations of bortezomib or MG132 caused cell death and thus resulted in reduced ubiquitination (Figure 6C).

Next, we tested whether molecules associated with osteoclast differentiation would also accumulate upon proteasome inhibition. As shown in Figure 6B, treatment with bortezomib dose-dependently increased c-Fos, NF-ATc1, and TRAF-6 protein levels, which are crucial for osteoclast differentiation, at concentrations stimulating osteoclasts while decreasing them at toxic concentrations. Thus, bortezomib altered the transcriptional profile of osteoclasts, leading to accumulation of proteins relevant for osteoclast differentiation, which leads to increased osteoclastogenesis.

DISCUSSION
In this study, we investigated the effects of proteasome inhibition on inflammatory bone destruction. Bortezomib, a potent inhibitor of the proteasome, increased synovial osteoclast formation and worsened arthritic bone erosions in an animal model of chronic arthritis. Furthermore, our data suggest a direct stimulatory effect of bortezomib on osteoclasts.

Bortezomib, a selective inhibitor of the 26S proteasome, is in clinical use for the treatment of multiple myeloma and mantle cell lymphoma. Bortezomib effectively prevents osteolytic bone destruction in multiple myeloma. Suggested mechanisms of myeloma cell killing by bortezomib include inhibition of NF-κB, modulation of the tumor microenvironment, and activation of apoptotic cell death as a result of the terminal unfolded protein response. Recent reports suggested that proteasome inhibition may primarily affect cells with high protein synthesis, such as normal and neoplastic plasma cells (8). Indeed, bortezomib efficiently and reversibly depletes short- and long-lived plasma cells in mice. Consequently, treatment with bortezomib abrogates disease in mouse models with lupus-like disease (13), thus suggesting therapeutic options for the treatment of several autoantibody-mediated autoimmune diseases.

In our study, we could not detect a beneficial effect of bortezomib on the course of inflammation in TNF-mediated arthritis. In fact, bortezomib treatment slightly worsened TNF-mediated arthritis. This result is consistent with previous observations suggesting that B cells, and now also plasma cells, are not essential for inflammation in arthritis triggered by transgenic overexpression of TNF (14). This enabled us to directly explore the role of proteasome inhibition in inflammatory bone disease.

Recent investigations indicated a direct inhibitory effect of bortezomib on human osteoclasts (9,15). Continuous treatment of osteoclasts with bortezomib inhibited osteoclast formation. However, in vivo, bortezomib is given as a cyclic treatment to reduce toxicity. Thus, continuous exposure to bortezomib might not adequately mimic the situation in humans. When bortezomib was added to preosteoclasts for a limited period of time (<24 hours), it effectively stimulated osteoclastogenesis at concentrations that are achieved during treatment. Moreover, we demonstrated an increased resorptive capacity of bortezomib-treated osteoclasts in bone resorption assays. These functional changes were associated with an altered transcriptional profile: NF-ATc1 and c-Fos mRNA levels, both of which are important regulators of osteoclast development, increased upon inhibition of the proteasome.

Bortezomib causes potent proteasome inhibition leading to the accumulation of ubiquitinated proteins. We observed that bortezomib-treated osteoclasts showed an increase in the expression of proteins such as TRAF-6, NF-ATc1, and c-Fos, all of which are crucial for osteoclast differentiation. We confirmed these findings in osteoclast precursors in vitro (data not shown). Most likely, decreased proteolysis by the ubiquitin–proteasome pathway explains the increased protein levels of osteoclast-associated genes and enhanced differentiation of cells into the osteoclast lineage. Our results contrast with those of previous in vitro studies, which showed inhibitory effects on osteoclasts (9). However, most of the studies are based on cell culture experiments, and current in vivo data are derived from observations in patients with myeloma, in whom bortezomib exerts multiple effects on various cell lineages including tumor cells themselves. Myeloma cells may activate osteoclasts and thereby induce bone resorption (16). Also, we tested the effects of stimulatory and inhibitory bortezomib concentrations on osteoclast apoptosis and cell counts. In support of our other findings, bortezomib strongly induced osteoclast apoptosis and decreased cell counts at inhibitory concentrations, while it has no effect on cell viability at stimulatory concentrations.

We investigated the effects of bortezomib on bone turnover in an animal model of chronic arthritis.
Surprisingly, bortezomib consistently increased the osteoclast number and worsened arthritic bone erosions. Although there was also an increase in synovial inflammation and cartilage damage, this increase was less pronounced. Thus, both direct and indirect effects may mediate the effect of proteasome inhibition on osteoclast precursors. Interestingly, bortezomib did not alter systemic bone turnover in arthritic mice or in WT mice. Consistent with these results, serum levels of type I collagen cleavage products were not increased following bortezomib treatment. However, we observed a significant increase in synovial and systemic RANKL but not OPG levels. Thus, bortezomib may directly affect not only osteoclasts but also RANKL expression in inflamed synovial tissue.

A possible explanation for the divergent local and systemic responses of bone to bortezomib may be based on a highly increased bone turnover in inflamed joints, with a massive accumulation of monocyte/macrophages, which serve as osteoclast precursors. This fact could render bone that is close to inflammatory infiltrates more susceptible to osteoclast-mediated bone resorption, because monocytes are forced to undergo osteoclast differentiation upon proteasome inhibition. In support of this hypothesis, the expression of monocyte/macrophages was strongly decreased in the synovium of hTNF-transgenic mice treated with bortezomib, suggesting a shift toward osteoclast differentiation. Also, bone turnover and metabolic activity may be much higher within inflamed joints, which potentially render osteoclast precursors more susceptible to bortezomib treatment.

Our results provide evidence against a direct protective role of proteasome inhibition in inflammatory bone disease. Bortezomib was recently shown to inhibit collagen-induced arthritis (CIA) and associated bone disease (17). In contrast, bortezomib could not exert beneficial effects on local and systemic bone disease in TNF-mediated arthritis but rather increased structural damage in this animal model of arthritis. The divergent outcomes may be caused by differences in the immunopathogenesis of these arthritis models. CIA is driven by an immune response against type II collagen driven by T and B lymphocytes involving the generation of autoantibodies. Bortezomib may protect bone in CIA indirectly by depletion of pathogenic plasma cells, leading to less autoantibody production. Interference with immune activation in CIA mitigates the inflammatory response and ultimately bone resorption. In the case of CIA, bortezomib appears to directly target the early phase of this autoantibody-mediated inflammatory process.

In contrast, inflammation in the TNF-transgenic mouse model does not involve an autoimmune response and also does not depend on T and B lymphocytes. As a consequence, bortezomib does not affect inflammation in TNF-transgenic mice. Production of TNF and other proinflammatory cytokines is usually under control of the transcription factor NF-κB, which is inhibited by bortezomib. However, transgenic TNF production is not sensitive to NF-κB inhibition, which allowed revealing of the direct stimulatory effect on osteoclast precursors. This sharp contrast between the hTNF-transgenic mouse model and the CIA model is interesting. Proteasome blockade inhibits immune activation and in consequence arthritis and bone damage in CIA, whereas chronic TNF-driven inflammation is not affected, and bone destruction is even worsened. This could be important if considering proteasome inhibition for the treatment of established human RA.

In conclusion, these data suggest that proteasome inhibition can induce osteoclast differentiation and might enhance bone erosion during inflammatory diseases, particularly when the inflammatory process itself is not suppressed.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Zwerina had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.


Acquisition of data. Polzer, Neubert, Meister, Frey, Baum, Distler, Gückel, Schett, Voll.

Analysis and interpretation of data. Schett, Voll, Zwerina.

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