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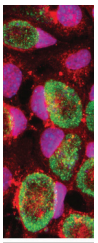

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RESEARCH ARTICLE

Elevated suppressor of cytokine signaling-1 (SOCS-1): a mechanism for dysregulated osteoclastogenesis in HIV transgenic rats

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The major point of this report is the identification of a putative mechanism for HIV-induced osteopenia/osteoporosis via disregulation of osteoclastogenesis. Although the *in vitro* osteoclastogenesis model used here may be different from what is happening in the *in vivo* situation, this constitutes a necessary step in the development of the model.

Keywords
SOCS-1; HIV-1; osteoclast; osteoporosis.

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Abstract

Accelerated bone loss leading to osteopenia, osteoporosis, and bone fracture is a major health problem that is increasingly common in human immunodeficiency virus (HIV)–infected patients. The underlying pathogenesis is unclear but occurs in both treatment naïve and individuals receiving antiretroviral therapies. We developed an HIV-1 transgenic rat that exhibits many key features of HIV disease including HIV-1-induced changes in bone mineral density (BMD). A key determinant in the rate of bone loss is the differentiation of osteoclasts, the cells responsible for bone resorption. We found HIV-1 transgenic osteoclast precursors (OCP) express higher levels of suppressor of cytokine signaling-1 (SOCS-1) and TNF receptor–associated factor 6 (TRAF6) and are resistant to interferon-gamma (IFN-γ) mediated suppression of osteoclast differentiation. Our data suggest that dysregulated SOCS-1 expression by HIV-1 transgenic OCP promotes osteoclastogenesis leading to the accelerated bone loss observed in this animal model. We propose that elevated SOCS-1 expression in OCP antagonizes the inhibitory effects of IFN-γ and enhances receptor activator of NF-kB ligand (RANKL) signaling that drives osteoclast differentiation and activation. Understanding the molecular mechanisms of HIV-associated BMD changes has the potential to detect and treat bone metabolism disturbances early and improve the quality of life in patients.

Introduction

The introduction of antiretroviral drugs has dramatically improved both survival and quality of life of HIV patients. However, improved survival has resulted in the emergence of long-term complications associated with HIV-1 disease. In many respects, HIV infection recapitulates conditions of accelerated senescence (Deeks, 2009). Two manifestations of natural senescence are skeletal degradation and decreases in immune competence. The two may be closely related; recent studies suggest that immune cells regulate bone homeostasis and cause changes in bone mineral density (BMD) during inflammatory conditions (Gao *et al.*, 2007; Li *et al.*, 2007; Weitzmann & Pacifici, 2007). Recent studies of HIV-1-infected patients show abnormalities in BMD and in bone turnover markers. The prevalence of bone loss in HIV patients range from 22% to 50% for osteopenia and from 3% to 20% for osteoporosis (Tebas *et al.*, 2000;

Carr *et al.*, 2001; Amiel *et al.*, 2004; Madeddu *et al.*, 2004; Yin *et al.*, 2005). Of concern are recent studies that suggest fractures occur more commonly in HIV-positive men and women, especially in older patients, contributing to HIV-associated morbidity and mortality (Ofotokun & Weitzmann, 2010; Stone *et al.*, 2010; Dolan *et al.*, 2006; Prior *et al.*, 2007; Triant *et al.*, 2008; Womack *et al.*, 2011; Young *et al.*, 2011).

The etiology of HIV-1 bone loss is likely multifactorial. Known risk factors for accelerated bone loss are common in HIV-1-infected patients such as low body weight, low vitamin D levels, hypogonadism, and alcohol abuse (McComsey *et al.*, 2010; Yin *et al.*, 2010). A meta-analysis of several cross-sectional studies reports that the odds ratio for osteoporosis in HIV patients is 3.7 compared with age-matched controls (Brown & Qaqish, 2007). In addition, HIV disease and antiretroviral therapies are risk factors for accelerated bone loss. Cross-sectional studies have shown a correlation between antiretroviral therapy itself and bone loss (Nolan *et al.*, 2001; Mondy *et al.*, 2003; Dolan *et al.*, 2006). However, longitudinal studies of patients on antiretroviral therapy show BMD stabilizes over time (Dolan *et al.*, 2006; Bolland *et al.*, 2007). Further, HIV-1 infection is associated with bone loss in both treated and treatment naïve patients and the HIV-1 proteins gp120 and Vpr *in vitro* increase expression of receptor activator of NF-kB ligand (RANKL), the key osteoclastogenic cytokine (Paton *et al.*, 1997; Fakruddin & Laurence, 2003, 2005; Madeddu *et al.*, 2004; Brown & Qaqish, 2007; Gibellini *et al.*, 2007; McComsey *et al.*, 2010).

The adult skeleton continuously undergoes bone remodeling to shape and repair damaged and worn bone (Manolagas & Jilka, 1995). Osteoblasts and osteoclasts are the primary cells responsible for bone formation and bone resorption, respectively. The breakdown of bone by osteoclasts is a critical function in bone homeostasis but is also implicated in the pathogenesis of various bone diseases including postmenopausal osteoporosis and inflammatory conditions such as periodontitis (Teitelbaum, 2000). Osteoclasts are large multinucleated hematopoietic cells of the myeloid lineage that develop from precursors following stimulation with macrophage/monocyte-colony-forming factor (M-CSF) and RANKL (Boyle *et al.*, 2003), which bind to their receptors c-Fms (also called CSF-1R) and RANK, respectively. M-CSF supports survival and proliferation of myeloid progenitors and promotes generation of osteoclast precursors (OCP) that express RANK (Arai *et al.*, 1999). RANKL, a member of the TNF superfamily of cytokines, provides the critical signal that drives development of OCP and activation of mature osteoclasts (Lacey *et al.*, 1998; Yasuda *et al.*, 1998b; Arai *et al.*, 1999; Kong *et al.*, 1999b). RANKL binding RANK induces recruitment of the adaptor protein TNF receptor–associated factor 6 (TRAF6) and activation of the transcription factors nuclear factor κB (NF-κB), activation protein 1 (AP-1) and nuclear factor of activated T cells and cytoplasmic 1 (NFATc1), which transactivate osteoclastogenic genes (Wong *et al.*, 1998; Takayanagi *et al.*, 2000, 2002). RANKL is expressed by osteoclasts, chondrocytes, osteocytes, osteoblasts, stromal cells, T cells, and B cells in either a membrane bound or soluble form (Lacey *et al.*, 1998; Kong *et al.*, 1999b; Takayanagi *et al.*, 2000; Vikulina *et al.*, 2010; Nakashima *et al.*, 2011; Xiong *et al.*, 2011). Expression is upregulated by vitamin D_3, prostaglandin E_2, parathyroid hormone, TNF-α, IL-1, IL-6, IL-11, and IL-17 (Wong *et al.*, 1997; Kong *et al.*, 1999b; Kotake *et al.*, 1999; Wada *et al.*, 2006; Nakashima & Takayanagi, 2008; Vikulina *et al.*, 2010).

Osteoclastogenesis is inhibited by IFN-γ and osteoprotegerin, a soluble decoy receptor of RANKL that blocks osteoclast formation *in vitro* and bone resorption *in vivo* (Simonet *et al.*, 1997; Yasuda *et al.*, 1998b; Teitelbaum, 2000). IFN-γ strongly suppresses osteoclastogenesis *in vitro*, which may be attributable to multiple inhibitory mechanisms. IFN-γ induces apoptosis, suppresses expression of RANK by OCP, down-regulates cathepsin K expression, and blocks RANKL-RANK downstream-signaling events (Takahashi *et al.*, 1986; van't Hof & Ralston, 1997; Wong *et al.*, 1998; Kamolmatyakul *et al.*, 2001; Takayanagi *et al.*, 2002, 2005; Gao *et al.*, 2007). IFN-γ inhibits RANK signaling by accelerating the proteasome-mediated degradation of the key adaptor molecule TRAF6 (Takayanagi *et al.*, 2000). Upon binding to its receptor, IFN-γ activates the Janus kinases Jak1 and Jak2 and phosphorylates the transcription factor signal transducer and activator of transcription (STAT)-1, which results in the induction of IFN-responsive gene transcription (Dalpke *et al.*, 2003). IFN-γ and STAT-1 induce expression of SOCS-1, a potent feedback inhibitor of IFN-γ signaling that also cross-inhibits signaling by type 1 IFN receptors and the IL-4 receptor in many lineages of immune cells (Hu & Ivashkiv, 2009).

We have reported that HIV-1 transgenic (Tg) rats have both reduced type 1 cytokine production (IFN-γ and IL-2) and type 1 cytokine responses and a concomitant increase in IL-10 production, which are also observed in patients during progression to AIDS (Clerici & Shearer, 1993; Reid *et al.*, 2004; Yadav *et al.*, 2006, 2009). We have shown that IL-10 induces over-expression of SOCS-1 in HIV-1 Tg rat $CD4^+$ T cells and dendritic cells, thereby disrupting the IL-12-IFN-γ-signaling axis (Yadav *et al.*, 2009). We showed that SOCS-1 is likewise elevated in $CD4^+$ T cells from HIV-1-infected patients and is correlated with defective IFN-γ signaling (Reid *et al.*, 2001; Reid *et al.*, 2004; Yadav *et al.*, 2006, 2009). It was recently reported that the HIV-1 Tg rat undergoes severe osteoclastic bone resorption and shows an imbalanced ratio of RANKL to osteoprotegerin in B cells (Vikulina *et al.*, 2010). Here, we demonstrate that along with dysregulated induction of SOCS-1 by OCP, the OCP are resistant to the suppression of osteoclast differentiation by IFN-γ. Therefore, we propose that elevated SOCS-1 expression by OCP abrogates IFN-γ-mediated control of osteoclastogenesis in the HIV-1 Tg rat and hypothesize that overproduction of SOCS-1 during HIV-1 infection is an important mechanism by which osteoclastogenesis is augmented, leading to an increase in bone loss. This study will help to understand the pathogenesis of HIV-1-induced bone loss in infected patients.

Materials and methods

HIV-1 Tg and non-Tg rats

The construction of the HIV-1 transgene and production of the Tg rats have been described (Reid *et al.*, 2001). Mature (12–15 months) pathogen-free Tg rats and age-matched Fisher 344/NHsd non-Tg rats were used in our analysis and were housed under pathogen-free conditions in microisolator cages on HEPA-filtered ventilated racks. The University of Maryland School of Medicine Animal Care and Use Committee approved the experimental protocol.

OCP and splenic mononuclear cell isolation and flow cytometry

OCP were isolated from the bone marrow, and RBCs were removed by osmotic lyses. Splenic mononuclear cells were isolated using Histopaque-1083 (Sigma-Aldrich). OCP were stained with anti-CD11b-FITC (Antigenex America) and isolated using positive selection (Miltenyi Biotec) under conditions described by the manufacture. The positive population was stained for RANK by staining with anti-RANK-PE (Imgenex). Flow cytometry was as described previously (Reid *et al.*, 2004), and data were analyzed by FLOWJO software. RANK surface expression levels were quantified using QuantiBRITE PE beads (BD Biosciences).

Biochemical indices of bone resorption

Serum C-terminal telopeptide of collagen, a marker of bone resorption, and serum osteocalcin, a specific marker for bone formation, were measured in rats 12–14 months of age using RATlaps and Rat-MID ELISAs, respectively (Immunodiagnostic Systems). Samples were measured in triplicate and averaged for each rat.

Real-time PCR

Relative levels of specific mRNA were quantified by real-time RT-PCR analysis using the IQ5 Multicolor Real-time PCR Detection System (Bio-Rad Laboratories). Isolated OCP from control and Tg rats were stimulated at 1.0×10^6 cells mL^{-1} at indicated times with IFN-γ or 5 h with 50 ng mL^{-1} sRANKL. Total cellular RNA was prepared using an RNeasy mini kit (Qiagen). First-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) and analyzed using IQ SYBR Green PCR kit (Bio-Rad Laboratories). Rat-specific primer sets for RANKL, osteoprotegerin, SOCS-1, 18S, tartrate-resistant acid phosphatase 5, and cathepsin K were synthesized (Bio-polymer core, University of Maryland, Baltimore or www.Realtimeprimers.com): RANKL forward primer: 5′-TTT GCT CAC CTC ACCATC AA; reverse primer: 5′-TCC GTT GCT TAA CGT CAT GT; osteoprotegerin forward primer: 5′-TCC GGA AAC AGA GAA GCA AC; reverse primer: 5′-TGT CCA CCA GAA CACTCA GC; SOCS-1 forward primer: 5′-AGC CAT CCT CGT CCT CGT C; reverse primer: 5′-GCG GAA GGT GCG GAA GTG; 18S forward primer: 5′-GCC TTT CTT CAT TGT CCA GA; reverse primer: 5′-AAA CTT TGG ACG CAG TCT TG; tartrate-resistant acid phosphatase 5 forward primer: 5′-CAA CTT CAT GGA CCC TTC TG; reverse primer: 5′-ACC CAT TAG GGG ATA AGC AG and cathepsin K forward primer: 5′-CTT GGC TCG GAA TAA GAA CA; reverse primer: 5′-GAG GCC ACA ACT CTC AGA AA. Samples were run in triplicate and the yield of PCR product was normalized to 18S ribosomal RNA. To control for DNA contamination, equal amounts of RNA were used without reverse transcriptase.

In vitro osteoclastogenesis

Bone marrow cells were collected from the femurs and tibias of 12–15-month old rats. These cells were suspended in a culture dish with αMEM containing 10% FBS for 24 h at 37 °C. Nonadherent cells were collected without contaminating RBC and washed in αMEM. Cells were cultured in 24-well plates (1.0×10^6 cells mL^{-1}) in the presence of 20 ng mL^{-1} mouse M-CSF (R&D Systems) for 3 days. Change media and culture adherent cells in 20 ng mL^{-1} M-CSF and 100 ng mL^{-1} rat RANKL (Antigenix America) for an additional 5–7 days with or without rat IFN-γ (BD Pharmingen). The cells were fixed and stained for tartrate-resistant acid phosphatase 5 (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich) according to manufacturer's instructions. The number of TRAP-positive multinuclear cells (> 3 nuclei/cell) were determined by counting. RNA was also isolated from adherent cells as described previously.

Immunoblotting

Osteoclasts were cultured with or without IFN-γ (10 ng mL^{-1}) for 2 h. Cells were lysed with RIPA buffer (Sigma-Aldrich) containing 0.1 mM PMSF, 1x EDTA-free protease inhibitor cocktail (Calbiochem), 1x Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich). Total protein concentration was determined by DC Protein Assay (Bio-Rad Laboratories), and equal amounts of total protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The primary antibodies included anti-SOCS-1 (Cell Signaling Technology), TRAF6 (Santa Cruz Biotechnology), and anti–β actin (Thermo Scientific).

Cell proliferation assay

Cell proliferation was determined by [^{3}H]-thymidine uptake.

Statistics

Statistical significance was determined using GRAPHPAD INSTAT (GraphPad Software). Simple comparisons were made using unpaired or paired 2-tailed Student's *t*-test for parametric data or Mann–Whitney U-test for unpaired nonparametric data. Multiple comparisons were made using one-way ANOVA with Tukey–Kramer post-test. $P < 0.05$ was considered statistically significant. All data are presented as Mean ± SEM.

Results

HIV-1 Tg rats express increased serum markers of bone resorption

We previously reported that HIV-1 Tg rats have low number of T cells with Th1 effector/memory phenotype, reduced production of IFN-γ and high levels of SOCS-1 expressed in lymphoid and myeloid cells, which led us to hypothesize that IFN-γ signaling is compromised in this animal model, interfering with suppression of osteoclastogenesis (Takayanagi *et al.*, 2000; Hayashi *et al.*, 2002; Ohishi *et al.*, 2005). Consistent with these findings, osteoclastogenic abnormalities include an uncoupling of serum biochemical indices of bone resorption and formation (C-terminal telopeptide of collagen (CTx) and osteocalcin, respectively) and an abnormal ratio of RANKL to the decoy receptor osteoprotegerin, a key determinant of the rate of osteoclastogenesis and bone resorption (Yasuda *et al.*, 1998a, b; Haskelberg *et al.*, 2011). Therefore, we measured serum levels of CTx and osteocalcin. Serum CTx (a bone resorption marker) was significantly increased in HIV-1 Tg rats relative to age-matched non-Tg controls (13 ± 1.5 and 8.2 ± 0.8 ng mL^{-1}, respectively, *t*-test; $P = 0.018$; $n = 5$; Fig. 1a), while differences in serum osteocalcin (a bone formation marker) levels in HIV-Tg rats relative to age-matched non-Tg controls did not reach significance (Fig. 1b).

HIV-1 Tg rat PBMC express an increased ratio of RANKL to osteoprotegerin

An increase in the ratio of RANKL to osteoprotegerin accelerates the rate of osteoclastic bone resorption. To further assess osteoclastic bone resorption, we measured levels of RANKL and osteoprotegerin mRNA from HIV-1 Tg and non-Tg control PBMC using real-time RT-PCR. Higher levels of RANKL mRNA was measured in HIV-1 Tg rats compared with controls. In contrast, the relative expression of osteoprotegerin mRNA did not differ significantly. This increase in the ratio of RANKL to osteoprotegerin mRNA levels for HIV-1 Tg and non-Tg controls (4.4 ± 0.09 and 1.1 ± 0.38, respectively; Mann -Whitney; $P = 0.02$; Fig. 2) suggests enhanced osteoclastogenesis as the basis for the accelerated bone resorption identified in the HIV-1 Tg rat. Similar results were also observed in mononuclear cells isolated from the spleen. HIV-1 Tg splenic mononuclear cells express elevated levels of SOCS-1 (Mann–Whitney; $P = 0.018$; $n = 3$) and RANKL (Mann–Whitney; $P = 0.0005$; $n = 3$) mRNA (Supporting Information, Fig. S1a and b, respectively) compared to control. Similar levels of osteoprotegerin mRNA (Fig. S1c) were observed resulting in an increase in the ratio of RANKL to osteoprotegerin (Mann–Whitney; $P = 0.0005$; $n = 3$; Fig. S1d).

HIV-1 Tg rats express increased SOCS-1 mRNA and protein

We hypothesized that compromised IFN-γ signaling mediated by SOCS-1 prevents effective suppression of osteoclast differentiation. Therefore, we analyzed SOCS-1 expression in HIV-1 Tg and control OCP. HIV-1 Tg and non-Tg control OCP were treated with IFN-γ for 2 h. Figure 3a shows that HIV-1 Tg OCP had a twofold greater basal levels of SOCS-1 mRNA relative to non-Tg controls and a highly significant 14.7-fold increase (ANOVA; $P = 0.008$) following IFN-γ stimulation. Treatment with IFN-γ-induced higher SOCS-1 protein expression in HIV-1 Tg OCP compared with non-Tg control OCP (Fig. 3b). In the absence of IFN-γ treatment, HIV-1 Tg and non-Tg control OCP express similar levels of the RANK receptor and no significant difference in proliferation was observed (Fig. S2a–c).

HIV-1 Tg rats are resistant to IFN-γ-mediated suppression of osteoclast differentiation

We tested whether the elevated SOCS-1 expression correlated with lack of suppression of osteoclast differentiation following treatment with exogenous IFN-γ. As shown in Fig. 4a, significantly more HIV-1 Tg OCP differentiated into tartrate-resistant acid phosphatase 5 $(TRAP)^+$ multinucleated cells, in the presence of 500 and 1000 pg mL^{-1} IFN-γ (Mann–Whitney; $P = 0.008$ and 0.032, $n = 5$, respectively) than non-Tg controls. Resistance to IFN-γ suppression by HIV-1 Tg OCP was confirmed by measuring expression of mRNA for the osteoclast-specific enzyme, TRAP and the predominant protease in bone-resorption, cathepsin K, by real-time RT-PCR. As shown in Fig. 4b, there was a significant 3.7- and 2.8-fold increase in TRAP (*t*-test; $P = 0.048$; $n = 4$) and cathepsin K (*t*-test; $P = 0.048$; $n = 3$) mRNA detected in developing osteoclasts from HIV-1 Tg rats relative to non-Tg controls following treatment with 1000 pg mL^{-1} of IFN-γ, respec-

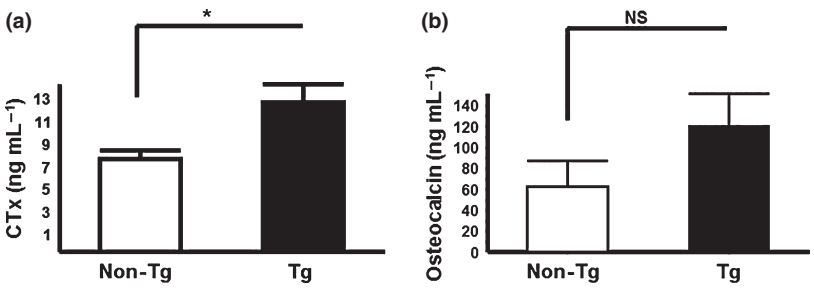

Fig. 1 Bone resorption in HIV-1 Tg rats. (a) C-terminal telopeptide (CTx) and (b) osteocalcin, markers of bone resorption and bone formation respectively, were quantified in the serum of non-Tg and HIV-1 Tg rats by ELISA. Samples were analyzed in triplicate.

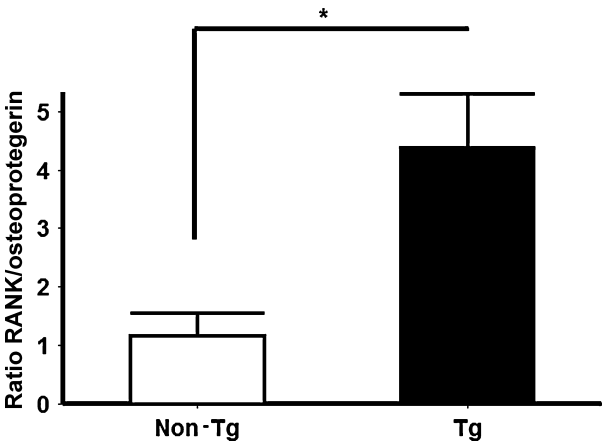

Fig. 2 Increased RANKL and osteoprotegerin ratio in HIV-1 Tg rat. RANKL and osteoprotegerin mRNA was quantified using real-time RT-PCR from PBMCs for HIV-1 Tg ($n = 4$) and non-Tg ($n = 6$) as described in the Material and methods section and the ratio determined for relative expression. Samples were analyzed in triplicate and data normalized to the 18S ribosomal RNA.

tively. Our findings that SOCS-1 is elevated in HIV-1 Tg OCP (Fig. 3a and b) and that HIV-1 Tg OCP are resistant to IFN-γ suppression of the RANKL induced bone-resorbing enzymes, TRAP and cathepsin K (Fig. 4a and b), suggest that increased SOCS-1 expression attenuates antiosteoclastogenesis mediated by IFN-γ. Therefore, we tested whether IFN-γ-mediated degradation of TRAF6, an adaptor protein critical in RANKL signaling, is disrupted in HIV-1 Tg OCP. HIV-1 Tg and non-Tg control OCP were treated with RANKL and IFN-γ for 24 h. RANKL-treated HIV-1 Tg OCP express higher levels of TRAF6 protein compared with non-Tg control OCP as determined by Western blot (Fig. 4c). Further, RANKL-treated HIV-1 Tg OCP express higher levels of TRAF6 following treatment with 500 pg mL^{-1} of IFN-γ compared with non-Tg controls (Fig. 4c). These data suggest that increased SOCS-1 expression by HIV-1 Tg OCP is associated with attenuated IFN-γ inhibition of osteoclastogenesis.

RANKL induction of SOCS-1 in non-Tg control OCP

IFN-γ inhibits osteoclastogenesis of OCP by suppressing the expression of c-fms and/or RANK signaling through TRAF6. RANKL induces SOCS-1 in OCP (Hayashi *et al.*, 2002); therefore, we measured SOCS-1 mRNA levels in non-Tg control rat OCP following 5 h of treatment with 50 ng mL^{-1} of sRANKL. SOCS-1 mRNA increased 2.8-fold (paired *t*-test; $P = 0.0159$; $n = 5$) relative to unstimulated controls (Fig. 5). These data suggest that the increased RANKL expression by HIV-1 Tg PBMC (Fig. 2) plays a role in increased SOCS-1 expression by HIV-1 Tg OCP and the reduced ability of INF-γ to attenuate osteoclast differentiation (Fig. 4).

Discussion

In the past, few HIV-infected patients lived long enough to experience the morbidity and mortality of bone loss. Measurement of BMD as a routine test in elderly HIV-infected patients has not previously been recommended. A detailed history and physical to assess individual risk for osteopenia/osteoporosis is now recommended. Abnormal clinical laboratory values obtained during the course of HIV treatment (i.e., an elevated alkaline phosphatase or low testosterone) suggest the need to test for changes in BMD. No studies, however, have thus far addressed the underlying mechanism(s) for abnormal BMD reported in HIV-1 disease; consequently, the pathogenesis remains poorly understood. In this study, we have identified a potential pathological mechanism of HIV-1-induced bone loss mediated by SOCS-1 enhancement of osteoclastogenesis. The differentiation of osteoclasts is dependent on signals from RANK, stimulated by its ligand RANKL (Vikulina *et al.*, 2010; Wong *et al.*, 1997; Kong *et al.*, 1999a). Up-regulation of RANKL by inflammatory cytokines such as TNF-α contributes to osteoclastogenesis (Lam *et al.*, 2000; Zhang

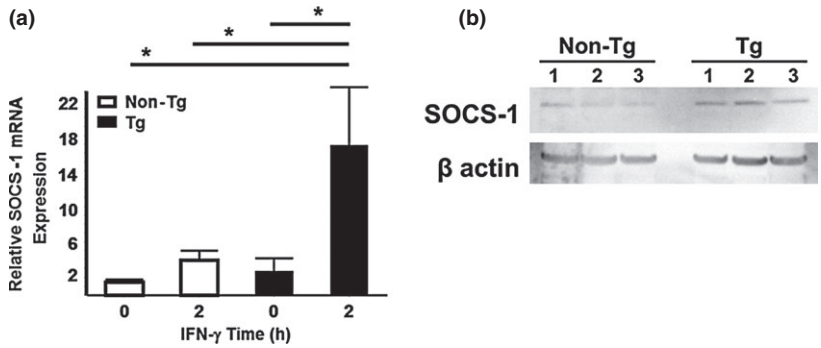

Fig. 3 SOCS-1 mRNA and protein expression are elevated in HIV-1 Tg rats. (a) OCP (1.0×10^6/mL) from non-Tg and HIV-1 Tg rats were stimulated with for 2 h with 10 ng mL^{-1} of IFN-γ and levels of SOCS-1 mRNA were determined by real-time quantitative RT-PCR. Samples were analyzed in triplicate and data normalized to the expression of 18S ribosomal RNA. (b) OCP from non-Tg ($n = 3$) and HIV-1 Tg ($n = 3$) rats were stimulated for 2 h with 10 ng mL^{-1} of IFN-γ. SOCS-1 and β actin were detected by Western blotting.

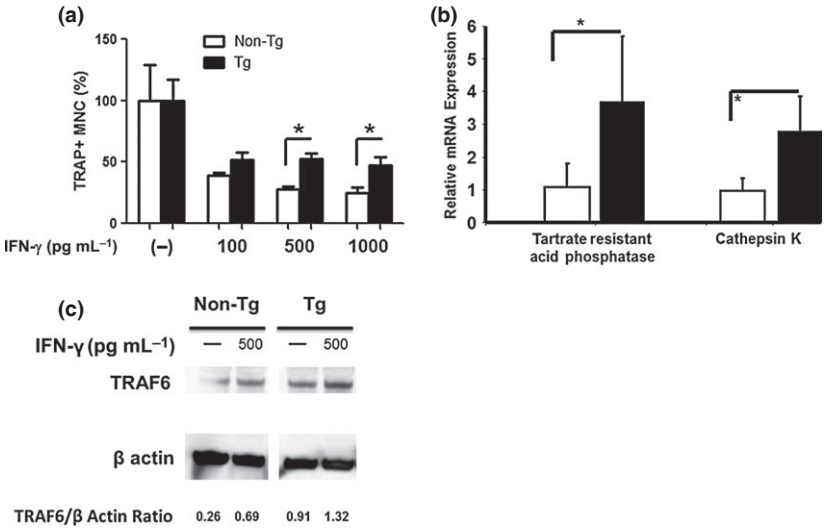

Fig. 4 SOCS-1 over-expression confers resistance to suppression of osteoclast differentiation by IFN-γ. (a) OCP (1.0×10^6/mL in a 24 well plate) were cultured with 20 ng mL^{-1} of M-CSF and 100 ng mL^{-1} RANKL with various concentrations of IFN-γ for 7–8 days. Osteoclast numbers [$TRAP^+$ multinuclear cells (MNC)] were determined from duplicate samples. $TRAP^+$ MNC cells are represented as a percent of osteoclast produced without added IFN-γ. Total RNA was isolated from developing osteoclast cultured for 5-days with IFN-γ (1000 pg mL^{-1}), and RT-PCR performed for (b) TRAP and Cathepsin K. Shown are expression levels of TRAP and Cathepsin K in HIV-1 Tg cells compared with non-Tg. Samples were analyzed in triplicate and data normalized to the 18S ribosomal RNA. (c) Non-Tg and HIV-1 Tg OCP were cultured in a 20 ng mL^{-1} of M-CSF and stimulated with 100 ng mL^{-1} RANKL and IFN-γ for 24 h. TRAF6 and β actin were detected by Western blotting.

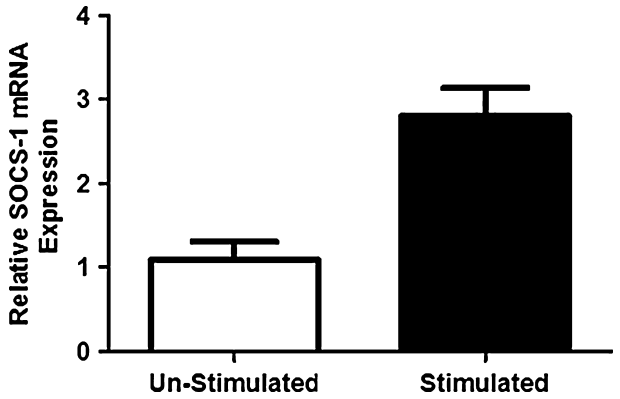

Fig. 5 SOCS-1 mRNA induction by RANKL in non-Tg control OCP. OCP from non-Tg controls stimulated with soluble (s) RANKL (50 ng mL^{-1}) for 5 h. Shown is the expression of SOCS-1 mRNA in control OCP. Levels of SOCS-1 mRNA were determined by real-time RT-PCR as described in Material and methods. Samples were analyzed in triplicate and data normalized to the expression of 18S ribosomal RNA.

et al., 2001). TNF-α has not, however, been implicated in increased RANKL expression in HIV-1 Tg rats (Vikulina *et al.*, 2010). The HIV-1 proteins Vpr and gp120 enhance expression of RANKL (Fakruddin & Laurence, 2003, 2005). We have previously shown elevated serum levels of gp120 in the HIV-1 Tg rat (Reid *et al.*, 2001); therefore, increased RANKL expression may be a consequence of the expression of this HIV-1 transgene protein. Relevantly, we have demonstrated that the HIV Tg rat expresses elevated levels of SOCS-1 and that IFN-γ treatment results in increased levels of TRAF6 and impaired suppression of osteoclastogenesis. Along with reduced production of IFN-γ by CD4+ T cells (Reid *et al.*, 2004; Yadav *et al.*, 2009) and increased RANKL expression, these results suggest that SOCS-1 amplifies the osteoclastogenic activity of RANKL, thereby enhancing bone loss in the HIV-1 Tg rat. In this model, IL-10 induction of SOCS-1 by CD4+ T cells and dendritic cells inhibits both LPS and IFN-γ signaling. Additionally, we reported that increased SOCS-1 expression by HIV-1-infected patients altered IFN-γ signaling by CD4+ T cells (Yadav *et al.*, 2009).

Here, consistent with previous findings, we demonstrate that similar to HIV-1-infected patients, the HIV-1 Tg rat undergoes pathological bone resorption. From a mechanistic viewpoint, we demonstrate that HIV-1 Tg rats have increased levels of the serum bone resorption protein CTx and higher expression of RANKL mRNA resulting in an increased ratio of RANKL to osteoprotegerin in PBMC and mononuclear cells isolated from the spleen. However, we did not observe a concurrent decrease in osteoprotegerin mRNA, as previously reported in bone marrow and total splenic cells (Vikulina *et al.*, 2010). We now report that HIV-1 Tg rat OCP express higher levels of SOCS-1 and TRAF6 which in conjunction with elevated RANKL expression enhances osteoclastogeneis and resistance to the suppression of osteoclast differentiation by IFN-γ signaling. The molecular mechanism of suppression of RANK signaling by IFN-γ has not been clarified; Takayanagi *et al.* (2000) have demonstrated that IFN-γ induces the ubiquitination and degradation of TRAF6 by a STAT1-depedent mechanism in murine cells while Ji *et al.* (2009) demonstrated inhibition of

RANK expression and osteoclastogenesis by TLR-4 and IFN-γ-signaling synergy, likely through a down-regulation of c-Fms expression. In conclusion, our data suggest a link between high levels of SOCS-1 by OCP and enhanced RANK signaling and resistance to IFN-γ-induced suppression of osteoclastogenesis. Understanding the mechanisms of HIV-1-induced bone loss and the role played by over-expression of SOCS-1 will be critical for early detection of changes in BMD and in developing effective therapy. We speculate that elevated SOCS-1 levels may be predictive for reduced BMD and an increased likelihood of HIV-1 induced fragility fractures.

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Authors' contribution

W.R., N.W., and M.K.L. conceived the study. W.R. and M.K.L. designed the experiments, performed the experiments, analyzed the data and wrote the manuscript. All coauthors interpreted the data and reviewed the manuscript.

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Supporting Information

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

Fig. S1. Increased SOCS-1, RANKL and osteoprotegerin ratio in HIV-1 Tg rat splenic mononuclear cells.

Fig. S2. (a) Representative flow cytometry dot plots of non-Tg and HIV-1 Tg OCP co-stained with CD11b and RANK. (b) The frequency of RANK on non-Tg and HIV-1 Tg OCP. (c) Proliferation was monitored [^{3}H]-thymidine incorporation in RANKL treated non-Tg and HIV-1 Tg OCP.