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Ovariectomy Activates Chronic Low-Grade Inflammation Mediated by Memory T Cells, Which Promotes Osteoporosis in Mice

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Abstract

The loss of estrogen (E_2) initiates a rapid phase of bone loss leading to osteoporosis in one-half of postmenopausal women, but the mechanism is not fully understood. Here, we show for the first time how loss of E_2 activates low-grade inflammation to promote the acute phase of bone catabolic activity in ovariectomized (OVX) mice. E_2 regulates the abundance of dendritic cells (DCs) that express IL-7 and IL-15 by inducing the Fas ligand (FasL) and apoptosis of the DC. In the absence of E_2, DCs become long-lived, leading to increased IL-7 and IL-15. We find that IL-7 and IL-15 together, but not alone, induced antigen-independent production of IL-17A and TNFα in a subset of memory T cells (T_{MEM}). OVX of mice with T-cell–specific ablation of *IL15RA* showed no IL-17A and TNFα expression, and no increase in bone resorption or bone loss, confirming the role of IL-15 in activating the T_{MEM} and the need for inflammation. Our results provide a new mechanism by which E_2 regulates the immune system, and how menopause leads to osteoporosis. The low-grade inflammation is likely to cause or contribute to other comorbidities observed postmenopause.

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Authors' roles: RA conceived and designed experiments with advice from AB, DV, and ACS. RA and ACS collated all data. Data was collected by ACS, AA, E.S., M.C., J.S., P.P., and M.P. in a blinded manner. Micro-CT analysis was performed by L.C. who was blinded to genotype, treatments and surgery. R.A. wrote the manuscript with input from D.V. and A.B. R.A. takes responsibility for the integrity of the manuscript.

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

Disclosures

The authors confirm they have no conflicts of interest.

Introduction

Osteoporosis increases the risk of bone fracture after minimal trauma. Increase in circulatory system disease, malignant neoplasms, and dementia postfracture are a significant cause of mortality among the elderly.[1,2] It was recognized nearly eight decades ago that involutional osteoporosis in postmenopausal women is mediated by loss of estrogen (E_2).[3] Since the studies by Reifenstein and Albright,[4] several hypotheses were proposed for how aging and E_2 loss lead to reduced bone mass. Some reports suggested that decreased calcium absorption,[5,6] decline in renal function,[7] and impaired vitamin D metabolism,[8,9] along with aging and menopause, cause osteoporosis. In general, the early studies focused on the effect of E_2 on nutrient absorption and metabolism as a cause of osteoporosis. At homeostasis, during remodeling the activity of bone resorbing cells, osteoclasts, and bone forming cells, osteoblasts, are coupled to maintain skeletal mass.[10] At menopause women undergo an early rapid phase, followed by a slower and extended period of bone loss. In the early phase, more cancellous bone is lost, whereas in the slower phase both cortical and cancellous bone are equally eroded.[11–13] Here we propose a new model based on our data for the events that lead to the early phase of bone loss.

The finding four decades ago, that sex steroid receptors are expressed in human, rat, and mouse osteoclasts, osteoblasts, and growth plate chondrocytes indicated that sex hormones directly regulate these cells to maintain bone mass.[14] In detail, experiments showed that E_2 regulates the expression of the Fas ligand (FasL) in osteoclasts and osteoblasts inducing apoptosis in osteoclasts to limit bone resorption in premenopausal women.[15–18] At the same time E_2 extends the lifespan of osteoblasts and osteocytes[15,19,20] to favor bone formation. Therefore, E_2 loss leads to increased osteoclast numbers and concurrent decreased osteoblast numbers, in line with the notion that E_2 regulates the bone remodeling unit (BRU) directly. This paradigm led to drug treatments for osteoporosis that have to date also focused on restoring the balance between bone resorption and formation by targeting cells in the BRU. For instance, antiresorptives such as bisphosphonates[21] and denosumab[22] (anti-RANKL antibody) suppress osteoclasts, whereas teriparatide[23] ($PTH_{1–34}$) and romosozumab[24] (anti-sclerostin antibody) target osteoblasts.

Nearly two decades ago, the recognition of the effect of T-cell–produced cytokines on osteoclasts[25] led to coining of the term osteoimmunology.[26] Some cytokines, such as TNFα and IL-17A, lead to increased osteoclastogenesis.[27] Other cytokines, such as interferon-gamma (IFN-γ), suppress osteoclastogenesis.[25,28] Pioneering studies by Roggia and colleagues[29] showed that in mice, ovariectomy (OVX) leads to increased TNFα-producing T cells in the bone marrow. Furthermore, whereas postmenopausal women with normal bone density have undetectable levels of IL-17A, postmenopausal osteoporotic women have detectable levels of IL-17A in peripheral blood.[30] Together, these results suggest that low-grade inflammation promotes or is associated with bone loss in some postmenopausal women. Consistent with these findings, men and women with chronic inflammatory diseases such as rheumatoid arthritis (RA), Crohn’s disease, and some viral (ie, human immunodeficiency virus [HIV]) infections develop osteoporosis.[31–36] Despite these observations, questions as to the nature and the contribution of inflammation to postmenopausal osteoporosis have lingered. In some cases, how the immune system is

activated is starting to be unraveled. For instance, self-reactive T cells play a central role in the pathogenesis and pathophysiology of RA.[37,38] Although controversial, antigen presentation by myeloid dendritic cells (mDCs) appears to play an important role in initiating joint damage.[39] OVX-induced oxidative stress has been suggested as an activator of DCs.[40] However, the mechanism(s) of how TNFα and IL-17A are induced in T cells postmenopause has not been identified to date.

It is accepted that E_2 is an "anti-inflammatory" that works through a number of mechanisms to suppress inflammation.[41] The first Framingham study seven decades ago showed that premenopausal women were protected against osteoporosis and cardiovascular morbidity in comparison with age-matched men and postmenopausal women (or with oophorectomy).[42–44] Because osteoporosis often occurs in the sixth decade of life, age has been also suggested as the cause of skeletal frailty.[45] The molecular mechanisms by which E_2 regulates the immune system, and conversely, how menopause effects the immune system, have not been elucidated. Here we describe a novel and coherent pathway by which E_2 suppresses the immune system and, conversely, our studies show how E_2 loss promotes a low-grade inflammation by T cells that leads to osteoporosis.

We previously discovered that osteoclasts are antigen-presenting cells that cross-present antigens to CD8 T cells. Osteoclasts induce FoxP3, CD25, and CTLA4, as well as interferon (IFN)-γ, IL-6, IL-10, and IL-2 in the CD8 T cells that are immunosuppressive. These osteoclast-induced regulatory T cells (Tc_{REG}s) also suppress bone resorption by osteoclasts to form a negative feedback loop. The mechanism by which Tc_{REG}s suppress osteoclasts has been detailed by our laboratory, but the mechanism by which they suppress the immune system in OVX mice is not well understood. We recently initiated studies to reveal the immune cells that Tc_{REG}s target. In the process of discovering the targets of Tc_{REG}s we determined how the loss of E_2 by OVX leads to activation of memory T cells (T_{MEM}) to secrete TNFα and IL-17A, leading to a low-grade persistent inflammation that is the focus of this work.

Materials and Methods

Mice

C57BL/6J (model 000664), IL15RA-floxed (model 022365),[46] and Lck-Cre Tg540-I (model 006889)[47] mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in house. Breeding trio of IL-7^{CFP} reporter mice[48] were generously provided by Dr. Scott Durum (NIH-NCI, Bethesda, MD, USA).

OVX

Mice were randomly assigned to sham surgery or OVX groups. Each cage had at least one sham or one OVX mouse to minimize alterations of the microbiome between groups. Bilateral OVX was performed on 12-week-old mice. Mice were anesthetized using 2.5% isoflurane to initiate anesthesia, and 1% for maintenance. The ovaries were accessed through a single incision in the skin and exteriorized through the muscle wall on each side. Each ovary was clamped using a hemostat and removed by a single cut. Skin staples (3M) were

used to close the skin incision. To minimize discomfort postsurgery, three doses of 0.1 mg/kg buprenorphine HCl over2 days or a single dose of 1 mg/kg buprenorphine SR was administered subcutaneously. No adverse responses or effects were observed in any of the mice used in these experiments.

Animal use statement

All animals were maintained in the Department of Comparative Medicine, Saint Louis University School of Medicine in accordance with institutional and Public Health Service Guidelines. The mice were housed in microisolator caging, tested and found to be specific pathogen free (SPF). The mice used in experiments described in this study were maintained on rodent chow (Lab Diets cat# 5LOB). Saint Louis University School of Medicine Institutional Animal Care and Use Committee (IACUC) approved all procedures performed on mice (protocol numbers 2072 and 2184).

Antibodies and FACS

Anti-mouse antibodies for FACS were as follows: Brilliant violent (BV) 711-conjugated anti-mouse CD45 (clone 30-F11; BD Biosciences, San Jose, CA, USA); AF700-conjugated anti-mouse CD44 (IM7; BD Pharmingen, San Jose, CA, USA); BV605-conjugated CD62L (MEL-14; BD Biosciences); PE-Cy7-cojugated anti-CD3e (500A2; BioLegend, San Diego, CA, USA); R700-conjugated anti-CD8a (5H10; Caltag); anti-CD4 (RM4–5; BD Pharmingen), PE-conjugated TNFα (MP6-XT22; BD Biosciences); and V450-cojugated IL-17A (TC11–18H10; BD Biosciences). For FACS, cells were blocked with anti-mouse FcgRIII/IIR (Fc-block; BD Pharmingen) for 10 min and then stained for 45 min on ice with fluorophore-conjugated antibody. Stained cells were washed, fixed with 3% paraformaldehyde, and analyzed on LSRII instrument with CellQuest (BD Biosciences) software. Gates were determined using combination of single-color and fluor-minus-one controls. For live cell sorting, cells for isolation T_{MEM} or BMDCs (Fig. 2 through Fig. 4), bone marrow cells (BMCs) were first incubated with Fc-block for 5 min, then stained with fluor-conjugated antibodies against cell-surface proteins (see figure legends or text for details) for 30 min in PBS + 1% FBS (PBS + 1%), washed and resuspended in PBS + 1% for sorting on a FACS-DIVA (BD Biosciences) The sorted cells were collected directly in 15-mL tubes containing 4 mL RPMI +10% FBS to improve viability. Data analyses were performed with FlowJo software (version 8.73; FlowJo, LLC, Ashland, OR, USA).

Magnetic bead–aided cell sorting

CD45-positive bone cells were isolated using positive selection; the flow through was considered to comprise CD45-negative cells. Anti-CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat # 130-052-301) were used to isolate cells from single-cell suspension of BMCs. Cells were lysed and RNA was isolated (Agilent Technologies, Santa Clara, CA, USA).

Culturing of BMDC and memory T cells

After FAC sorting, both T-cells and BMDC were pelleted and resuspended in complete T-cell growth media (RPMI +10% heat-inactivated FBS + Penicillin + Streptomycin +2mM

glutamine +1mM sodium pyruvate that was supplemented with non-essential amino acids and 55μM β-mercaptoethanol; all from Sigma-Aldrich, St. Louis, MO, USA) and cocultured for 2 to 5 days, as indicated in the figure legends.

Immunofluorescence staining

Tibias were fixed in 10% neutral buffered formalin for 24 hours. The bones were washed briefly with water, placed in 70% ethanol, and submitted to Washington University Musculoskeletal Histology and Morphometry Core. Tibias were decalcified for 2 weeks in EDTA and embedded in paraffin, then sectioned (5 μm), mounted, and stored. Prior to immunofluorescence (IF) staining, slides were baked at 55°C for 2 hours, washed with Xylene, and decreasing concentrations of ethanol (100% to 30%) in Coplin jars. Citrate buffer was used for antigen retrieval and 30% BSA in PBS was used for block. The following primary antibodies were used: rabbit anti-mouse IL-7 (Abcam, Cambridge, MA, USA; cat# ab9732); rat anti-mouse IL-15 (eBioscience, Santa Clara, CA, USA; cat# MAB477), and Armenian hamster anti-mouse CD11c (Abcam; cat# ab33483). Binding of primary antibody was visualized using fluor-conjugated secondary antibodies: goat anti-rabbit-AF594; donkey anti-Armenian hamster-AF555; and chicken anti-rat-AF488 (for triple labeling) or Texas Red (in Fig. 1D). The slides were imaged, and cell counts were obtained using Image J/Fiji[49] (NIH, Bethesda, MD, USA; https://imagej.nih.gov/ij/) by an operator blinded to treatments. No region of interest was selected, instead a total of 96 fields, four randomly selected fields (using 10× objective) from each slide, and eight mice/group in two groups (sham or OVX) were used for quantitation.

TUNEL assay

Formalin-fixed, paraffin-embedded bone sections were TUNEL stained (In situ cell death detection kit TMR red) per the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Quantitation was performed as described for IF.

Cytokines

Recombinant murine IL-7 and IL-15 (PeproTech, Rocky Hill, NJ, USA; cat # 217–17 and 210–15, respectively) were used at 20 and 50 ng/mL, respectively. 17-β estradiol (Sigma-Aldrich; cat # E8875) powder was dissolved in DMSO and used in culture assays. 17β-estradiol pellets (Innovative Research of America, Sarasota, FL, USA; 0.1 mg/pellet 21 day-release; cat #SE-121) or placebo pellets (Innovative Research of America; cat# C-111) were implanted 3 days post-OVX subcutaneously using a 10G trocar.

CTX assay

Mice were fasted overnight prior to blood collection. Blood (100 to 200 μL), obtained via submandibular vein, was allowed to clot for 1 hour at room temperature and serum was collected by spinning down the cell pellet (1000*g* for 10 min). Serum was flash frozen and stored at −80°C. Serum C-terminal telopeptide of type 1 collagen (serum CTX) was measured using ELISA according to the manufacturer's instructions (Immunodiagnostic Systems, Gaithersburg, MD, USA; cat # AC-06F1)

Micro–CT data collection and analysis

Scanning settings and thresholding used for analysis were determined empirically to account for known age differences in bone microarchitecture. The bones, left proximal tibia, were scanned in μCT40 (Scanco Medical) at 55 kV, 72 μA, 4 W, at a resolution of 10 μm. Gauss sigma of 0.8, Gauss support of 1, lower threshold of 220 permilles for cancellous bone and 240 permilles for cortical bone, and upper threshold of 1000 permilles were used for all analyses. These thresholds correspond to 468.8, 592.2, and 3000 mg hydroxyapatite (HA)/cm^3. Regions of interest were selected 50 slices below the growth plate of the proximal tibia to evaluate the trabecular compartment. Bone mineral density was obtained by quantitative micro–CT (μCT) using Phantoms for calibration.[50] All μCT data was collected and analyzed by LC, who was blinded to the treatment performed.

Statistical analysis

Statistical significance (*p* values) was assessed in all cases using unpaired two-tailed Mann-Whitney *U* test in GraphPad Prism8.2 (GraphPad Software, Inc., La Jolla, CA, USA) because no assumption of normal distribution is assumed. ANOVA was also performed in Prism 8.2.

Results

Increased IL-7 and IL-15 by DCs in the bone marrow of OVX mice

To characterize the bone marrow environment, we harvested bone marrow cells from sham-operated and OVX mice and sorted cells into CD45-positive (hematopoietic origin) and CD45-negative populations. We analyzed the cytokine expression in each cell subset by quantitative PCR (qPCR). As shown in Fig. 1A, the number of cytokines were found to be upregulated in OVX mice relative to sham-operated mice, including IL-6, IL-7, IL-15, RANKL, and TNFα. These cytokines were upregulated in the Cytokine Super Array (Qiagen, Valencia, CA, USA). With the exception of TNFα, all of the cytokines are differentially increased in CD45+ cells. Although these five cytokines have been previously observed to increase in bone marrow cells of OVX animals and in serum from postmenopausal women,[51–56] the regulation of these cytokines by ovarian hormones has not been established. Furthermore, IL-7 and IL-15 play an important role in T-cell homeostasis and in the formation and reactivation of T_{MEM} responses. Data in Fig. 1A suggest that T_{MEM} may play an important role following loss of ovarian function, so we focused our attention on the source of IL-7 and IL-15.

To identify the source of IL-7, IL-7^{eCFP} reporter mice (cyanofluorescent protein [CFP])[48] were OVX (ovariectomized) or sham-operated and euthanized 10 days postsurgery. Bone marrow cells were examined by flow cytometry to assess the change in CFP-positive cells between mice with sham surgery and OVX. Consistent with the observations of Mazzucchelli and colleagues,[48] two populations of CFP-expressing cells were observed: one minor population that were CD45-negative (most likely stromal cells), and a second $CD45^+$ $CD11b^+$ $CD11c^+$ population (Supplementary Fig. 1). Of the two CFP^+ populations in the bone marrow, only the $CD11c^+$ population increased post-OVX (Fig. 1B), confirming the qPCR data. The CFP-positive (ie, IL-7–expressing) cells also produce IL-15, as shown in

Western blots (Fig. 1C). Because the CFP^+ cells are $CD11b^+$ and $CD11c^+$ we refer to them here as bone marrow myeloid dendritic cells (BMDCs). Further characterization (Supplementary Fig. 1) of the CFP^+ BMDCs showed that in addition to CD11b these BMDCs express IRF4 and IRF8 and both CD8 and CD4. Based on these markers, these myeloid DCs do not fit into the "classical pDC/DC1/DC2" paradigm.[57–59]

We confirmed the results of FACS analysis by immunofluorescence (IF) of bone sections from OVX and sham-operated mice. The IF results (Fig. 1D; Supplementary Fig. 2) show that the number of ($CD11c^+$) DCs increased by 2.5-fold/mm^2 in OVX mice, in agreement with FACS data (Fig. 1B). Also, consistent with the FACS (Fig. 1B) the IF data shows that 40 to 50% of DC express IL-7 or IL-15 (Fig. 1D; see also Supplementary Fig. 2 for representative images and Materials and Methods for details of quantification). The quantitation of triple-positive cells (CD11c, IL-7, and IL-15) in bone sections shows a 2.5-fold/mm^2 increase in OVX relative to sham surgery, with one-half of the $CD11c^+$ also IL-7–postivite and IL-15–positive (Fig. 1D). In summary, data in Fig. 1 show increased levels of DCs that express IL-7 and IL-15 post-OVX relative to sham-operated mice.

Estrogen regulates the lifespan of the DCs via inducing FasL

E_2 regulates osteoclast lifespan by autocrine FasL-induced apoptosis.[15,16,60] As both osteoclasts and BMDC are derived from myeloid lineage, we hypothesized that increased BMDC numbers in the absence of E_2 is due to their increased lifespan. To test this hypothesis, first we isolated BMDC and cultured them with vehicle (DMSO) or 10nM 17-β estradiol for 4 hours. We quantitated expression of FasL by PCR (qPCR) using BMDCs (Fig. 2A) and found increased expression of FasL, which led to increased apoptosis in the presence of E_2 relative to vehicle (Fig. 2B). Increased apoptosis, as assessed by annexin V, was also observed with recombinant FasL, confirming that DCs express the receptor (Fas for FasL; Fig. 2B). Etoposide, a topoisomerase inhibitor that induces apoptosis by creating DNA breaks, was used as a positive control (Fig. 2B). Second, to test the hypothesis that E_2 regulates the lifespan of the BMDCs in vivo, IL-7^{eCFP} reporter mice were sham-operated or OVX. Three-weeks post-OVX, 17β-estradiol pellets were implanted subcutaneously in one-half of the OVX mice. Mice were euthanized 10 days post-pellet implantation and the number of $CD11c^+CFP^+$ cells assessed by flow cytometry of freshly isolated bone marrow cells in all three groups. CFP-expressing BMDCs increased in OVX (Fig. 2C), relative to sham-operated mice; additionally, E_2 replacement restored the percent of CFP^+CD11c^+ cells to levels in sham-operated mice (Fig. 2C). FasL mRNA expression by qPCR in the three groups was also assessed in freshly isolated BMDCs. OVX mice had nearly undetectable levels of FasL, but significantly higher levels of FasL were detected in both sham-operated and 17β-estradiol–treated OVX mice. Together, these results show that increase in CFP^+ BMDC and decrease in FasL expression was attributable to E_2 loss (Fig. 2D).

Finally, to measure E_2-induced apoptosis in vivo, we used TUNEL staining of histological sections of long bones collected from sham-operated and OVX mice. As shown in Fig. 2E, a number of TUNEL-positive foci (per mm^2) are observed in sham-operated mice, including BMDCs (Fig. 2E lower panel). Examination of the micrographs indicates that cells proximal to DCs also stain TUNEL-positive (Fig. 2E; Supplementary Fig. 3), suggesting that either E_2

induces apoptosis in cells other than DCs or FasL produced by DCs induces paracrine apoptosis. In contrast, very few foci are observed in OVX mice (Fig. 2E center panel). Furthermore, no TUNEL staining is observed in OVX mice in the proximity of the BMDCs. In summary, these experiments show that in estrogen-replete animals, DC lifespan is shortened by induction of FasL. Next, we examined the effect of IL-7 and/or IL-15, as well as BMDCs in the presence or absence of E_2, on bone marrow T cells.

Together IL-7 and IL-15 induced IL-17A and TNFα in a subset of bone marrow T cells

Previous reports have shown that IL-7 and IL-15 together, but not individually, lead to the proliferation of human T_{MEM}.[61,62] Here, to assess cytokine production, bone marrow CD4 and CD8 were cultured with IL-7, IL-15, or both for 48 hours. TNFα and IL-17A are found in abundance in postmenopausal women with osteoporosis, and TNFα and IL17A null mice do lose bone post-OVX.[29,63,64] Therefore, these two cytokines were assessed in the T cells. The data show that T_{MEM} ($CD44^{high}$ $CD62L^{int}$) not only proliferate (as assessed by Ki-67) but also produce TNFα and IL-17A when treated with both IL-7 and IL-15 for 48 hours, but not when treated with each cytokine individually (Fig. 3A; Supplementary Fig. 4). No IFN-γ or IL-4 expression was detected in the T_{MEM} (not shown). Although prior studies have shown that these cytokines lead to proliferation of T_{MEM},[61,62] the induction of proinflammatory cytokines has not been previously shown and was unexpected. To assess whether IL-7 and IL-15 produced by BMDCs also activate T_{MEM} to induce TNFα and IL-17A, we cocultured the two cell populations in the presence and absence of 10nM 17β-estradiol for 48 hours. The DCs and T_{MEM} were isolated by FACS: BMDC were sorted using CD45, CD11c and CFP fluorescence and thus are the only source of IL-7 and IL-15 in the well; the T cells were isolated using CD45, CD44 and CD62L signals. Use of anti-CD3 was avoided because CD3 crosslinking can activate T cells.[65] In the absence of E_2, a fourfold expansion of T_{MEM} and a twofold decrease in effector T cells (Fig. 3B) was observed in cocultures with BMDC that was not observed in the presence of E_2. The increase in T_{MEM} is consistent with the effects of IL-7 and IL-15 produced by the BMDCs (Fig. 1B,C). We also observed induction of TNFα, IL-17A, or both in T_{MEM} cocultured with BMDCs in the absence of E_2 but not in the presence of E_2 (Fig. 3B lower panel). Although the effect of BMDCs in inducing TNFα or IL-17A (Fig. 3B) reproduce the results with recombinant IL-7 and IL-15 (Fig. 3A), to determine whether IL-7 and IL-15 from the BMDCs was responsible for this effect we titrated several anti-mouse IL-7 or IL-15 antibodies to neutralize these cytokines. At low (<25 μg/mL) concentrations we observed no effect. At higher concentrations of either IL-7 or IL-15 antibody (≥50 μg/mL), with two different antibody clones for each cytokine, we observed reduced numbers of BMDCs, suggesting that these cytokines may also have an autocrine or paracrine function for the BMDC.

To verify the role of E_2 on T_{MEM} in vivo we compared the T_{MEM} population in sham, OVX, and OVX mice with 17β-estradiol replacement. The T_{MEM} population increased in OVX mice relative to sham-operated mice and 17β-estradiol replacement restored T_{MEM} to sham-surgery levels (Fig. 3C). Based on the observation that BMDCs express FasL in the presence of E_2, we checked if T-cell subsets express Fas (CD95). Culturing bone marrow T cells with IL-15 (Fig. 3D) but not IL-7 (not shown), induced Fas in T_{MEM} (Fig. 3D). These results

indicate that in E_2-replete females, BMDCs regulate memory T cells via IL-7 and IL-15, but also by inducing Fas via IL-15 to prevent expansion of T_{MEM}. In the absence of E_2, BMDCs use IL-7 and IL-15 to activate T_{MEM} to proliferate and secrete proinflammatory cytokines.

Summarizing, our data show IL-7 and IL-15 together lead to production of IL-17A, TNFα, or both in T_{MEM}. Recombinant IL-7 and IL-15 activated a subset of T_{MEM}, leading to expression of TNFα, IL-17A, or both. The induction TNFα and IL-17A was recapitulated using CFP^+BMDCs (ie, IL-7–expressing and IL-15–expressing DCs, as shown in Fig. 1B,C) and T_{MEM} cocultures (Fig. 3B). E_2 induces FasL and apoptosis in the BMDCs (Fig. 2A,D), leading to fewer BMDCs, low levels of IL-7 + IL-15, and low-level proliferation of T_{MEM}; IL-15–induced FAS in T_{MEM} thus maintains T_{MEM} homeostasis (Fig. 3D). In the absence of E_2, BMDCs have longer lifespans because they do not produce FasL, leading to higher levels of IL-7 and IL-15, which induces TNFα, IL-17A, or both in a subset of T_{MEM}. We hypothesized that these proinflammatory cytokines contribute to bone loss (Fig. 3E).

Mice with a T-cell–specific deletion of IL15RA fail to activate T_{MEM} post-OVX

To test our hypothesis and validate the model shown in Fig. 3E in vivo we developed a new mouse model. As our results showed that IL-7 and IL-15 produced by DCs (Fig. 1), whose abundance is controlled by E_2 (Fig. 2), are primarily responsible for activation of T_{MEM} in the bone marrow (Fig. 3), one possible approach was to delete either of these cytokines. IL-7 is required for T cell development, and for generation of T_{MEM},[66] deleting IL-7 would lead to depletion of T cells and T_{MEM}. We then considered deleting IL-15 because the role of IL-15 in reactivating T_{MEM} in the context of menopause has not been defined. A global knockout or antibody-mediated depletion of IL-15 was rejected because this cytokine plays an important role in NK cell homeostasis that would make interpretation of the results questionable and because anti-IL-15 antibodies have been shown to produce adverse neurological effects.[67–69] Further, IL-15 has been shown to directly regulate osteoclastogenesis[70] and mineralization by osteoblasts.[71] Because we do not know the developmental lineage of the CFP^+DC cell-specific deletion, IL-15-blockade was deemed to not be a feasible approach. Therefore, we chose to delete the IL-15 receptor-α (*IL15RA*) gene in T cells by crossing Lck-Cre with IL15RA-floxed ($IL15RA^{f/f}$) mice. *Lck*, a kinase, is constitutively expressed primarily in CD4 and CD8 T cells.[47] High expression of Lck-Cre has been reported to have off-target effects[72]; therefore, we used a mouse strain with low-expression of Cre (see Materials and Methods). We first compared levels of bone marrow T cells between C57BL/6J (WT) and mice with T-cell–specific deletion of the *IL15RA* gene ($IL15RA^{\Delta T\text{-cells}}$) to assess changes due to IL15RA deletion. Our results show that in 8-week-old mice, there are modestly higher levels of memory (T_{MEM}), but no significant change in naïve (T_N) or effector (T_{EFF}) populations of T cells in the bone marrow (Fig. 4A). The moderate increase in T_{MEM} in these E_2-replete mice may be attributed to the observation that IL-15 induces Fas in T_{MEM} (Fig. 3D). The absence of the IL-15 receptor leads most likely to low levels of Fas expression in these cells and allowing the cells to live longer even with FasL expression in E_2-replete mice.

Next, to determine the effect of E_2 deficiency on the immune and skeletal systems, 12-week-old female $IL15RA^{f/f}$ Cre^+ or control $IL\text{-}15^{+/-}$ (Cre^+ $IL15RA^{f/+}$) littermates were OVX or

sham-operated. A significant increase in T_{MEM} was observed in (control) $IL15RA^{f/+}$ mice, but not in $IL15RA^{\Delta T\text{-cells}}$ (Fig. 4B). The levels of T_N decrease in both the $IL15RA^{f/f}$ and control mice to similar levels (Fig. 4B). We also observed TNFα and IL-17A expression in control littermates but not in mice with IL15RA-deficient T cells (Fig. 4C). Consistent with expression of IL-17A and TNFα in the control littermates, these mice also lost bone volume and trabecular bone and had decreased bone mineral density as assessed by μCT measurements of the tibias in the four groups (Fig. 4D; Table 1). While the number of osteoclasts increased in control mice, a modest increase in osteoclasts was observed in OVX $IL15RA^{f/f}$ mice post-OVX (Table 1). We also noted a slight increase in osteoclasts at baseline between the control and $IL15RA^{f/f}$ mice. We examined bone formation in both control and $IL15RA^{\Delta T\text{-cells}}$ and, as expected, the control mice had increased bone formation but no change in mineral apposition rate (MAR) or bone formation rate per unit of bone surface (BFR/BS) was observed in the $IL15RA^{f/f}$ mice (Table 1). Finally, we studied the kinetics of bone resorption post-OVX in both groups of mice. Our results show (Fig. 4E) that no increase in CTX is observed in the absence of TNFα and IL-17A in the $IL15RA^{f/f}$ Cre^{+} mice. These results also confirm that IL-7 and IL-15 together are needed to induce TNFα and IL-17A in T cells, which then promote bone loss.

Summarizing, the results show that T_{MEM} develop normally (at baseline) in absence of the IL-15 receptor. However, no TNFα and IL-17 is induced by OVX in the T_{MEM}, relative to littermate controls that express the IL-15 receptor. Furthermore, in the absence of TNFα, IL-17A, or both, we observed no bone resorption, decrease in bone mass, change in trabecular parameters, or increase in osteoclast numbers (Fig. 4D,E; Table 1). These studies confirm or validate the model proposed in Fig. 3E and show that inflammation is needed for bone loss leading to osteoporosis.

Discussion

Involutional osteoporosis is mediated by E_2 loss at menopause. Osteoporosis leads to effete bone that is prone to fracture with minimal trauma. Although the effect of E_2 was recognized decades ago, a number of causes of osteoporosis have been proposed. Early studies by Avioli and others suggested that aging and metabolic changes associated with menopause lead to osteoporosis.[73–76] Later, molecular studies suggested the direct effect of E_2 on maintaining balance in the cells of BRUs.[20,60,77,78] The prevailing view has been that E_2 loss leads to uncoupled increase in bone resorption by osteoclasts and deficit in osteoblasts.[79,80] Most strategies to treat osteoporosis have focused on rebalancing the activities of cells in the BRU.

Elegant studies from Pacifici, Weitzmann, Takayanagi, and colleagues have highlighted the role of proinflammatory cytokines produced by T cells as a cause for promoting bone loss. These studies laid the foundations of osteoimmunology.[26,29,81–84] A number of studies have shown both in humans and mice, that loss of E_2 leads to T-cell–dependent inflammation, but the underlying mechanism is undetermined. Here, we have identified for the first time a definitive mechanism by which E_2 suppresses the activation of T cells. Conversely, we also show how loss of E_2 leads to activation of T_{MEM}, unexpectedly converting them to TNFα-secreting and IL-17–secreting T_{MEM} that lead to bone loss. The

activation of T_{MEM} post-OVX is mediated by IL-7 and IL-15 produced by increased numbers of bone marrow DCs (Fig. 1). To the best of our knowledge, no previous study has identified the critical role of BMDCs in induction of TNFα, IL-17A, or both by T cells in the context of postmenopausal osteoporosis. Although a previous study showed that OVX causes the maturation of BMDCs (CD11c) to express costimulatory signal CD80,[40] the authors did not demonstrate that the expression of proinflammatory cytokines in the T cells was due to antigen-dependent activation by BMDCs. The steady state levels of BMDCs is maintained by E_2 by inducing apoptosis via FasL in the DCs (Fig. 2). With declining E_2 levels postmenopause, the levels of FasL also decline, leading to increased lifespan and levels of DCs and hence to higher levels of IL-7 and IL-15 (Figs. 1 and 2).

DCs are a heterogenous population of professional antigen-presenting cells that arise from different hematopoietic lineages.[85,86] The DCs that produce IL-7 and IL-15 in the bone marrow are closest in lineage to plasmacytoid DCs (pDCs) because these express the transcription factor IRF4 and IRF8, but they also express CD11b and CD11c, which are typically expressed by myeloid DCs. In addition, these DCs expressed both CD4 and CD8 markers, which is unusual for tissue-resident DCs (Supplementary Fig. 1). Because these DCs express novel marker combinations that have not been previously described, additional studies are needed because they are important for maintaining T_{MEM} that reside in the bone marrow and because the role of this DC population in inducing proinflammatory T cells in postmenopausal osteoporosis has not been previously described.

IL-7 is required for T cell homeostasis, because loss of functional IL-7 leads to thymic hypoplasia, decreased numbers of B cells and T cells.[87] Importantly, IL-7 is needed for development of T cell memory.[88,89] Similarly, IL-15 is also important for the development of NK cells and memory CD8 T cells.[90] Resting memory CD4 and CD8 T cells are dependent on signals from IL-7–producing and IL-15–producing cells for their survival and intermittent homeostatic proliferation.[91] For this reason, IL-7 and IL-15 are considered to be redundant in their functions. Furthermore, Geginat and colleagues[61,92] have shown that IL-7 and IL-15 together, but not alone, lead to antigen-independent proliferation of human T_{MEM}. Therefore, increased levels of IL-7 and IL-15 in OVX mice, which lead to T_{MEM} proliferation, could be anticipated. Indeed, we observed 12-fold expansion due to proliferation (assayed by Ki-67 staining) of T_{MEM} in culture in presence of IL-7 and IL-15 (Supplementary Fig. 4). However, we were not successful in measuring proliferation in vivo by Ki-67 or BrdU pulsing (at 1 and 3 days postsurgery). The lack of success may be due to several factors: first, it is possible that the T_{MEM} proliferated very rapidly (<24 hours) after surgery, prior to pulsing with BrdU or isolation of BMC for Ki-67 staining. We selected days 1 and 3 post-OVX because we observed increased serum CTX 5 days post-OVX (Fig. 4E). Alternatively, the levels of E_2 post-OVX decrease progressively over days, leading to buildup of IL-7–producing and IL-15–producing DCs over this time. This is the only population of IL-7–producing and IL-15–producing cells that reacted to E_2 loss and replacement (Figs. 1B and 2C). The buildup of IL-7 and IL-15 over days leads to induction and accumulation of TNFα-expressing and IL-17A–expressing T_{MEM}. If initial levels of T_{MEM} that respond to IL-7 and IL-15 represent a small subset that divide nonsynchronously over days, BrdU or Ki-67 will not be sufficiently sensitive to detect proliferation. To increase sensitivity additional tools are needed to identify the IL-7 and IL-15 responsive subset of

T_{MEM}. Nonetheless, we observed a 3.8-fold expansion of T_{MEM} in vivo (Fig. 4B) post-OVX over 3 weeks. Importantly and not previously shown, we found that together these two cytokines *also* induce IL-17A, TNFα, or both, in a subset of T_{MEM} (Fig. 3A; see also Supplementary Fig. 4). Because these TNFα$^+$ or IL-17A$^+$ cells do not alter the expression of CD44 and CD62L (Fig. 3B), we refer to them as effector memory T cells (T_{EM}). IL-15 has also been shown to directly regulate osteoclasts and osteoblasts, either alone or in combination with RANKL.[70,71,93–96] Together with the CTX time course (Fig. 4E), our results are most consistent with the notion that the acute (early) phase of bone loss stems from the accumulation of BMDCs, IL-7 and IL15 expressing, and TNFα and IL-17–expressing T_{MEM} over ~3 weeks of time. In support of our data, polymorphisms in IL-15 are associated with bone mineral density in postmenopausal women.[97] Additionally, Djaafar and colleagues[70] have shown that IL-15$^{-/-}$ mice have a high bone mass; furthermore, OVX of the IL-15 null mice did not increase osteoclast numbers in distal femur relative to control mice. In our study, IL-15 also induced the Fas receptor in the T_{MEM}, so in the presence of E_2, which induces FasL, these T cells undergo apoptosis (Fig. 3C). Notably, both DCs and proliferating T_{MEM} would undergo apoptosis consistent with the foci of the TUNEL stain (Fig. 2E). The dying cells display annexin V (Fig. 2B), an “eat-me” signal that would allow safe removal of apoptotic cells by phagocytes.[98] We note that although the current paradigm of T_{MEM} activation requires antigen exposure, E_2 loss leads to a cytokine-dependent but antigen-independent activation of T_{MEM} by exposure to high levels of IL-7 and IL-15.

Due to the pleiotropic nature of IL-15, the effect on NK cells, on osteoclasts, endothelial cells, epithelial cells, and myocytes,[99] to assess the role of IL-15 in T_{EM} activation in vivo, we ablated IL-15Rα specifically in T cells by crossing mice engineered with loxP sites flanking exons 2 and 3 of the *IL15RA* gene with Lck-Cre (a pan αβ T-cell expressed gene). Fortunately, no effect on the development of T_{MEM} was observed in E_2-replete Cre$^+$IL15RA$^{f/f}$ female mice (Fig. 4A). However, although an increase in T_{MEM} levels was observed in control (IL15RA$^{f/+}$Cre$^+$) mice post-OVX, no such increase in T_{MEM} was observed in IL15RA$^{\Delta T\text{-cells}}$ mice, confirming that T_{MEM} expansion is dependent on IL-15 (with IL-7; Fig. 4B). Consistent with lack of expansion, no TNFα or IL-17A expression was observed in T_{MEM} (Fig. 4C) in the IL15RA$^{\Delta T\text{-cells}}$. In summary, we show that OVX leads to the expansion of BMDC which produce IL-7 and IL-15 (we note that only CFP$^+$ BMDC express IL-15; Fig. 1) because E_2 directly induces apoptosis of the CFP$^+$ BMDC in vivo (Fig. 2). We show that coculturing CFP$^+$ BMDC with BM T cells leads to the expansion of T_{MEM} and because the CFP$^+$BMDCs are the only source of IL-7 and IL-15, this result confirms that these cytokines also induce TNFα, IL-17, or both in the T_{MEM} in absence of E_2 (Fig. 3B). We observed similar expansion of BM T_{MEM} (Fig. 4B) and induction of TNFα, IL-17A, or both in T_{MEM} post-OVX (Fig. 4B,C). One limitation of the current study is that we cannot directly show in vivo that IL-7 and IL-15 produced by the BMDCs are the cause of TNFα and IL-17A induction in T_{MEM}. As discussed, blockade of IL-7 and IL-15 using antibodies is not feasible and genetic ablation of these cytokines will require identification of cell-specific promoters for Cre expression. Nonetheless, the increase in T_{MEM} post-OVX is consistent with the increase in IL-7 and IL-15 and expression of CD127 (IL-7 receptor) on the T_{MEM}.[100,101] Our data with T-cell–specific deletion of the IL-15Rα

gene (*IL15RA*$^{\Delta T\text{-cell}}$) shows that IL-15 is required for expansion and induction of TNFα, IL-17A by T_{MEM}.

Because *IL15RA*$^{\Delta T\text{-cell}}$ mice do not express the proinflammatory cytokines post-OVX, this mouse model allows us to assess the contribution of inflammation and the direct effect of E_2 loss on the BRU and on bone loss. Our results show that although OVX of control mice leads to induction of TNFα and IL-17 produced by T_{EM}s and to significant trabecular bone loss by activating bone resorption, the *IL15RA*$^{\Delta T\text{-cell}}$ mice did not lose bone volume and no increase in serum CTX or loss of trabecular bone was observed (Fig. 4; Table 1). We observed a modest but statistically significant increase in osteoclast surface/bone surface (Oc.S/BS) (a modest increase in number of osteoclasts/bone surface [N.Oc/BS] is also observed but did not achieve statistical significance) post-OVX showing that E_2 loss does lead to increased osteoclasts, even in the absence of inflammation, but interestingly this increase does not translate to a significant decrease in bone mass (Fig. 4D) or bone resorption as measured by CTX (Fig. 4E) in the absence of TNFα and IL-17A. Further, we note based on our previous experience, that exposure to IL-7 and IL-15 activation of T_{MEM} leads to low levels of proinflammatory cytokines, relative to infection (antigen-dependent activation). Both IL-17A and TNFα promote osteoclastogenesis and increased bone resorption.[27] Neutralization of IL-17A[102] and TNFα[103] have been shown to decrease osteoporosis in response to OVX in mice. Additionally, no bone loss is observed in OVX in TNFα-deficient mice,[104] or in transgenic mice expressing soluble TNF receptor.[105] In humans, polymorphisms that effect TNFα[106–108] or IL-17A[109] function are also modulators of osteoporosis. Therefore, our results place inflammatory signals upstream or epistatic to the direct effect of E_2 on the BRU via FasL and/or other mechanism. Taken together, our results show that production of these proinflammatory cytokines is required for acute phase bone loss leading to osteoporosis.

The finding that inflammation is due to activation of T_{MEM} may provide a population-level explanation. Because T_{MEM} encode the lifetime of exposure to commensal and pathogenic microbes, the levels of T_{MEM} that can be induced to produce TNFα and IL-17A are likely to vary in a population. Nearly one-half of postmenopausal women develop osteoporosis, but the remaining one-half do not. Based on current knowledge, T cells that produce TNFα, IL-17A, or both in the initial activation by antigen and that develop into the memory repertoire are reactivated by IL-7 + IL-15 to produce TNFα, IL-17A, or both.[110–112] The levels of T_{MEM} in individuals in a population that have the potential to produce TNFα and IL-17A will vary because each individual's exposure to these microbes vary. We hypothesize that postmenopausal women having healthy bone mass have low levels of T_{MEM} capable of producing TNFα and IL-17A or both. In contrast, there are more TNFα, IL-17A–producing T_{MEM} in postmenopausal women with osteoporosis. Thus, our pathway (Fig. 3E) may explain a population-level phenomenon. In this context, we note that many studies have implicated the gut microbiome as a factor in maintaining bone health in postmenopausal women.[113,114] Our studies implicate the role of T cells in the crosstalk between the immune system maintaining the gut microbiota and the gut microbiota affecting the immune system.[115,116] The gut microbiota is likely to influence bone marrow–resident T_{MEM} populations.[112,117] In mice, recent studies have shown that segmented filamentous bacteria (SFB) increase T_H17 response in the gut and that this response protects against infection by

pathogenic *Citrobacter rodentium*.[118] Although, it is not clear if SFB colonization leads to T_{MEM} development, OVX of SFB-colonized mice may produce increased bone loss relative to mice lacking SFB. Additional studies are needed to test this hypothesis. We have tested for, and find no evidence for, presence of SFB in our colony of mice (originally purchased from The Jackson Laboratory, Bar Harbor, ME, USA).[118]

Although aspects of these findings such as the increase in IL-7,[82] IL-15,[51,97] and IL-17A[30,119] have been noted to increase post-OVX in mice and in postmenopausal women with osteoporosis, our study connects the cellular and signaling components into a coherent description. The role of DCs and FasL have also been previously noted.[120] However, the connection between these individual cells and molecules into a pathway that is activated at menopause has not been described previously. The model described in Fig. 3E is entirely consistent with all of the previous studies including genetic engineering, cellular, epidemiological, and population genetic studies in both rodents and humans. A number of features are novel, including the antigen-independent activation of T_{MEM}, the role of IL-7 and IL-15–producing DCs, and the requirement for T-cell–dependent inflammation for bone loss.

The physiological (and evolutionary) basis for activation of this low-grade inflammation associated with loss of E_2 may be reconciled by considering the link between pregnancy, immune system, and bone as proposed by Pacifici.[81] During pregnancy calcium is stored in the bones because of increased weight and high levels of placenta-produced E_2, which promotes osteoblast activity while blocking osteoclast activity. High E_2 levels during pregnancy also decreases the immune response to protect the fetus, because it is partially self. E_2 levels drop when the placenta is delivered, leading to an acute increase in osteoclast numbers, bone resorption, and to release of stored calcium in bone. The calcium is supplied to the rapidly growing skeleton of the neonate via lactation. In contrast to postpartum, where these events are limited in duration (months in humans and weeks in mice), in postmenopausal women inflammation persists (for decades), because the ovaries restart E_2 production postlactation but not postmenopause. Unpublished data show that the same panel of cytokines shown in Fig. 1A are upregulated 2 days postpartum, relative to age-matched nulliparous females in the bone marrow.

Nearly one-half of postmenopausal women develop osteoporosis, but menopause also increases incidence of other comorbidities including coronary artery disease, certain cancers, chronic obstructive pulmonary disease, stroke, and dementia.[121–124] It is tempting to speculate that the proinflammatory T cells produced by E_2 loss also contribute to the pathogenesis of these conditions because all these conditions are known to be caused by both chronic inflammation and metabolic syndrome.[125–129] In conclusion, our studies establish a new regulatory circuit between E_2, the immune system, and the skeletal system. Our data shows IL-7 and IL-15 together activate T_{MEM} to induce TNFα, IL-17A, or both (referred to as T-effector memory cells or T_{EM} for short). In mice that have a T-cell–specific deletion of IL-15 receptor, T_{MEM} do not produce TNFα and IL-17A and no bone loss is observed. These results show that the uncoupled activity of the BRU in osteoporosis is due to T-cell–dependent inflammation and not due to the direct effect of E_2 on the BRU. Because bone is a mechanosensing organ, a store for calcium and phosphate, and the bone marrow a

site for hematopoiesis and a nest for immune memory cells, the BRU is regulated by a number of different feedback loops. Knowledge of this pathway will, most likely, lead to additional discoveries on how bone homeostasis is maintained and how these feedback loops may intersect in health and pathological states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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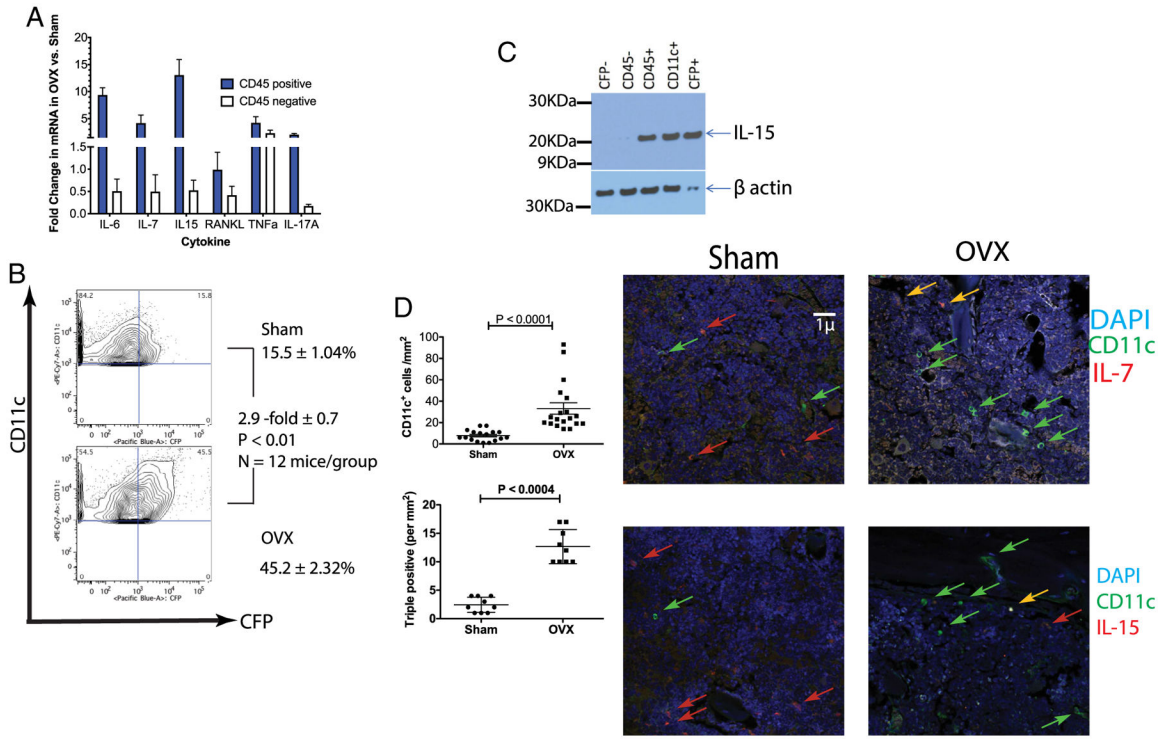

Fig. 1.

Ovariectomy increases IL-7 and IL-15 levels that are produced by bone marrow CD11c^{+} cells. (*A*) Increased IL-7 and IL-15 mRNA expression in OVX mice: BMCs from sham-operated and OVX mice were separated by magnetic beads into CD45-positive and CD45-negative populations using magnetic beads. RNA was isolated from each subset and expression of cytokines analyzed by qRT-PCR (SYBR green; ABI). The data is expressed as the fold difference between OVX and sham-surgery, after normalizing to GAPDH expression (data is from 5 mice/group with 3 technical replicates/mouse). (*B*) Identification of IL-7–expressing cells: BMCs from OVX or sham-operated from IL-7eCFP reporter mice were analyzed by flow cytometry. Although two cell populations were found to express CFP in the bone marrow, only the CD45+ CD11b + CD11c + population changed in ovariectomized mice. (*C*) IL-7–expressing cells co-express IL-15: expression of IL-15 was examined using Western blots in sorted BMC CD45 negative (lane 2), hematopoietic (CD45^{+}), CD45^{+} CD11c^{+}, CD45^{+}CFP^{+}, and CD45^{+}CFP$^{−}$ (lane 1) cells. Representative Western blot of four experiments. (*D*) OVX leads to increased IL-7^{+} IL-15^{+} and CD11c^{+} cells: increased CD11c^{+} cells are observed post-OVX (top left panel) in bone sections, as well as triple-positive CD11c^{+} IL-7^{+} and IL-15^{+} (bottom-left) cells, but not all CD11c^{+} cells express IL-7 and IL-15 concurrently as shown in representative photomicrographs (right panels; yellow arrows are DAPI, CD11c, and cytokine positive). BMC = bone marrow cell; CFP = cyan-fluorescent protein; OVX = ovariectomized.

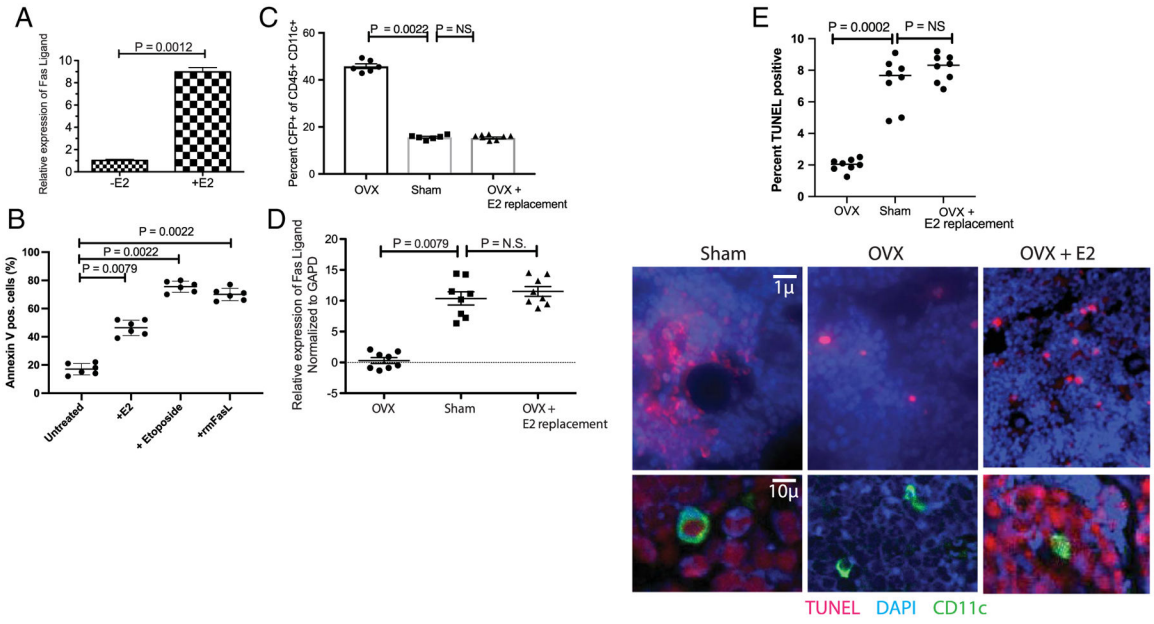

Fig. 2.

E_2 regulates dendritic cell lifespan by inducing apoptosis via FasL. (*A*) Estrogen induced FasL in BMDCs: BMDC ($CD45^+$ $CD11c^+$ CFP^+) cells isolated by FACS were cultured for 4 hours with 10nM 17β-estradiol. FasL mRNA was quantified by qPCR relative to GAPDH message (average of six independent experiments [BMC preparations] are shown). (*B*) FasL induced apoptosis in BMDC: apoptosis was assessed by Annexin V staining in cells used in *A* after 24 hours. Etoposide (10^{-8}M) was used as a positive control and recombinant murine FasL (10 ng/mL) was used to assess expression of Fas (receptor). (*C*) E_2 replacement in OVX mice restores CFP^+ BMDC: Increased number of CD45 CD11c and CFP positive cells are observed in OVX mice (cf. Fig. 1B) but decrease with E_2 replacement (0.1 mg/pellet 21-day release). The decrease is observed in BMDC when 17β-estradiol pellets are implanted 1 or 3 weeks post-OVX. Data from 8 mice/group. (*D*) E_2 regulates FasL in vivo: RNA was isolated 3 weeks postsurgery from bone marrow cells of OVX, sham-operated, and OVX with 17β-estradiol pellets implanted mice. FasL expression was assessed by qRT-PCR. (Same mice from *C*). (*E*) E_2 loss limits apoptosis: foci of apoptotic cells are observed in E_2-replete mice (sham-surgery), but fourfold to fivefold fewer foci are observed in OVX mice (also see Supplementary Fig. 3). As the ovary produces multiple factors/hormones, we performed E_2 replacement in OVX mice, and show that 17β-estradiol restored the levels of apoptotic foci. BMDC = bone marrow dendritic cell; E_2 = estrogen; FasL = Fas ligand.

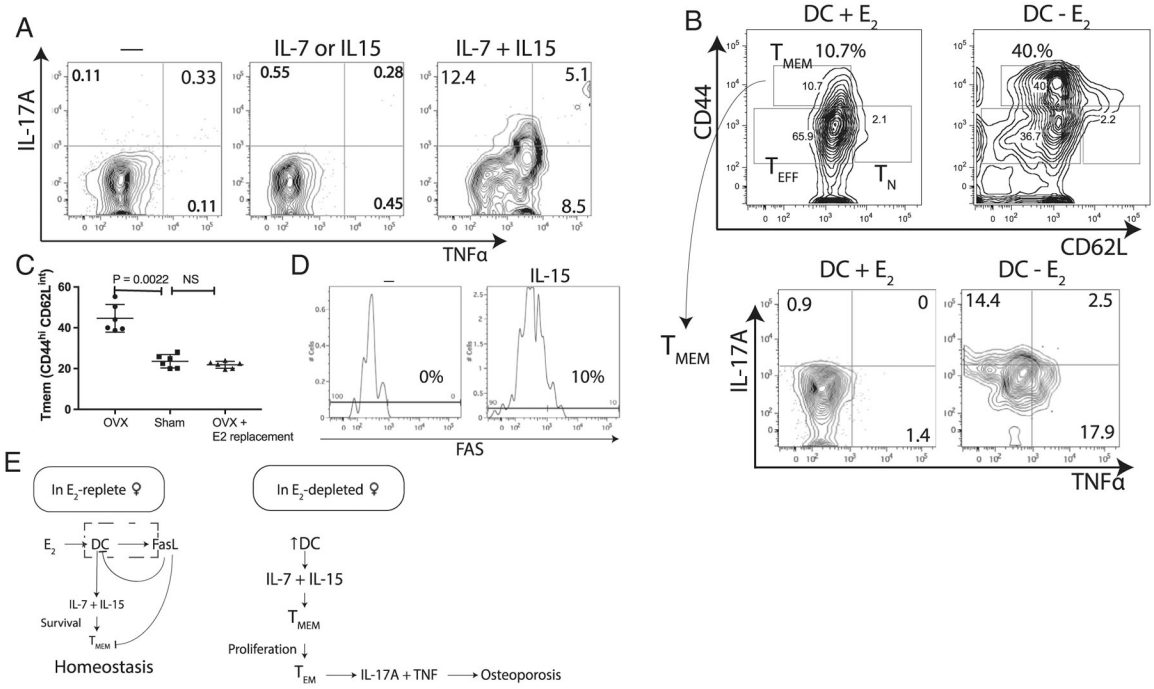

Fig. 3.

IL-7 and IL-15 together lead to induction of IL-17A, TNFα or both in memory T-cells. (*A*) Cytokine-dependent conversion of T_{MEM} to T_{EM}: culturing memory T-cells (T_{MEM}: CD3 $CD44^{high}$ $CD62L^{low}$) with IL-7 and IL-15 together induces TNFα and IL-17 in the absence of antigen stimulation. Representative flow contour plot of six experiments is shown. (*B*) Co-culturing T_{MEM} with BMDCs induced TNFα and IL-17A in T_{MEM}: culturing T with CFP^+ BMDC in the absence of E_2 results in a fourfold increase in T_{MEM} and twofold decrease in T_{EFF} levels relative to DC in the presence of 10nM E_2; BMDC induced IL-17A and TNFα in T_{MEM} in the absence of E_2 mimicking OVX. Representative flow contour plot from six experiments is shown. (*C*) E_2 replacement in OVX mice restores T_{MEM} levels to that of sham-operated mice. Data from six mice/group. (*D*) IL-15 induces FAS in T_{MEM}: culturing BM-T_{MEM} with IL-15 induced FAS in this subset. (*E*) A model summarizing the data from Fig. 1 through Fig. 3D.

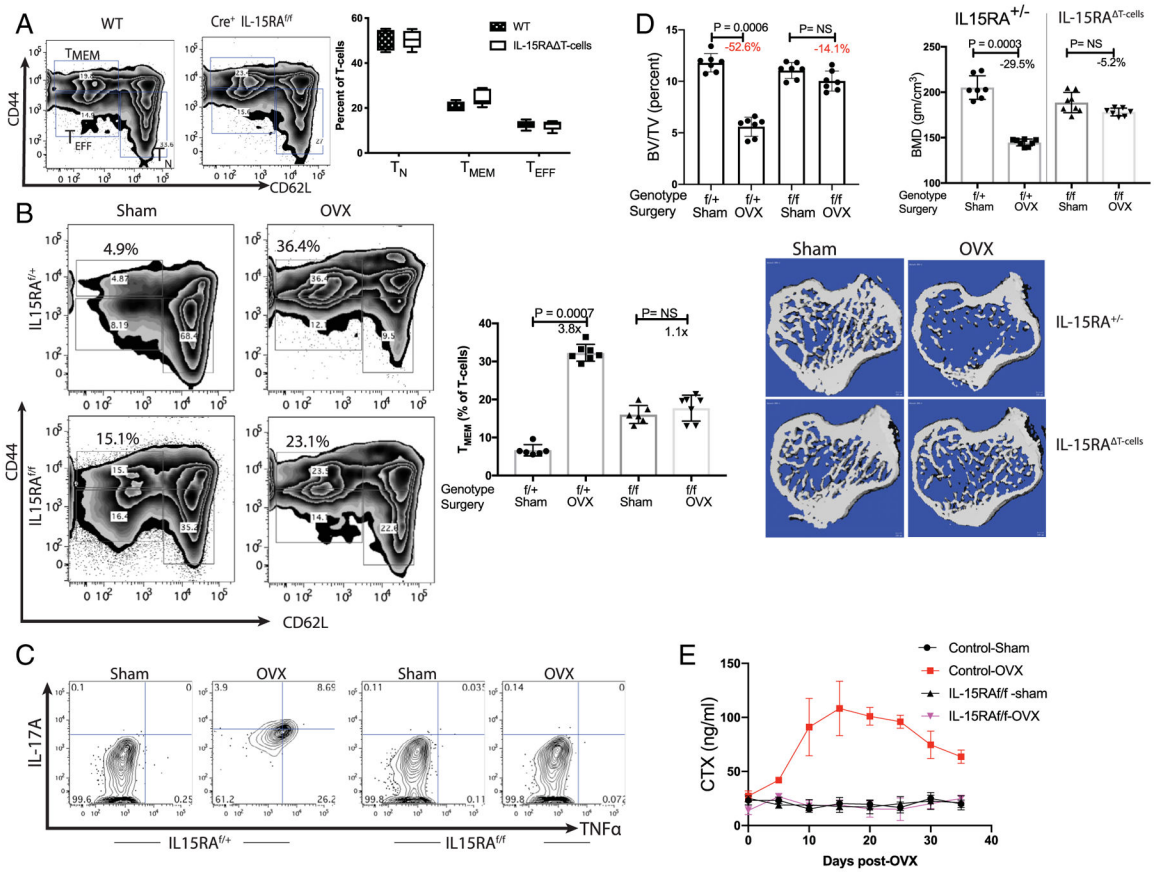

Fig. 4.

Ablation of IL-15 receptor α chain in T-cell prevents bone loss post-ovariectomy: *IL15RA*$^{f/f}$ mice were mated with Lck-Cre mice in two rounds of crosses to obtain Cre-positive *IL15RA*$^{f/f}$, *Cre*$^{+}$ *IL15RA*$^{f/+}$ and *Cre*$^{+}$ *IL15RA*$^{+/+}$ littermates. The control (IL15RA sufficient) mice included both Cre^{+} homozygote (IL15RA$^{+/+}$) and heterozygotes (*IL15RA*$^{f/+}$) as they were phenotypically similar, if not identical, in assays used here (i.e. T-cell populations, IL-17A, TNFα induction and bone parameters). *IL15RA*$^{f/f}$ *Cre*$^{+}$ mice are labeled as *IL15RA*$^{\Delta T\text{-}cells}$ in some panels. (*A*) No significant change in T-cell populations in *IL15RA*$^{\Delta T\text{-}cells}$ mice: Percent of T-cell subsets in the bone marrow of WT C57BL/6J and Cre^{+} IL15RA$^{f/f}$ in 10-week-old female mice were compared. No significant difference is observed in in bone marrow T-cells indicating that all T-cell subsets develop normally in the absence of IL15RA. Data representative of six mice/group is shown. (*B*) No change in T_{MEM} subset is observed in IL15RA-deficient mice post-OVX: 12-week-old Cre-positive flox/flox (*IL15RA*$^{f/f}$) or *1L15RA*-sufficient mice were OVX or sham-operated. Mice were euthanized 3 weeks postsurgery and bone marrow T cells analyzed by flow-cytometry. Two-factor ANOVA (genotype and surgery) indicates that genotype (baseline values) account for 1.29% of the variance ($p < .03$) and surgery accounts for 55.6% of the variance ($p < .0001$); the interaction term accounts for 37.8% ($p < .0001$) indicating that OVX had a larger effect in WT but not in mutant mice. These results are consistent with the model in Fig. 3D, where an increase in T (CD45^{+} CD3^{+} CD44^{high} CD62L^{low}) is observed in the control mice, the T_{MEM} in IL15RA$^{\Delta T\text{-}cells}$ mice do not change. (*C*) T-cells with IL15RA deficiency do not

express IL-17A and TNFα: While $Cre^{+}IL15RA^{f/+}$ mice express IL-17A and TNFα, these cytokines are not observed in OVX $Cre^{+}IL15RA^{f/f}$ mice. (*D*) $IL15RA^{\Delta T\text{-cell}}$ mice maintain bone mass post-OVX: 12-week-old female $Cre^{+}IL15RA^{f/f}$ and control liter-mates ($Cre^{+}IL15RA^{f/+}$) were sham-operated or OVX. Mice were sacrificed 3.5 weeks post-OVX and were evaluated by measuring bone-volume over total-volume (% BV/TV) and BMD. For BV/TV a two-factor ANOVA indicates that genotype contributed 13.15% ($p < .0001$) of the variance, surgery accounted for 50.3% ($p < .0001$) of the variance and the interaction term accounted for 25.9% ($p < .0001$). While control mice lost bone mass, mice with T-cell deficient in IL15RA maintained bone mass. Trabecular parameters by μCT of tibias is shown in Table 1. (*E*) Time course of CTX post-OVX in IL15RA sufficient and T-cell specific 1L15RA-deficient mice: Although control IL15RA-sufficient mice lost bone, no bone loss is observed in IL15RA-deficient mice, demonstrating the role of inflammation in promoting bone loss.

Table 1.

Summary of Trabecular and Histomorphometry Data in *IL15RA*$^{\Delta T\text{-}cells}$ and Control Mice

		Control (*IL15RA*$^{f/+}$ Cre^{+})		*IL15RA*$^{\Delta T\text{-}cells}$(*IL15RA*$^{f/f}$ Cre^{+})	
Experiment	**Parameter**	**Sham (*n* = 10)**	**OVX (*n* = 10)**	**Sham[1] (*n* = 10)**	**OVX (*n* = 12)**
Cancellous µCT (proximal tibia)	Tb.N	4.338 ± 0.1194	3.561 ± 0.3040 ***	4.489 ± 0.2939 NS	4.298 ± 0.1022 NS
	Tb.Th (mm)	0.04895 ± 0.000169	0.04896 ± 0.000119 NS	0.04930	0.04595
				0.0002268 NS	0.004272 NS
	Tb.Sp (mm)	0.2521 ± 0.004573	0.2423 ± 0.005556 **	0.2646 ± 0.003119 ***	0.2615 ± 0.004100 NS
	Conn.D (mm^{-3})	17.53 ± 0.2804	13.30 ± 0.5949 ***	18.44 ± 1.113 NS	17.85 ± 0.4840 NS
Histomorphometry (proximal tibia)	N.Oc/BS (mm^{-1})	3.335 ± 1.153	4.360 ± 0.4840 **	3.568 ± 1.025 NS	3.955 ± 0.3771 NS
	Oc.S/BS (%)	11.80 ± 0.6541	24.84 ± 0.9230 ***	12.28 ± 0.7114 NS	14.22 ± 0.7162 ***
Dynamic histomorphometry (proximal tibia)	MAR (µm/day)	1.60 ± 0.2418	2.092 ± 0.3687 **	1.628 ± 0.3005 NS	1.5890 ± 0.2630 NS
	BFR/BS (µm^3/µm^2/day)	0.546 ± 0.112	0.773 ± 0.0540 ****	0.525 ± 0.077 NS	0.565 ± 0.091 NS

Mean ± SD is shown for all parameters for each genotype. All measurements are from 3 weeks post-OVX. Unpaired two-tailed Mann-Whitney *p* values were computed.

Conn.D = connectivity density; NS = nonsignificant; Tb.N = trabecular number; Tb.Sp = trabecular spacing; Tb.Th = trabecular thickness.

[1]Values of *p* were computed using values between control and *IL15RA*$^{f/f}$Cre^{+} to assess the effect of genotype.

** $p < .01$.

*** $p < 1 \times 10^{-3}$.

**** < 1E-4.