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IL-17 Mediates Estrogen-Deficient Osteoporosis in an Act1-Dependent Manner

Carl J. DeSelm,[1,2] Yoshifumi Takahata,[1] Julia Warren,[1,2] Jean C. Chappel,[1] Taimur Khan,[1] Xiaoxia Li,[3] Caini Liu,[3] Yongwon Choi,[4] Youngmi Faith Kim,[4] Wei Zou,[1] and Steven L. Teitelbaum[1,5*]

[1]*Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri*
[2]*Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri*
[3]*Department of Immunology, Cleveland Clinic, Cleveland, Ohio*
[4]*Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*
[5]*Division of Bone and Mineral Diseases, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri*

ABSTRACT

Estrogen-deficient osteoporosis may be an inflammatory disorder and we therefore asked if IL-17 participates in its pathogenesis. Deletion of the principal IL-17 receptor (IL-17RA) protects mice from ovariectomy (OVX)-induced bone loss. Further supporting a central role of IL-17 in its pathogenesis, OVX-induced osteoporosis is prevented by a blocking antibody targeting the cytokine. IL-17 promotes osteoclastogenesis by stimulating RANK ligand (RANKL) expression by osteoblastic cells, mediated by the IL-17RA SEFIR/TILL domain. Estrogen deprivation, however does not enhance IL-17RA mRNA expression by osteoblasts or in bone, but augments that of Act1, an IL-17RA-interacting protein and signaling mediator. Similar to IL-17RA$^{-/-}$ mice, those lacking Act1 are protected from OVX-induced bone loss. Also mirroring IL-17RA-deficiency, absence of Act1 in osteoblasts, but not osteoclasts, impairs osteoclastogenesis via dampened RANKL expression. Transduction of WT Act1 into Act1$^{-/-}$ osteoblasts substantially rescues their osteoclastogenic capacity. The same construct, however, lacking its E3 ligase U-box or its SEFIR domain, which interacts with its counterpart in IL-17RA, fails to do so. Estrogen deprivation, therefore, promotes RANKL expression and bone resorption in association with upregualtion of the IL-17 effector, Act1, supporting the concept that post-menopausal osteoporosis is a disorder of innate immunity. J. Cell. Biochem. 113: 2895–2902, 2012.

KEY WORDS: INTERLEUKIN 17; CYTOKINES; OSTEOPOROSIS; OSTEOCLAST/OSTEOBLAST BIOLOGY

Periarticular osteolysis is a crippling complication of rheumatoid arthritis. As in all forms of pathological bone loss, the osteoclastogenesis of this disorder requires RANK ligand (RANKL). Inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-1, also promote osteoclast recruitment via enhanced RANKL expression by osteoblasts and related cells. Additionally, TNF and IL-1 synergize with the osteoclastogenic cytokine to directly stimulate precursor differentiation into the mature resorptive polykaryon [Lam et al., 2000; Wei et al., 2005].

For many years, it had been assumed that Th1 CD4$^+$ T cells, which produce pro-inflammatory cytokines, fuel osteoclast recruitment in rheumatoid joints. This concept has been challenged by discovery of the Th17 subset of CD4$^+$ lymphocytes and their product, IL-17. In fact, IL-17 is both necessary and sufficient to promote inflammatory osteolysis [Lubberts et al., 2001; Nakae et al., 2003; Koenders et al., 2006].

Bone loss attends menopause because estrogen insufficiency stimulates formation of both osteoblasts and osteoclasts, with the

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*Correspondence to: Dr. Steven L. Teitelbaum, MD, Department of Pathology and Immunology, Washington University School of Medicine, Campus Box 8118, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail: teitelbs@wustl.edu

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latter predominating. Despite the demographic significance of post-menopausal osteoporosis, the mechanisms by which estrogen deficiency promotes osteoclastogenesis are controversial. Interestingly, estrogen-deficient osteoporosis exhibits features of an immune inflammatory disease. This conclusion is derivative of observations that post-menopausal, osteoporotic women have deranged distribution of their T-cell subsets and elevated serum concentrations of inflammatory cytokines, which promote osteoclast differentiation of bone marrow macrophages (BMMs) [D'Amelio et al., 2008]. These findings evolved into a model wherein estrogen deficiency enhances T-cell reactivity to a pool of self and foreign antigens, physiologically present in healthy individuals [Pacifici, 2010]. The antigen-activated T cells expand following estrogen deprivation, and in so doing, express TNF, which stimulates stromal cells and osteoblasts to produce osteoclastogenic cytokines, particularly RANKL. While controversial [Lee et al., 2006], some investigators report immune-compromised mice and those lacking TNF or its receptor are protected from ovariectomy (OVX)-induced osteoporosis [Cenci et al., 2000; Roggia et al., 2001].

With this in mind, we posited that IL-17, which is central to the periarticular bone loss attending severe inflammation, may also participate in the pathogenesis of estrogen-deficient osteoporosis. This hypothesis is strengthened by the synergy between TNF and IL-17 in promoting intracellular signaling [Miossec, 2003]. We find that mice lacking the principal IL-17 receptor (IL-17RA) or its effector, Act1, are protected from the skeletal effects of OVX. In keeping with a central role of IL-17 in the pathogenesis of post-menopausal osteoporosis, OVX-induced bone loss is also prevented by an antibody targeting the cytokine. IL-17RA and Act1 are essential for IL-17-stimulated osteoclastogenesis and each exerts its effect by promoting RANKL production by osteoblast lineage cells. These observations support the concept that post-menopausal bone loss is a disorder of innate immunity and anti-inflammatory agents may retard its progression.

MATERIALS AND METHODS

MICE

All animals were housed in the animal care unit of the Washington University School of Medicine and were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques.

REAGENTS AND PLASMIDS

The following reagents and suppliers were used: IL-17 (R&D Systems), 1,25-dihydroxyvitam D3 (Biomol), collagenase type 2 (Worthington Biochemical), monoclonal anti-human IL-17 blocking antibody (Dendritics Clone 408H6.01) [Tajima et al., 2008]. IL-17R [Maitra et al., 2007] and Act1 [Liu et al., 2009; Swaidani et al., 2009] mutant constructs were described previously.

QUANTITATIVE RT-PCR

For ex vivo RNA analysis, bone marrow was removed from femora by centrifugation. The remaining bone was frozen in liquid nitrogen and pulverized using a Braun Mikrodismembrator (Sartorius BBI Systems Inc., Bethlehem, PA) in an RNAase-free environment. RNA was extracted using Trizol (Invitrogen) following manufacturer's protocol. Quantitative PCR was performed using TaqMan probes to IL-17RA (Invitrogen, Mm00434214) Act1 (Invitrogen, Mm00506094), RANKL (Invitrogen, Cat #4331182) and as a control, Cyclophillin (Invitrogen) or GAPDH as described.

CALVARIA OSTEOBLAST ISOLATION AND CO-CULTURE

Osteoblasts were mobilized from calvaria of E14 fetuses by 3 min × 20 min of collagenase treatments at 37°. BMMs were maintained in M-CSF for 4 days prior to being lifted and then co-cultured with osteoblasts for 5–6 days in α-MEM media containing pen/strep, 10% FBS, and 10 nM 1,25 dihyroxycholecalciferol, ±IL-17. Prior to osteoclast fixation and TRAP staining, osteoblasts were removed with collagenase.

RETROVIRAL TRANSDUCTION

IL-17R constructs were cloned into the pMXs-IRES-BSR retroviral vector (Cell Biolabs, Inc., San Diego, CA) and ACT1 constructs were cloned into the pMSCV-GFP retroviral vector. Empty vector was used as controls. Plat-E retrovirus packaging cells were purchased from Cell Biolabs, Inc. and maintained in DMEM media (Cellgro, Manassas, VA) containing 10% fetal calf serum (Hyclone, Waltham, MA), and 2 mM L-glutamine (Gibco, Carlsbad, CA). Retroviral transduction was performed as previously described [DeSelm et al., 2011]. Briefly, 10 μg of retroviral DNA was transfected into Plat-E cells using the Fugene HD transfection reagent (Roche, Basel, Switzerland). Plat-E media was replaced 24 h later and virus harvested at 48 and 72 h for transduction of 50% confluent osteoblasts. At 96 h, the transduction media was replaced with alpha10 and selected with antibiotic. After 5 days of selection, cells were harvested and plated for experiments, at which time non-transduced control cells were killed by the antibiotic.

MICROCOMPUTED TOMOGRAPHY AND OVARIECTOMY

Trabecular volume in the distal femoral metaphysis (right leg) was measured using in vivo microcomputed tomography (vivaCT 40, Scanco Medical, Brüttisellen, Switzerland), while the mice were anesthetized with isofluorane. A threshold linear attenuation coefficient of 1.2 cm^{-1} was used to differentiate bone from non-bone. A threshold of 220 was used for evaluation of all scans. Thirty slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible. Measurements were made 14 and/or 28 days after sham operation or OVX. Mice were 8-week old at time of OVX. Sham operated mice were anesthetized and similarly incised but ovaries were not removed.

HISTOLOGY

Tibias were harvested, cleaned of soft tissue, and fixed in 10% buffered formalin overnight, then rinsed in water then incubated in 14% EDTA (pH 7.2) for 7 days, changing the solution after 3.5 days. Following decalcification, tibias were rinsed in water then incubated in an ethanol gradient (20%, 30%, 50%, and 70% ethanol, for 30 min each). Bones were then embedded in paraffin, cut into 4 μm longitudinal sections, and stained with TRAP or hematoxylin and

eosin for visualization of osteoclasts or osteoblasts, and measurements were made using BioQuant software (Bioquant; Nashville, TN).

SERUM CTX MEASUREMENTS

Blood was collected by cheek puncture after 6 h starvation. Plasma was obtained using plasma separator tubes with lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Serum CTx-I, a specific marker of osteoclastic bone resorption, was measured using a RatLaps ELISA kit from Nordic Bioscience Diagnostics A/S (Herlev, Denmark).

STATISTICS

All data were analyzed with Graphpad Prism 5.0d software for Macintosh (Graphpad, San Diego, CA), using two-tailed unpaired Student's *t*-test. Error bars represent standard deviation.

RESULTS

IL-17 MEDIATES ESTROGEN-DEFICIENT OSTEOPOROSIS

To determine if IL-17 mediates estrogen-deficient osteoporosis, we subjected WT mice and those lacking the IL-17RA, to OVX or sham surgery. Four weeks of estrogen deficiency substantially diminished trabecular bone in WT mice whereas those lacking IL-17RA were completely protected, whether determined by μCT or histomorphometry (Fig. 1A,B). Confirming that IL-17RA depletion spares estrogen-deficient bone by inhibiting osteoclastic resorption, serum CTx was elevated in WT OVX mice but not those lacking the receptor (Fig. 1C).

These data suggest IL-17 mediates OVX-induced bone loss. This being the case, one would expect reduced IL-17 to protect against experimental post-menopausal osteoporosis. To address this issue we selected an anti-human IL-17 mAb for its ability to block murine IL-17-induced osteoclastogenesis in murine co-cultures of WT osteoblasts and osteoclast precursors, in the form of BMMs (not shown). We administered the antibody or isotype control to sham operated or OVX WT mice. Two weeks following OVX, trabecular bone was significantly diminished in control mice whereas those receiving anti-IL-17 mAb experienced no such change (Fig. 2A). These differences were more dramatic after 4 weeks wherein an approximate 40% decline in control OVX animals was completely abrogated by the antibody (Fig. 2B).

THE IL-17RA SEFIR/TILL DOMAIN IS NECESSARY FOR OSTEOBLAST-INDUCED OSTEOCLAST FORMATION

Having established IL-17RA mediates estrogen-deficient osteoporosis we turned to the osteoclastogenic components of the receptor's intracellular domain. While controversial, IL-17 likely exerts its osteoclastogenic properties by stimulating RANKL and M-CSF production by osteoblast lineage cells [Sato et al., 2006; Adamopoulos et al., 2010]. We confirmed that the cytokine does not directly promote osteoclast formation, as it has no effect on M-CSF and RANKL-stimulated differentiation of pure populations of precursor cells (Fig. 3A). Therefore, to determine the components of IL-17RA mediating osteoclastogenesis, we transduced IL-17RA$^{-/-}$ osteoblasts with WT and mutated constructs of the receptor's 520 amino acid cytoplasmic tail (Generously provided by Dr. Sarah Gaffen; Fig. 3B). Each construct binds ligand confirming conformational integrity [Sato et al., 2006]. The IL-17RA$^{-/-}$ transductants were co-cultured with WT BMMs, and osteoclasts identified by tartrate resistant acid phosphatase (TRAP) activity 5 days later. IL-17 equally stimulates the osteoclastogenic capacity of WT osteoblasts and IL-17RA$^{-/-}$ cells expressing the WT construct (Fig. 3C). Vector-bearing IL-17RA$^{-/-}$ osteoblasts, however, are incapable of generating osteoclasts in response to the cytokine.

In other circumstances, IL-17RA's SEFIR and overlapping Toll/IL-1R (TIR)-like loop (TILL) motifs are essential for its activation [Maitra et al., 2007]. Similarly, IL-17RA lacking its SEFIR domain (IL-17RA$^{\Delta SEFIR}$) fails to rescue the osteoclast-stimulating capacity of IL-17RA$^{-/-}$ osteoblasts. IL-17RA$^{-/-}$ osteoblasts bearing IL-17RA$^{\Delta 527}$, which deletes the C-terminal 337 amino acids including the distal SEFIR and entire TILL domains, also generate no osteoclasts in response to IL-17. In contrast, normal IL-17-mediated osteoclast formation occurs in co-cultures containing IL-17RA$^{-/-}$

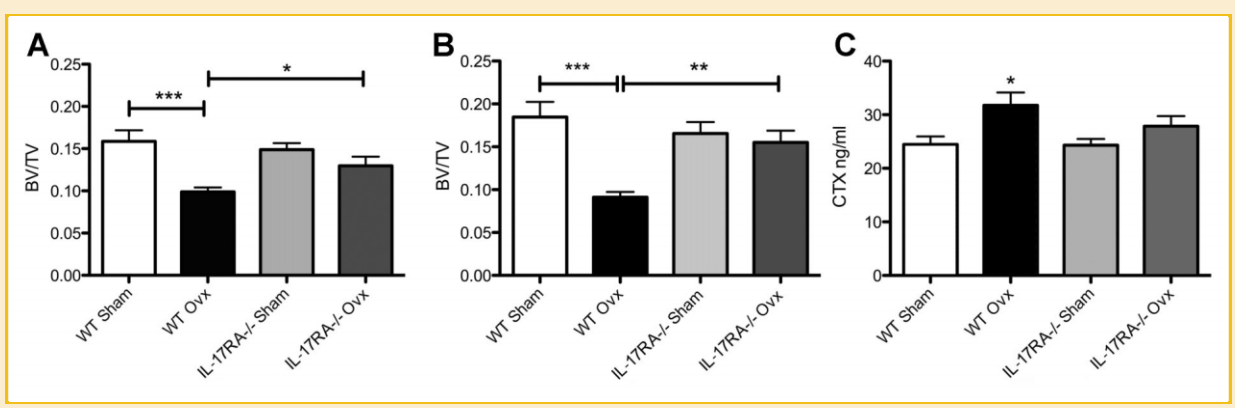

Fig. 1. IL-17 mediates estrogen-deficient osteoporosis. A,B: WT or IL-17RA$^{-/-}$ mice were subjected to sham operation or OVX. Four weeks later trabecular bone volume was determined by (A) μCT or (B) histomorphometry. C: Serum collected, at sacrifice, from mice described in (A and B) was analyzed for CTx content ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

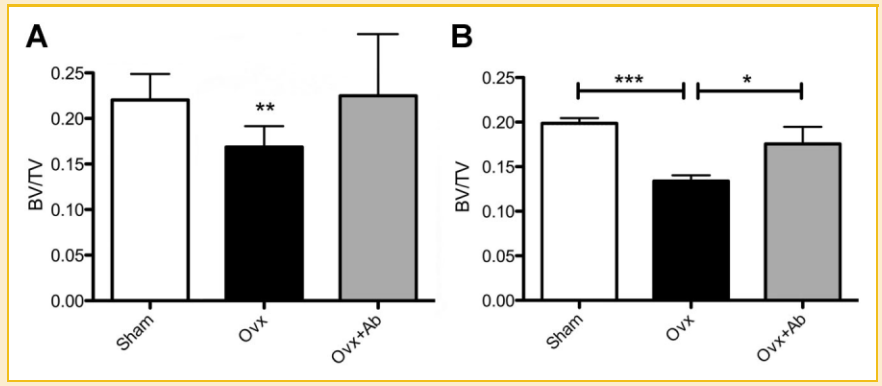

Fig. 2. Anti-IL-17 mAb prevents OVX-induced osteoporosis. WT mice were subjected to sham operation or OVX. OVX animals were injected with anti-human IL-17 mAb (37 μg IP every 3 days) or isotype control. A: Two and (B) 4 weeks later trabecular bone volume was sequentially determined in the same mice by viva μCT ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

osteoblasts bearing IL-17RA^{R549A}, which forms a salt bridge within the TILL motif. Hence, the SEFIR/TILL domain governs IL-17RA-stimulated osteoclast induction by osteoblasts in a TILL salt bridge-independent manner. Importantly, parallel expression of RANKL occurs under the influence of IL-17 by the same transductants and WT IL-17RA completely restores the RANKL-producing capacity of receptor-deficient osteoblasts, which requires a functional SEFIR/TILL domain (Fig. 3D).

ACT1 MEDIATES ESTROGEN-DEFICIENT OSTEOPOROSIS

Act1 is an adaptor protein required for IL-17's induction of inflammatory cytokine expression [Qian et al., 2007]. It is also the means by which TRAF6 is recruited to the receptor's cytoplasmic domain [Liu et al., 2009]. A number of IL-17-mediated biological events are, however, independent of Act1 [Linden, 2007].

This being the case we asked if Act1 regulates IL-17-mediated osteoclastogenesis. Similar to their IL-17RA$^{-/-}$ counterparts, Act1-deficient BMMs undergo normal osteoclast differentiation when exposed to RANKL and M-CSF (Fig. 4A). In contrast, osteoblasts lacking the adaptor protein are incapable of increased osteoclastogenesis in response to IL-17 (Fig. 4B).

Act1 also contains a SEFIR motif [Novatchkova et al., 2003], which recognizes its equivalent in IL-17RA. To determine if this region of Act1 participates in osteoclast formation, osteoblasts lacking the docking protein were transduced with WT Act1 and that devoid of its SEFIR domain located at its C-terminal (Act1$^{\Delta SEFIR}$;

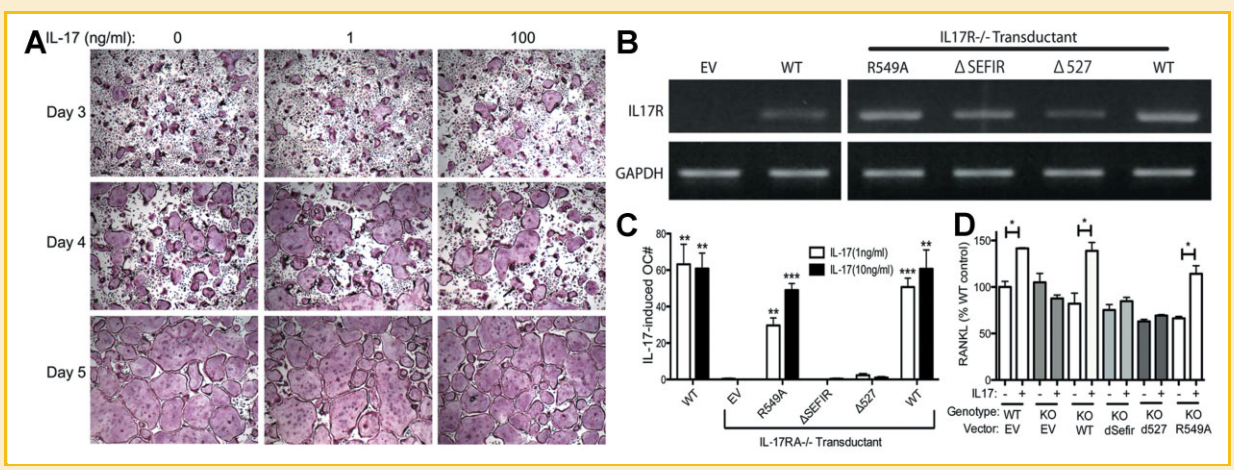

Fig. 3. The IL-17RA SEFIR/TILL domain is necessary for osteoblast-induced osteoclast formation. A: Bone marrow macrophages were cultured in the presence of RANKL (100 ng/ml), M-CSF (20 ng/ml), and increasing concentrations of IL-17. The cells were stained for TRAP activity with time. B: Expression of IL-17RA construct mRNA in transduced IL-17RA$^{-/-}$ osteoblasts determined by RT-PCR. Naïve WT cells serve as positive, and empty vector (EV) as negative controls. GAPDH serves as loading control. C: IL-17RA$^{-/-}$ osteoblasts, transduced with specified IL-17RA constructs were co-cultured with WT BMMs with increasing amounts of IL-17. WT osteoblasts cultured with WT BMMs serve as control. After 5 days the cells were stained for TRAP activity and osteoclasts counted. Data are expressed as the number of osteoclasts present in IL-17-containing, minus IL-17-free cultures. Statistical significance is relative to empty vector (EV). D: Osteoblasts detailed in (C) were exposed to IL-17 (10 ng/ml) (+) or carrier (−). After 48 h RANKL mRNA was quantified by qPCR. GAPDH serves as loading control. Data are normalized to WT/EV(−) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

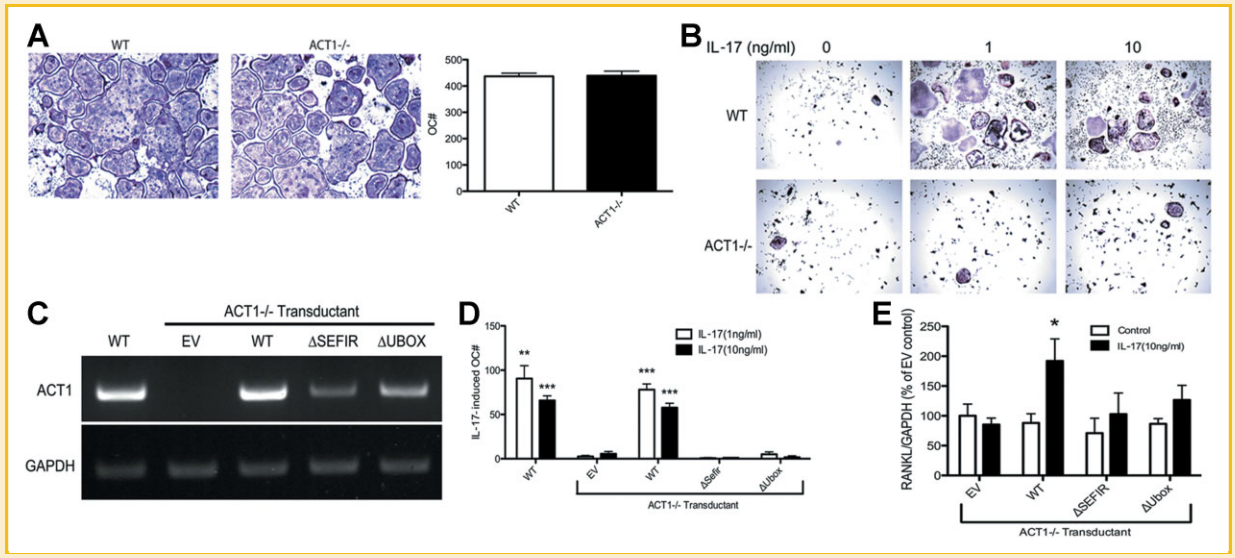

Fig. 4. A: Act1 mediates estrogen-deficient osteoporosis. WT and Act1$^{-/-}$ BMMs were cultured in RANKL (100 ng/ml) and M-CSF (20 ng/ml). After 5 days the cells were stained for TRAP activity and counted. B: WT or Act1$^{-/-}$ osteoblasts were cultured with WT BMMs. After 5 days the cells were stained for TRAP activity. C: Expression of Act1 construct mRNA in transduced Act$^{-/-}$ osteoblasts determined by RT-PCR. Naïve WT cells serve as positive and EV as negative controls. GAPDH serves as loading control. D: WT or Act1$^{-/-}$ osteoblasts transduced with specified Act1 constructs or EV were cultured, ±IL-17, with WT BMMs. After 5 days, the cells were stained for TRAP activity and counted. Data are expressed as the number of osteoclasts present in IL-17-containing, minus IL-17-free cultures. Statistical significance is relative to IL-17-free cultures. E: Act1$^{-/-}$ osteoblasts transduced with specified Act1 constructs or EV were treated with IL-17 (10 ng/ml). After 48 h RANKL mRNA was quantified by qPCR. GAPDH serves as loading control. Data are normalized to EV control. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Fig. 4C) [Swaidani et al., 2009]. Like its counterpart in IL-17RA, Act1$^{\Delta SEFIR}$-brearing osteoblasts fail to induce osteoclast formation in response to IL-17 (Fig. 4D). While IL-17 enhances RANKL expression by Act1$^{-/-}$ cells transduced with WT Act1, absence of the SEFIR domain obviates such rescue (Fig. 4E). Thus, IL-17 only promotes osteoclast formation, indirectly, stimulating RANKL expression by osteoblastic cells via its key effector, Act1.

In addition to being a docking protein, Act1, via its U Box, is an E3 ubiquitin ligase [Liu et al., 2009]. To determine if Act1 requires its ubiquitinating capacity to promote osteoclast formation, we generated co-cultures containing WT BMMs and Act1$^{-/-}$ osteoblasts transduced with WT or U-Box-deleted Act1 [Liu et al., 2009]. As seen in Figure 4D, Act-1's U Box is indispensable for IL-17-stimulated, osteoblast-mediated, osteoclast differentiation, and RANKL expression.

These data raise the possibility that estrogen exerts its bone-sparing effects, at least in part, by suppressed expression of IL-17 effectors, such as Act1. To determine if such is the case, we first established that estrogen supplementation does not significantly affect expression of IL-17RA and Act1 mRNA by WT osteoblasts (Fig. 5A,B). We then exposed osteoblasts to estrogen for 5 days followed by 5 days in the absence of the steroid. As control, the osteoblasts were maintained in the presence of the steroid for the entire 10 days of culture. Estrogen withdrawal does not affect IL-17RA mRNA expression but increases that of Act1 (Fig. 5C,D). To determine physiological relevance, we extracted RNA from bone of OVX and sham-operated WT mice. In keeping with our in vitro observation, estrogen deprivation in vivo, significantly enhances Act1 mRNA but not that of IL-17RA (Fig. 5E,F).

Because these experiments suggest Act1 mediates IL-1RA's osteoclastogenic properties, we asked if absence of the adaptor protein also inhibits post-menopausal bone loss. Whereas WT mice lose approximately 40% of trabecular bone within 4 weeks of OVX, those lacking Act1 are unaffected (Fig. 6). Thus, IL-17 promotes estrogen-deficient osteoporosis in an IL-17RA/Act1-dependent manner.

DISCUSSION

As in inflammatory osteolysis, TNF and T cells may be central to the development of post-menopausal osteoporosis [Ammann et al., 1997; Kimble et al., 1997; Cenci et al., 2000; Roggia et al., 2001]. In fact, the perceived relationship between T-cell-produced TNF and increased RANKL expression provided the principal foundation for immune regulation of the skeleton in the context of estrogen deprivation. While it synergizes with TNF, IL-17, has emerged as a key mediator of systemic bone loss in the context of inflammation. The cytokine is produced not only by the Th17 subset of CD4+ T cells but also the other members of the immune system including a subset of NK and γδ T cells [Gaffen, 2009].

Evidence of shared immune mechanisms governing inflammatory and estrogen-deficient osteolysis raised the possibility that IL-17 also mediates post-menopausal bone loss. This hypothesis assumes that estrogen's anti-inflammatory properties include suppressing the effects of IL-17 [Straub, 2007]. This issue is clinically relevant, as IL-17 is a current therapeutic target.

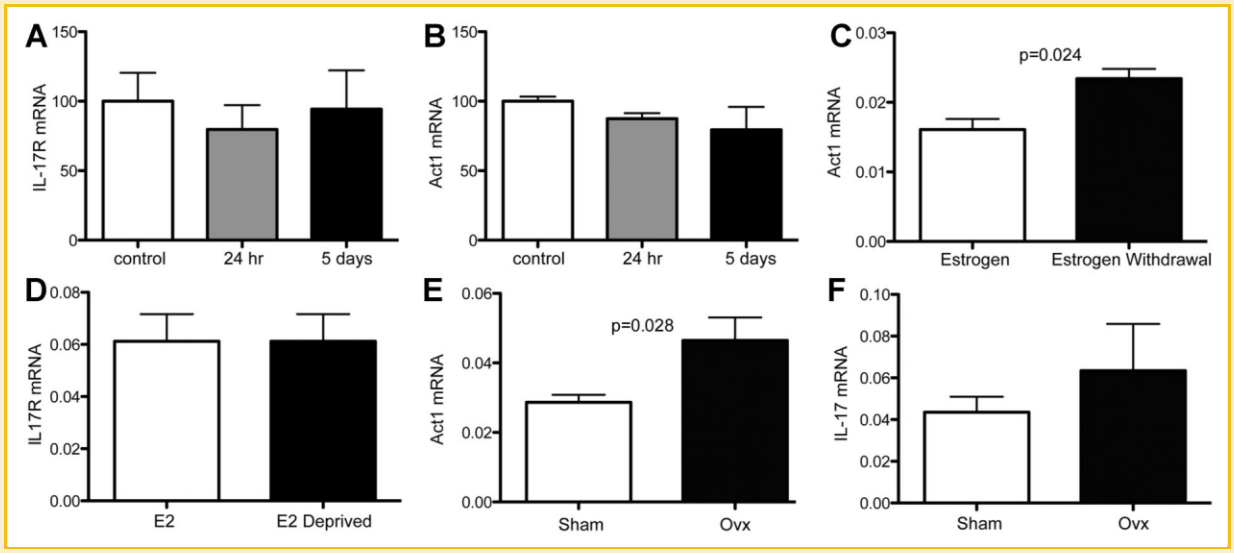

Fig. 5. Estrogen deprivation promotes Act1 expression. A,B: WT osteoblasts, cultured in phenol-free medium containing charcoal-stripped serum, were exposed to estrogen (10 nm) with time. A: Act1 and (B) IL-17RA mRNA were measured by qPCR and expressed as percent of control. C,D: WT osteoblasts were cultured for 10 days. Estrogen was added for the entire culture period (E2) or for only the first 5 days (E2 withdrawal). C: Act1 and (D) IL-17 mRNA was determined by qPCR. E,F: RNA of bone of OVX or sham operated mice was extracted 2 weeks after surgery. E: Act1 and (F) IL-17RA mRNA was determined by qPCR. Cyclophillin serves as loading control ($^{*}P < 0.05$).

To determine if IL-17 participates in the pathogenesis of post-menopausal osteoporosis, we first utilized receptor-deleted mice. There are five known IL-17 receptor isoforms but IL-17RA, in conjunction with IL-17RC, is the principal entity mediating the cytokine's inflammatory properties. Hence, mice lacking IL-17RA are protected from severe inflammatory diseases, including those which are bone destructive. Substantiating that the receptor is central to the pathogenesis of post-menopausal osteoporosis, its absence completely ablates the substantial bone loss occurring within 4 weeks of OVX and precludes the consequent rise in the collagen degradation product, CTx. Thus, arrest of IL-17 signaling prevents estrogen-deficient osteoporosis by retarding osteoclastic resorption. The cytokine's stimulatory effect on osteoblast precursor proliferation and differentiation indicates that impaired osteogenesis does not contribute to the low bone mass phenotype [Huang et al., 2009]. Most importantly, the bone-sparing effects of IL-17 receptor deletion are mirrored by a cytokine-blocking antibody, providing translational implications to these observations.

A previous study unexpectedly found IL-17R-deficient mice lose more whole-body bone density after OVX than do WT controls [Goswami et al., 2009]. While we cannot explain the discrepancy with our data it may reflect the use of DEXA versus μCT to determine bone mass. DEXA measurements of bone density are influenced by fat distribution [Hangartner and Johnston, 1990; Formica et al., 1995; Tothill et al., 1997, 1999; Wren et al., 2007], which is potentially affected by IL-17 [Goswami et al., 2009; Shin et al., 2009; Sumarac-Dumanovic et al., 2009]. Our conclusions are also supported by the multiple approaches we used to test the role of the IL-17 signaling cascade in the pathogenesis of post-menopausal osteoporosis. We find that not only does blockade of the cytokine protect skeletal mass but also the same obtains with elimination of its receptor and a key effector molecule.

There is substantial evidence that estrogens regulate immunity [Liu et al., 2003]. These steroids, however, may be stimulatory or inhibitory depending upon cell type and circumstance [Pernis, 2007]. Thus, whether the skeletal deficiency of menopause is an inflammatory disorder is controversial. Because IL-17 links innate and adaptive immunity, our observation that the cytokine initiates a signaling cascade necessary for estrogen-deficient bone loss buttresses the concept that post-menopausal osteoporosis has an autoimmune foundation. On the other hand, if post-menopausal osteoporosis is an autoimmune disease, the magnitude of inflammation is substantially less than that attending classical inflammatory osteolysis. Because IL-17 signaling has been documented to produce bone loss only in the context of severe inflammation, the fact that it is an essential component of estrogen-deficient osteoporosis is surprising.

While TNF and IL-17 partner in promoting inflammation, the means by which they stimulate osteoclastogenesis differ. TNF enhances the number of bone degrading polykaryons in a bi-functional manner. When present in abundance, as in rheumatoid joints, it directly targets osteoclast precursors to accelerate their differentiation, and also stimulating RANKL and M-CSF production by osteoblast lineage cells [Lam et al., 2000; Kitaura et al., 2004]. Less copious amounts of TNF, however, are incapable of directly activating osteoclast precursors and exert their effects, only indirectly, by promoting production of osteoclastogenic cytokines. Despite the fact that TNF expression by macrophages is stimulated by IL-17, the latter cytokine appears to enhance osteoclast number only by targeting RANKL-producing stromal cells. In keeping with

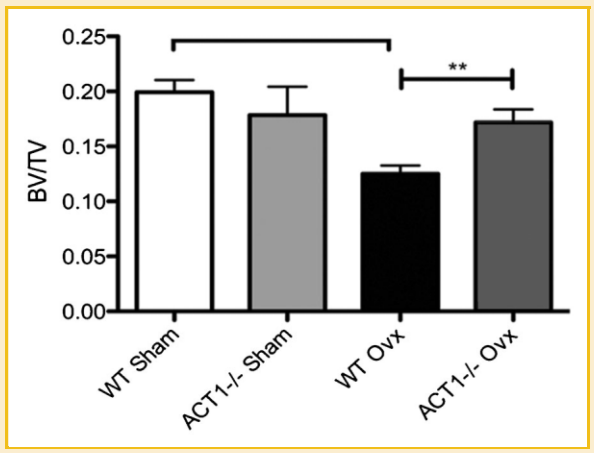

Fig. 6. Act1 mediates estrogen-deficient osteoporosis. WT or IL-Act1$^{-/-}$ mice were subjected to sham operation or OVX. Four weeks later trabecular bone volume was determined by μCT ($^{**}P < 0.01$).

this conclusion, RANKL gene activation parallels the magnitude of osteoclastogenesis mediated by all IL-17RA mutant constructs expressed in IL-17RA-deficient osteoblasts.

IL-1 and lipopolysaccharide (LPS) are osteoclastogenic and their receptors share a common TIR sequence. IL-17 receptors contain an intracellular SEFIR domain, homologous to the TIR motif, a docking site for adaptor proteins [Maitra et al., 2007]. A distal component of the SEFIR domain, known as TILL, is unique to IL-17RA and in other circumstances, its mutation disables the receptor's activity. The TILL-residing amino acid, R549, generates a key salt bridge in the homologous TIR domain. Recently, Onishi et al. [2010] established that signals emanating from IL-17RA require a large extension distal to the TILL component of the SEFIR domain. While deletion of the distal SEFIR and its entire TILL sequence, in IL-17RA, forestalls IL-17-stimulated osteoclastogenesis and RANKL expression by osteoblasts, the process occurs independently of R549 and its putative salt bridge formation. This observation is in keeping with the fact that IL-17RA^{R549A} does not impair IL-17/TNF-induced signaling in transformed cells [Maitra et al., 2007]. Thus, while the LPS, IL-1 and IL-17 are each osteoclastogenic, homologous components of their receptors may function differently.

Act1 is an adaptor protein which activates NF-κB and is central to the inflammatory signals emanating from IL-17RA [Qian et al., 2007]. In keeping with Act1's role as an IL-17 effector, its deficiency does not retard differentiation of osteoclast precursors directly, but by arresting osteoblast lineage cell RANKL expression. Moreover, like those deleted of IL-17RA, Act1$^{-/-}$ mice are protected from estrogen-deficient osteoporosis. The fact that estrogen withdrawal enhances Act1 mRNA abundance and not that of IL-17RA or circulating IL-17 [Yasui et al., 2008] underscores the likely importance of the docking protein in post-menopausal osteoporosis. Act1's essential role in IL-17-stimulated osteoclast formation is mediated by its own SEFIR domain, which interacts with its counterpart in IL-17RA. Thus, absence of the SEFIR motif in either the receptor or docking protein arrests IL-17 induced osteoclast formation by suppressing RANKL expression. In keeping with the centrality of IL-17RA and Act1 SEFIR domains, a decoy peptide which blocks their interaction attenuates IL-17 induced signaling [Liu et al., 2011]. Importantly, the same construct inhibits pulmonary inflammation, in vivo, raising the possibility a similar strategy will retard post-menopausal osteoporosis.

RANKL-stimulated osteoclast formation requires TRAF6 recruitment to RANK in osteoclast lineage cells. In this circumstance, TRAF6, which has an N-terminal RING domain common to many E3 ubiquitin ligases, undergoes autoubiquitination. It then polyubiquitinates IKK, via Lys63, thus activating NF-κB [Deng et al., 2000; Wang et al., 2001]. Autoubiquitinated TRAF6 also mediates RANK activation of the key osteoclastogenic transcription factor, NFATc1 [Darnay et al., 1999]. Act1 contains binding sites for TRAF6 which it recruits to the IL-17RA complex with cytokine stimulation. Similar to its interaction with RANK, TRAF6 is also polyubiquitinated upon Act1 recognition leading to recruitment of TAK1 and subsequent activation of NF-κB. Whereas RANK-associated TRAF6 is auto-ubiquitinated, however, Act1 is the E3 ligase in the context of the IL-17RA complex [Liu et al., 2009]. Furthermore, deletion of the Act1 U-box, which is the protein's ubiquitinating domain, dampens RANKL expression by osteoblasts and their osteoclastogenic capacity. Although speculative, these observations raise the possibility that TRAF6, in addition to promoting osteoclast formation cell-autonomously, upon RANK activation, does so by stimulating RANKL expression by osteoblast lineage cells.

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