Accelerated bone resorption leading to osteopenia and osteoporosis has been noted in human immunodeficiency virus (HIV) seropositive, treatment-naive patients, but it may be greatly increased in incidence in those receiving highly active anti-retroviral therapies that incorporate certain protease inhibitors (PI). The pathophysiology of these processes is unclear. We have documented the induction of the primary cytokine responsible for osteoclast differentiation and bone resorption, the receptor activator of nuclear factor κB ligand (RANKL), in T cells exposed to soluble HIV-1 envelope glycoprotein gp120. Using a murine osteoclast precursor cell line as well as primary human osteoclast precursors, we demonstrate that pharmacologic levels of two PIs that are linked clinically to osteopenia, ritonavir and saquinavir, abrogate a physiological block to RANKL activity, interferon-γ-mediated degradation of the RANKL signaling adapter protein, TRAF6 (tumor necrosis factor receptor-associated protein 6) in proteasomes. In contrast, indinavir and nelfinavir, PIs that may promote or stabilize bone formation in vivo, had no impact on this system. These findings offer a molecular basis for the acceleration of bone resorption by certain PIs and provide the first example of clinically useful drugs that can interfere with the cross-talk between RANKL and interferon-γ via the proteasome. They also suggest a novel therapeutic approach to HIV osteopenia through modulation of these two molecules.

Loss of bone mineral density (BMD) leading to osteopenia has been observed among HIV seropositive patients naive to antiretroviral therapy, but it is infrequent and of unclear clinical significance (1–3). However, certain antiretroviral therapy regimens may accelerate bone resorption in HIV+ adults and children (4–8). For example, in a survey of 112 homosexual HIV+ men, the relative risk for bone resorption in those receiving highly active antiretroviral therapy treatment incorporating a protease inhibitor (PI) was 2.19 (1.13–4.23, p = 0.02) (5). In another recent longitudinal evaluation of BMD in HIV+ individuals, there was only a weak association between BMD and PI treatment, but there were too few patients to distinguish among different type of PIs (9). Indeed, in one study the PIs indinavir and nelfinavir were associated with augmented or stable bone resorption, respectively (10). A satisfactory pathophysiological mechanism for HIV-linked bone resorption must be able to account for all of these findings. We focused on a T cell cytokine linked to acceleration of bone resorption in vitro and in vivo, the receptor activator of nuclear factor κB ligand (RANKL) (11), and its interaction with a signaling pathway that is altered by therapeutic levels of certain PIs.

Specifically, RANKL activity is modulated through interferon (IFN)-γ-coupled proteasomal degradation of TNF receptor-associated factor 6 (TRAF6) (12). RANKL recruits TRAF6 to the cytoplasmic tail of RANK, resulting in the activation of NF-κB and certain mitogen-activated protein kinase pathways (13). Osteoclast differentiation and survival are based upon those signals. IFN-γ can inhibit RANKL-stimulated osteoclastogenesis by proteasomal degradation of TRAF6, and exacerbation of osteoclast formation and bone destruction occurs in knockout mice lacking the IFN-γ receptor (14–16). It is known that the PIs ritonavir and saquinavir can interfere with some proteasomal functions at suprapharmacological concentrations (17–19) and that general proteasome inhibitors such as MG132 can block cross-talk between RANKL and IFN-γ (12). We demonstrate, in physiologically relevant systems, the role of the soluble envelope glycoprotein gp120 of HIV-1 in receptor activation and the ability of pharmacologically relevant concentrations of certain PIs to suppress interaction between IFN-γ and RANKL, recovering the normal physiological block to RANKL function. These data suggest methods to mitigate the effect of both HIV and specific PIs on bone resorption in AIDS.

MATERIALS AND METHODS

Reagents—Human and mouse recombinant RANKL and IFN-γ were purchased from Peprotech and osteoprotegerin (OPG)-Fc from R&D Systems. Recombinant HIV-1 gp120, monoclonal antibodies to gp120 (b12) and CD4, and HIV PIs ritonavir, saquinavir, indinavir, and nelfinavir were obtained from the National Institutes of Health AIDS Research and Reference Reagents Program. The PIs were prepared as stock solutions of 27 μM in Me2SO, so that the final concentration of Me2SO in control (buffer) or drug-treated cultures was 0.1%. Polyclonal...
anti-TNF-α (R&D Systems) and anti-TRAF6 antibodies (Santa Cruz Biotechnology) were purchased. Sperm whale dentine slices (ALPCO Diagnostics) and osteologic discs (BD Biosciences) were purchased.

Cell Culture and Isolation and Propagation of PBMCs—RAW 264.7 murine monocytic cells were maintained in minimal essential medium plus 5% heat-inactivated FBS and 1% non-essential amino acid preparation. Human PBMCs were derived from heparinized venous blood by density gradient centrifugation using Ficol-Paque and cultured in RPMI 1640 plus 10% heat-inactivated FBS (20).

Isolation of CD3+ T Cells and Adherent Cells from PBMCs—Mature CD3+ T cells were obtained from PBMCs by incubation with Dynabeads M-450 CD3 at a ratio of 5 beads/target cell according to the manufacturer's directions (Dyana! Oslo, Norway). This resulted in a CD3+ T cell population of >95% purity as evaluated by fluorescence-activated cell sorter.

RANKL Protein Enzyme-linked Immunosorbent Assay—RANKL protein concentration was measured using a sandwich enzyme-linked immunosorbent assay that we developed. Briefly, 100 μl/well of capture protein OPG-Fc was coated in 96-well plates at final protein concentrations of 2 μg/ml in PBS, pH 7.4, and incubated overnight at 4 °C. The plates were blocked by adding 300 μl of PBS containing 1% bovine serum albumin, 5% sucrose, and 0.05% NaN3 to each well and incubating the plates at room temperature for 3 h. Plates were washed five times with wash buffer (0.05% Tween 20 in PBS, pH 7.4). Samples to be tested were diluted 1:1 in diluent (1% bovine serum albumin in PBS, pH 7.4) and incubated at room temperature for at least 2 h in final volumes of 100 μl. After washing, the plates were incubated with 100 μl of biotinylated mouse monoclonal anti-human RANKL IgG, 200 ng/ml (R&D Systems) for 2 h at room temperature. To detect biotinylated antibody, streptavidin horseradish peroxidase conjugate (R&D Systems) was used. The bound horseradish peroxidase was assayed with 3,3',5,5'-tetramethybenzidine (Sigma) as substrate, and the reaction was stopped with 1 M H2SO4. ODs were determined at 450 nm. The detection limit was 50 pg/ml, and the mean percent recovery of RANKL, spiked at three concentrations, was 92% in culture supernatant. Intra-assay coefficients were <12%.

Osteoclastogenesis Systems—RANKL induces osteoclast formation in human PBMC cultured for at least 3 days in the presence of macrophage colony-stimulating factor (21, 22). Following this model, we cultured PBMC for 3 days in chamber slides (5 × 105 cells in 0.3 ml of medium/1-cm2 well; Nalgene Nunc) in the presence of human macrophage colony-stimulating factor (M-CSF, 100 ng/ml; Peprotech). Non-adherent cells were removed before conditioned medium from control or gp120-treated cells was added.

RAW 264.7 cells or adherent cells derived from 1.5 × 106 total PBMCs were cultured for up to 7 days with supernatants from control or gp120-treated cultures. The ratio, by volume, of the various culture media was 4.5 cell medium:4.5 culture supernatant:1 FBS. One-half of the culture medium was replaced with fresh medium every 3 days. Prior to performing tr drag-resistant acid phosphatase (TRAP) assays to identify mature osteoclasts, cells were starved for 5 h in serum-free medium followed by 6 days of culture in test supernatants plus medium containing 2% FBS. To study the effect of HIV PIs and IFN-γ on osteoclast formation, RAW 264.7 cells were cultured for 6 days in the presence of RANKL and varying concentrations of IFN-γ and PIs. The medium was changed every 3 days.

TRAP Activity—Osteoclastogenesis was quantitatively assessed using TRAP activity as an index. p-Nitrophenol phosphate in acid phosphatase assay buffer served as a substrate. The assay was performed according to the manufacturer's instructions (Sigma), and TRAP activity was measured colorimetrically at OD 405 nm. For TRAP staining, cells were fixed in 10% formalin for 5 min followed by ethanol-acetone (50:50 v/v) fixation in the presence of acetate buffer (pH 4.8) containing naphthol AS-MX phosphate, fast red violet LB salt, and 50 mM sodium acetate for 10 min at room temperature, as previously described (23).

Bone Resorption Assays—5 × 105 RAW 264.7 murine cells or adherent cells derived from 1.5 × 106 human PBMCs were seeded onto calcium-phosphate-coated osteologic discs (BD Biosciences), placed in wells of 24-well tissue culture plates, and exposed to cell supernatants for from 7 to 15 days. The culture medium was changed every 3–4 days. Discs were then treated with bleach and washed in water, and the number of resorption lacunae per disc was visualized by light microscopy as described (23).

For pit formation in sperm whale tooth dentine slices, identical conditions were used except that the incubation time with PBMC-derived adherent cells was 21 days. The medium was then removed, and 1 M NH4OH was added to the wells for 30 min. The dentine slices were then cleaned by ultrasonication, stained with hematoxylin for 45 s, and washed with water. Resorption pits were visualized under transmitted light.

Analysis of TRAF6 by Metabolic Labeling, Immunoprecipitation, and Immunoblotting—2.5 × 105 RAW 264.7 cells at 0.5 × 106 cells/ml were incubated for 30 min with [35S]methionine in methionine-free Dulbecco's modified Eagle's medium supplemented with dialyzed FBS. Cells were then washed and incubated for 3 h with RANKL and IFN-γ in the presence or absence of ritonavir or saquinavir. Immunoprecipitation was performed using anti-TRAF6 polyclonal antibody and Staphylococcus protein A, products were resolved by SDS-PAGE, and results were quantified by IMAGEQUANT 5.0 (Amersham Biosciences).

For immunoblotting, 2 × 105 RAW 264.7 cells were cultured in the presence of RANKL and IFN-γ with or without anti-HIV PIs in 12-well tissue culture plates for 48 h. Cell extracts were prepared in lysis buffer (50 mm Tris-Cl, pH 7.5, 150 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, aprotinin 2 μg/ml, EDTA 5 μM, Pefabloc Sc 4 μM, peptatin 1 μM, and leupeptin 10 μg/ml) and loaded onto SDS-PAGE. Expression of TRAF6 was evaluated by probing the transferred proteins with anti-TRAF6 antibody (1:500 dilution).

RESULTS

Role of Soluble HIV-1 gp120 in RANKL-mediated Osteoclastogenesis—Given the small fraction of tissue-based or circulating HIV-infected cells in HIV+ patients, it is unlikely that direct HIV infection of T cells could be involved in RANKL regulation in vivo. However, exposure of CD3+ T cells derived from the PBMCs of two normal donors to levels of recombinant HIV-1 IIIB gp120 comparable with that detectable in vivo in untreated patients (100 ng/ml) resulted in marked induction of RANKL protein production (Fig. 1A). RANKL protein was up-regulated by gp120 in separate experiments utilizing PBMCs from three normal donors (Fig. 1A).

We investigated the biologic activity of gp120-induced RANKL in two established model systems for RANKL-mediated osteoclast differentiation: the macrophage-like osteoclast precursor murine line RAW 264.7 and precursor cells derived from human PBMCs. Both cell types can differentiate into osteoclast-like cells, recognized by morphology (giant cells containing >5 nuclei), TRAP staining, and resorption of dentine slices.

We first cultured precursor cells derived from human
PBMCs and RAW 264.7 with 1:1 dilutions of conditioned medium from control PBMC or PBMC exposed to gp120 for 72 h (Fig. 2, A and B). As shown in the middle panels of Fig. 2, A and B, the multinucleated cells (>5 giant cells containing 5 nuclei) induced in both types of osteoclast precursors by supernatants from gp120-treated PBMCs supported formation of resorption pits on dentine slices. The addition of RANK-Fc inhibited the formation of osteoclasts from gp120-treated conditioned medium (Fig. 2, A and B, bottom panels). Further experiments with PBMC-derived precursor cells exposed to supernatants from gp120-treated cultures showed a marked increase in the formation of TRAP⁺ multinucleated osteoclasts (Fig. 2C). The RANKL specificity of the active factor in gp120-exposed cultures was demonstrated by RANK-Fc-mediated suppression of TRAP⁺ cell induction (Fig. 2C). In addition, an anti-CD4 monoclonal antibody (b12; data not shown) completely blocked the effect of gp120 on the induction of osteoclastogenic activity in gp120-treated PBMCs. 

**Fig. 2.** Induction of biologically active RANKL by gp120-exposed PBMC culture supernatants. A, few TRAP⁺ multinucleated cells (top left) and no cells capable of forming pits in dentine slices (top right) were induced in normal donor PBMC exposed to supernatants of control human PBMCs. In the middle panel, multinucleated TRAP⁺ cells (left) and cells capable of forming pits in dentine slices (right, hematoxylin stain, 400×) were induced in PBMC cultures by supernatants from gp120-treated PBMCs. The bottom panel shows the RANK-Fc control. B, multinucleated TRAP⁺ cells were induced in 6-day RAW 264.7 cultures by supernatants from gp120-treated PBMCs (middle panel, left). Pit formation by these osteoclasts was seen, as marked by the arrow (middle panel, right, hematoxylin stain, 400×). The bottom panel shows the RANK-Fc control. C, RAW 264.7 murine cells were cultured for 6 days with supernatants from control or gp120-treated PBMCs along with buffer, RANK-Fc, anti-TNF-α antibody (10 ng/ml), or anti-CD4 monoclonal antibody. Osteoclasts, defined as TRAP⁺ cells with >5 nuclei, in all wells (1 cm²) of 8-well Lab-tek chamber slides were counted under the light microscope. The results represent the mean ± S.D. of triplicate wells. This experiment is representative of three independent experiments performed. The addition of RANKL (5 ng/ml) restored MNC formation blocked by both RANK-Fc and anti-CD4 antibody. *, p = 0.01 control versus gp120-treated PBMCs. D, RAW 264.7 murine cells were cultured with supernatants from control or gp120-treated PBMCs along with buffer, RANK-Fc, anti-TNF-α antibody (10 ng/ml), or anti-CD4 monoclonal antibody. After 4 days of incubation TRAP activity was quantified. *, p = 0.01 control versus gp120-treated PBMCs.
exposed PBMC supernatants. A TRAP activity assay was carried out in parallel with the above experiment as a measure of osteoclastogenesis (Fig. 2D). Consistent with the literature on osteoclast differentiation, the evaluation of osteoclast formation by TRAP⁺ multinucleated giant cell formation and by TRAP activity gave identical results (compare Fig. 2, C and D).

gp120 is known to induce secretion of TNF-α by PBMCs (24), and our gp120-exposed PBMC culture supernatants contained this cytokine at 230 pg/ml. However, TNF-α alone does not promote osteoclastogenesis in murine systems (25) or in our human precursor cell model (data not shown). In addition, although TNF-α can synergize with RANKL in induction of osteoclastogenesis, at least in a murine system (25), TRAP⁺ multinucleated giant cell formation driven by gp120-stimulated culture supernatants was only partially inhibited (35%) by the addition of 10 ng/ml anti-TNF-α antibody (Fig. 2, C and D), a concentration capable of inhibiting >1 ng/ml TNF-α.

Effect of IFN-γ and HIV PIs on gp120-induced, RANKL-mediated Osteoclastogenesis—At suprapharmacological concentrations (20–100 μM) the anti-HIV PIs ritonavir and saquinavir inhibit a variety of proteasomal functions (17–19). As RANKL-mediated osteoclast formation involves signaling through TRAF6, as well as its normal physiologic control by IFN-γ-mediated degradation of TRAF6 in proteasomes (12), we explored whether PIs, linked to accelerated loss of BMD when used at normal pharmacological concentrations, would have an impact on this pathway.

Adherent cells derived from PBMCs of HIV seronegative donors were exposed to medium from purified human CD3⁺ T cells that had been cultured in the presence of gp120 for 72 h. As expected based on studies of RANKL and IFN-γ in other model systems (12), osteoclastogenesis induced by these supernatants was inhibited by the addition of concentrations of recombinant IFN-γ normally found in HIV seronegative individuals (26) or in HIV⁺ individuals with low viral loads (27, 28). The addition of a therapeutically relevant concentration (5 μM) of ritonavir to cultures exposed to supernatants of gp120-treated T cells in the presence of 5 ng/ml IFN-γ resulted in a substantial recovery of osteoclast activity (Fig. 3). Saquinavir had a similar effect, whereas nelfinavir and indinavir at doses up to 10 μM had no impact on osteoclast activity (data not shown). As in our previous experiments with this system, RANK-Fc blocked the osteoclastogenesis induced by gp120-activated cell culture supernatants (data not shown). The direct addition of gp120 had no effect on PBMC-derived precursor cells with respect to osteoclastogenesis (Fig. 3).

We also examined the effect of these PIs on RANKL-induced osteoclastogenesis in the absence of basal levels of IFN-γ. In this nonphysiologic system, concentrations of nelfinavir and indinavir up to 10 μM continued to have no impact on osteoclast formation assessed by TRAP activity (data not shown). Ritonavir, at pharmacologic concentrations, also had no effect on osteoclast formation in the absence of IFN-γ (Fig. 3, A and B). Suprapharmacologic doses of ritonavir (10 and 25 μM) suppressed RANKL-induced osteoclastogenesis (data not shown), but this effect was related to the marked cytotoxicity induced by these concentrations during the 72 h of drug exposure in this assay.

Consistent with previous reports (12), TRAF6 expression was markedly inhibited by IFN-γ, as shown by Western blot analysis of RAW 264.7 osteoclast differentiation driven by RANKL (Fig. 4A). The addition of ritonavir or saquinavir resulted in significantly higher levels of TRAF6 expression, whereas indinavir and nelfinavir had no effect (Fig. 4A, top panel). Ritonavir, saquinavir, indinavir, and nelfinavir, in the absence of IFN-γ, had no effect on TRAF6 expression (Fig. 4A, bottom panel).

Metabolic labeling of RAW 264.7 cells and immunoprecipitation with anti-TRAF6 antibody showed that ritonavir and saquinavir protected TRAF6 from intracellular degradation, an effect comparable with that seen using the general proteasome inhibitor MG132 at lower concentrations (Fig. 4B). This result was confirmed in a time course experiment. TRAF6 was immunoprecipitated at 0, 3, and 6 h after ritonavir exposure. As expected, TRAF6 protein was rapidly degraded in the cells treated with RANKL (100 ng/ml) and IFN-γ (5 ng/ml). After 3 h of chase, a very low amount of labeled TRAF6 was observed, and it was undetectable by 6 h (Fig. 4C).

RANKL is known to affect osteoclast differentiation through TRAF6-mediated processes involving inhibition of NF-κB and JNK1 but not JNK2 (29). Consistent with an earlier report (12), RANKL markedly up-regulated phosphorylation of JNK1 but not JNK2 (no visible phosphorylated band), and IFN-γ com-
HIV-1 PIs restoring RANKL-meditated osteoclast formation and function inhibited by IFN-γ—To investigate whether the results seen at a molecular level translate to the level of biologically active product, we examined the effect of HIV PIs on the bone resorbing activity of RANKL-induced osteoclasts. Ritonavir was added to RAW 264.7 cells treated with RANKL alone or in the presence of IFN-γ, and osteoclastogenesis quantified by TRAP activity and TRAP⁺ MNC count (Fig. 5, A and B). Osteoclast differentiation promoted by RANKL was inhibited by IFN-γ, an effect overcome by ritonavir at pharmacologically relevant concentration (Fig. 5, A and B). Saquinavir also mitigated the block to osteoclast formation, but to a lesser extent (data not shown). Neither indinavir nor nelfinavir had an impact on this system (data not shown), consistent with the biochemical assays for osteoclast activity reported above. The fact that recovery of TRAP⁺ cells in the presence of 10 μM ritonavir was less than with 5 μM may relate to cytotoxicity with prolonged exposures at the higher concentrations (Fig. 5, C and D). Ritonavir or saquinavir treatment also led to recovery of the bone resorbing activity of multinucleated TRAP⁺ osteoclasts exposed to physiologic concentrations of IFN-γ (Fig. 5, C–E). This indicates that these PIs were able to permit all phases of osteoclastogenesis to occur in the presence of its normal physiologic block.

In contrast, the addition of pharmacologic concentrations of IFN-γ, which may vary from 2 to 300 ng/ml depending on the dose and route of administration (30), overcame the PI block to suppression of osteoclastogenesis (Fig. 6). There was a dose-dependent decline in RANKL-driven osteoclast differentiation mediated by IFN-γ, and levels of IFN-γ>5 ng/ml abrogated the ritonavir block to control of RANKL activity seen at physiologic levels of IFN-γ (Fig. 6A). The higher concentrations of IFN-γ had no effect on the viability of these cells. The marked increase in potency of the IFN-γ effect between 5 and 10 ng/ml (Fig. 6A) directly paralleled its effect on overcoming the ritonavir block to TRAP6 degradation as assessed by immunoblotting (Fig. 6B).

**DISCUSSION**

Hyperactivity of the osteoclast, the principal resorptive cell of bone, relative to the bone-forming osteoblast defines the pathophysiology of most cases of osteopenia and osteoporosis (31). Unlike TNF-α, RANKL alone induces osteoclast differentiation from bone marrow precursor cells in the absence of a stroma (32, 33). Deletion of OPG, the RANKL decoy receptor, in knockout mice leads to sustained RANKL activity and severe, early osteoporosis in the absence of changes in TNF-α or other cytokines involved peripherally in bone homeostasis (34). We explored the RANKL pathway in HIV infection and its treatment with PI-based regimens in vitro.

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**Fig. 4.** Effect of HIV PIs on the expression of RANKL signaling adapter protein TRAF6 during RANKL/IFN-γ signaling cross-talk. A, top, RAW 264.7 cells were treated with RANKL (100 ng/ml) and IFN-γ (5 ng/ml) alone or in the presence of varying concentrations (1 to 5 μM) of ritonavir (RTR), saquinavir (SQR), indinavir (INR), or nelfinavir (NFR) for 18 h. They were then immunoblotted with anti-TRAF6 polyclonal antibody. β-Actin was used to normalize for total protein concentration. A graphic representation of relative band intensities for TRAF6, determined by densitometry scanning, is provided below the TRAF6 immunoblot. Bottom, HIV PIs do not have an effect on the expression of TRAF6 in the absence of IFN-γ (5 ng/ml). These results are representative of four independent experiments. B, 35S-labeled RAW 264.7 cells were treated with RANKL and IFN-γ in the presence or absence of varying concentrations (5 and 25 μM) of ritonavir or saquinavir. MG132 (40 μg/ml), a general proteasomal inhibitor, served to completely suppress that activation (Fig. 4D). Indinavir and nelfinavir had no impact on IFN-γ-induced suppression of JNK1 activation, whereas ritonavir and saquinavir both overcame such suppression (Fig. 4D).

Certain HIV PIs Restore RANKL-mediated Osteoclast Formation and Function Inhibited by IFN-γ—To investigate whether the results seen at a molecular level translate to the level of biologically active product, we examined the effect of HIV PIs on the bone resorbing activity of RANKL-induced osteoclasts. Ritonavir was added to RAW 264.7 cells treated with RANKL alone or in the presence of IFN-γ, and osteoclastogenesis quantified by TRAP activity and TRAP⁺ MNC count (Fig. 5, A and B). Osteoclast differentiation promoted by RANKL was inhibited by IFN-γ, an effect overcome by ritonavir at pharmacologically relevant concentration (Fig. 5, A and B). Saquinavir also mitigated the block to osteoclast formation, but to a lesser extent (data not shown). Neither indinavir nor nelfinavir had an impact on this system (data not shown), consistent with the biochemical assays for osteoclast activity reported above. The fact that recovery of TRAP⁺ cells in the presence of 10 μM ritonavir was less than with 5 μM may relate to cytotoxicity with prolonged exposures at the higher concentrations (Fig. 5, C and D). Ritonavir or saquinavir treatment also led to recovery of the bone resorbing activity of multinucleated TRAP⁺ osteoclasts exposed to physiologic concentrations of IFN-γ (Fig. 5, C–E). This indicates that these PIs were able to permit all phases of osteoclastogenesis to occur in the presence of its normal physiologic block.

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**DISCUSSION**

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Our studies suggest a mechanism by which HIV-1 or soluble HIV-1 envelope gp120 can promote osteoclastogenesis and bone
Fig. 5. Ritonavir restores RANKL-mediated osteoclast formation blocked by IFN-γ. A, RAW 264.7 cells were cultured in the presence of 100 ng/ml RANKL and/or 5 ng/ml IFN-γ and/or 5 μM ritonavir. The results represent the mean ± S.E. of three independent experiments, each performed in duplicate. Statistical significance relative to RANKL/IFN-γ-treated cells; *, p = 0.033. B, ritonavir augments the TRAP⁺ MNC counts reduced by cultures of RAW 264.7 treated with both RANKL (100 ng/ml) and IFN-γ (5 ng/ml). The results represent the mean ± S.E. of three independent experiments, each performed in duplicate. Statistical significance relative to RANKL/IFN-γ-treated cells; *, p = 0.02. C, RAW 264.7
resorption via the induction of RANKL production by T cells, as could be seen in active HIV replication with shedding of gp120. gp120-mediated induction of RANKL may parallel the ability of soluble gp120 to induce secretion of TNF-α and other cytokines from both CD4+ T cells and monocytes (24). The latter appears to relate to induction of the mitogen-activated protein kinase pathway (35). As extracellular signal-regulated kinase (ERK) also plays a key role in RANKL expression (36), it is likely that gp120 induces RANKL via ERK signaling. However, given the low incidence of BMD loss in untreated HIV disease, even with additional findings of decreased bone formation in vivo in such patients (37), this must be a relatively modest process. However, certain PIs may remove the normal physiological block to RANKL overactivity, IFN-γ-mediated proteasomal degradation of the signal adapter protein TRAF6. We suggest that this leads to the accelerated bone resorption seen in some HIV+ patients being treated with highly active antiretroviral therapy. The ritonavir dose required for inhibition of the IFN-γ block to osteoclast formation, 5 μM, is in the range of peak serum levels in patients (38). In contrast, inhibition of proteasomal housekeeping functions, such as NF-κB degradation, requires much higher levels of ritonavir (38).

The most pronounced PI effect in terms of inhibition of IFN-γ-mediated osteoclast differentiation was exerted by ritonavir, and this is the first study to show that a clinically useful drug can block signaling cross-talk through the proteasome. Saquinavir had a more modest impact in this system; indinavir and nelfinavir had no effect. These results correlate with in vitro findings on the relative anti-proteasomal activity of the HIV PIs in the processing of major histocompatibility complex-I antigens (38). They are also consistent with findings of increased or stable BMD in HIV-infected patients treated with indinavir or nelfinavir, respectively, versus ritonavir or saquinavir, although the absence of direct comparisons among those PIs in randomized trials renders that conclusion tentative (12).

Our results emphasize the need to explore further the proteasome-inhibiting properties of different types of HIV PIs, in an attempt not only to interpret their role in the variety of metabolic disturbances that might be linked to HIV infection and its therapy but also to design new PIs that might mitigate those effects. They also emphasize the need to examine the effects of various PIs in the context or HIV. For example, a recent study utilizing rat calvaria explants alone, apart from HIV or immune cells, showed that all four PIs tested here had osteoclast activating activity, but the relevance of those findings to the current results is unclear (39). In terms of specific therapeutic options to retard HIV osteopenia, there are at least two possibilities. First, the RANKL decoy OPG can suppress bone resorption in vivo by blocking RANKL, as demonstrated in a study of osteopenia in postmenopausal women, but the development of anti-OPG antibodies limits its efficacy (40, 41). Use of RANK-Fc to block RANKL, as suggested by our in vitro studies, may offer another experimental therapeutic option. Second, our findings that physiologic levels of IFN-γ can reverse the effect of osteoclastogenic activity through RANKL production by gp120-stimulated human T cells and that pharmacologic levels of IFN-γ abolish the PI block to RANKL suppression suggest the exploration of possible clinical applications of this cytokine. IFN-γ is known to suppress the loss of BMD in animal models linked to T cell activation (42), although not in other models of accelerated bone resorption (43, 44). There is also the potential for synergy between RANKL and TNF-α in promotion of osteoclastogenesis. This has been shown in a murine model (25, 45) and duplicated in our primary human PBMC system (data not shown). However, the concentrations of TNF-α required for this synergy in murine or human systems, at least in vitro (>100 ng/ml), exceed those found in most HIV+ patients (46), and inhibition of TNF-α in our system had minimal effect on osteoclastogenesis.

The ability of certain PIs to interfere with IFN-γ function in bone physiology may be particularly damaging in AIDS. Receptors for IFN-γ are maintained on HIV+ patient PBMCs even in the setting of advanced disease (47), but there is a marked
defect in IFN-γ production to antigen challenge in PBMCs of untreated HIV+ patients, paralleling their clinical and immunologic stage of disease (48). Circulating levels of IFN-γ may be very low in these patients (49). Although antiretroviral therapy can restore IFN-γ production to normal levels in many (27, 28) or if not all (50) patients, its regulatory role may be abrogated by certain HIV PIs as demonstrated here. TRAF6 would be a more specific target than IFN-γ for the development of inhibitors to RANKL activity, and peptides capable of such activity have already been tested in murine systems (51).

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