An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency

Michaela Robbie Ryan*, Rebecca Shepherd†, Jennifer K. Leavey*, Yuhao Gao*, Francesco Grassi*, Frederick J. Schnell‡, Wei-Ping Qian*, Gilbert J. Kersh‡§, M. Neale Weitzmann*, and Roberto Pacifici*‡¶

*Division of Endocrinology, Metabolism, and Lipids, ‡Immunology and Molecular Pathogenesis Program, and §Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322; and †Division of Rheumatology, Washington University School of Medicine, St. Louis, MO 63110

Edited by David M. Kipnis, Washington University School of Medicine, St. Louis, MO, and approved September 17, 2005 (received for review June 20, 2005)

The bone wasting induced by estrogen deficiency is, in part, a consequence of increased T cell production of the osteoclastogenic cytokine TNF-α. This phenomenon is due to an expansion of T cells, but the responsible mechanism is unknown. We now show that ovariectomy (ovx) disregulates T lymphopoiesis and induces bone loss by stimulating, through a rise in IL-7 levels, both thymic-dependent differentiation of bone marrow-derived progenitors and thymic-independent, peripheral expansion of mature T cells. Attesting to the relevance of the thymic effects, thymectomy decreases by ≈50% the bone loss and the stimulation of T lymphopoiesis induced by ovx. In contrast, *in vivo* attenuation of the elevated IL-7 completely prevents the stimulation of T lymphopoiesis and the bone loss that follow ovx. Thus, the disruption of both T cell and bone homeostasis induced by ovx is mediated by IL-7 and due to both the thymic and extrathymic mechanisms. We conclude that IL-7 is a pivotal upstream target through which estrogen regulates hematopoietic and immune functions that are critical for bone homeostasis.

osteoporosis | ovariectomized | thymus | TNF-α

Estrogen (E) deficiency induces bone loss through a variety of mechanisms, the most relevant of which includes up-regulation of cytokine-driven osteoclastogenesis (1). Among the factors that up-regulate osteoclast formation in estroprevic humans and rodents is TNF-α (2). Although multiple cell lineages produce TNF-α in the bone marrow (BM), the major E-regulated source of this cytokine is the T cell (3). The pivotal role of T cell-produced TNF-α has been established by the failure of ovariectomy (ovx) to induce bone loss in mice with T cells lacking TNF-α (4).

ovx enhances T cell TNF-α production by causing an expansion of the T cell pool in the BM (4) through a complex mechanism driven by up-regulated IFN-γ production that leads to antigen-dependent stimulation of T cell activation (5). One mechanism by which ovx up-regulates T cell IFN-γ is by repressing the BM levels of TGF-β (6), an E-regulated factor (7) known to directly repress T cell activation (8).

Another mechanism by which E deficiency governs T lymphopoiesis and osteoclastogenesis is through enhanced production of IL-7 (9, 10). This cytokine is produced in the BM by stromal cells and osteoblasts (11) and is recognized as a bone wasting cytokine (9, 10) and as a pivotal, nonredundant regulator of multiple stages of T cell ontogeny and homeostasis (12–17). Several lines of evidence implicate IL-7 in the pathogenesis of ovx-induced bone loss. First, the BM levels of IL-7 increase after ovx, and *in vivo* attenuation of IL-7 levels completely prevents ovx-induced bone loss in mice (11). Second, IL-7 induces bone resorption and bone loss *in vitro* (9) and *in vivo* (10) by inducing T cell production of RANKL (receptor activator of NF-κB ligand) and TNF-α. Furthermore, IL-7 potently stimulates IFN-γ production (18), thus promoting antigen presentation and T cell activation.

Despite significant progress, the mechanism by which ovx causes an expansion of the number of TNF-α-producing T cells, and thus bone loss, remains largely unknown. In fact, cytokines other than TGF-β and IL-7 are likely to contribute to the regulation of IFN-γ production *in vivo*, and mechanisms unrelated to IFN-γ may contribute to the T cell expansion that is characteristic of estroprevic mice. Furthermore, the mechanism by which IL-7 induces the expansion of T cells observed in response to ovx remains to be defined.

In this study, we investigated the mechanism by which ovx expands the T cell pool and determined which of the multiple effects of IL-7 are relevant for ovx-induced bone loss. We report that ovx expands the T cell pool through stimulation of both peripheral expansion and thymic T cell output, that the bone loss induced by ovx is in part due to a resurgence of thymic function, and that IL-7 mediates the regulatory effects of E on immune functions critical for bone homeostasis.

Methods

Mice and Surgery. All animal procedures were approved by the Institutional Animal Care and Use Committee. C57BL/6 WT mice and C57BL/6 OTII transgenic mice were obtained from The Jackson Laboratory. Female mice were either sham-operated or ovx at 12 weeks of age, and total body bone mineral density (BMD) was determined 4 weeks after ovx as described in ref. 19. Endpoints were collected at 4 weeks after ovx with the exception of the progenitor and thymus studies, which were performed 2 weeks after surgery, a time previously determined to optimally measure the early phases of T cell development after ovx.

IL-7 mRNA RT-PCR. RNA was isolated from 20 mg of tissue by using the RNAlater RNA stabilization reagent (Qiagen, Valencia, CA) and RNeasy Mini kit (Qiagen). Reverse transcription was performed by using 2 μg of RNA and TaqMan Reverse Transcription reagents (Applied Biosystems). For real-time PCR, SYBR green master mix (Applied Biosystems), 250 nM primers, and 5 μl of cDNA were used on an Applied Biosystems 7700 real-time PCR machine at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. IL-7 forward primer was 5′-TCTGCTGCCT-GTCACATCATC, and the reverse primer was 5′-GGACATT-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: E, estrogen; ovx, ovariectomy/ovariectomized; THX, thymectomized; IRR Ab, irrelevant Ab; BM, bone marrow; HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; ETP, early thymic progenitor; RTE, recent thymic emigrant; CIITA, class II transactivator; TREC, T cell receptor excision circle; BMD, bone mineral density; SP, single-positive.

¶To whom correspondence should be addressed at: Division of Endocrinology, Metabolism, and Lipids, Emory University School of Medicine, 1639 Pierce Drive, Room 1309, Atlanta, GA 30322. E-mail: roberto.pacifici@emory.edu.

GAATTCTTCACTGATATTCA. Melting curves confirmed a single product. All samples were performed in triplicate. Input cDNA was normalized to actin. All values were normalized to sham.

***In Vivo* IL-7 Ab Neutralization.** ovx mice were treated with an anti-IL-7 Ab (M25) that was previously shown to neutralize IL-7 *in vivo* (11, 20). M25 hybridoma was generously provided by Immunex, and a protein A-purified Ab was generated by Zymed. Mice were injected i.p. with 2 mg/kg Ab (ovx IL-7 Ab) weekly for 2–4 weeks as described in ref. 11. The optimal dose of anti-IL-7 Ab was determined as the amount of IL-7 Ab that was sufficient to prevent the ovx-induced increase in thymic and splenic T cell numbers without reducing T cell numbers below homeostatic levels. Control ovx mice received an irrelevant isotype-matched (IgG2b) Ab (ovx IRR Ab).

Intracellular TNF-α and IFN-γ Staining. BM cells were cultured in the presence of phorbol 12-myristate 13-acetate (5 ng/ml, Sigma), ionomycin (500 ng/ml, Calbiochem), and Golgi plug for 5 h at 37°C. The cells were then labeled with CD4 and intracellularly stained for TNF-α, IFN-γ, or isotype control by using a Cytofix/Cytoperm kit (BD Pharmingen). The cells were analyzed by flow cytometry on a four-color FACScan flow cytometer (Becton Dickinson). All antibodies used for flow cytometry were purchased from BD Pharmingen.

T Cell Progenitors and T Cell Activation Markers. Cells were used immediately after harvest without purification or stimulation. To determine hematopoietic stem cell (HSC) (Lin^-IL-7Rα^-c-$Kit^+Sca\text{-}1^+$) and common lymphoid progenitor (CLP) cell (Lin^-IL-7Rα^+c-$Kit^-Sca\text{-}1^-$) numbers, BM cells were gated on Lin^- cells ($B220^+$, $CD11b^+$, GR-1^+, $Ly76^+$, $CD3^+$, and $THY1^+$). To quantitate early thymic progenitor (ETP) cell (Lin^-c-Kit^+IL-7R$\alpha^-CD44^+CD25^-$) numbers, thymocytes were gated on Lin^- cells ($CD8\alpha^+$, TCRβ^+, $CD4^+$, TCRγ^+, $NK1.1^+$, $CD11b^+$, GR-1^+, $B220^+$, and $CD25^+$) (21, 22). To examine T progenitor cell populations, thymocytes were gated on Lin^- cells ($CD4^+$, $CD8^+$, $CD3^+$, $\gamma\delta$TCR$^+$, GR-1^+, $B220^+$, and $CD11b^+$) and stained with CD4 and CD8. To determine T cell activation, BM cells were stained with CD69 and CD4.

T Cell Receptor Excision Circle (TREC) Assay. The TREC assay was performed as described in ref. 23 with modifications. DNA was harvested from total thymus, spleen, or whole blood by using the QIAamp DNA Mini Kit (Qiagen). Rearrangement of the T cell alpha gene was measured by signal joint excision circles containing the TCRD (T cell receptor delta) locus in a real-time PCR assay using primers located within the murine TCR$\alpha\beta$ locus [forward primer, 5′-CCAAGCTGACGGCAGGTTT (200 nM); reverse primer, 5′-GCATGGCAAGCAGCACC (200 nM); probe, FAM-TGCTGTGTGCCCTGCCCTGCC-TAM (300 nM)]. The real-time PCR was carried out by using TaqMan master mix (PE Biosystems) and 200 ng of input DNA at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (Applied Biosystems 7700). All samples were performed in triplicate and were normalized to sham.

FITC Injections. Two weeks after ovx, mice were anesthetized, the thorax was opened, and 10 μg of FITC (Calbiochem) was injected per thymic lobe (24). The thorax was closed, and, 48 h later, splenocytes were stained with CD4, CD8, and CD44 and analyzed by flow cytometry. The $FITC^+CD44^-$ cells were considered recent thymic emigrants (RTEs). The total number of $FITC^+$ cells in the spleen was divided by the percentage of FITC labeling observed in the thymus of each mouse to normalize for incorporation rates between mice (24).

T Cell Proliferation Assays. T cell proliferation was measured by *in vivo* incorporation of BrdUrd according to the manufacturer's instructions using the BD Pharmingen FITC BrdUrd flow kit. Mice were injected i.p. with BrdUrd (1 mg per mouse) 72 h before harvesting of cells, a time point previously determined to be necessary to see measurable T cell proliferation in the BM.

Thymectomy. C57BL/6 mice were either sham-operated (sham THX) or thymectomized (THX) at 11 weeks of age. Mice were anesthetized, the thymus was removed by suction, and the incision was closed by staples. One week later, the mice were either ovx or sham-operated. ovx mice were treated with either irrelevant Ab (IRR Ab) or IL-7 Ab, as described above. Four weeks after ovx, the THX mice were euthanized, and the chest cavity was checked for residual thymic tissue. THX mice, which contained thymic tissue, were excluded from analysis.

Measurement of Antigen Presentation and Class II Transactivator (CIITA) Expression. Assays were performed as described in ref. 5 except that T cells were isolated from splenocytes from 10-week-old OTII transgenic mice (CD4 TCR Tg mouse strain specific for ovalbumin 323–339 in the context of $I\text{-}A^b$), and BM antigen-presenting cells were harvested from sham, ovx IRR, or ovx IL-7 Ab mice. Antigen presentation was assessed by measuring T cell incorporation of [^{3}H]thymidine by liquid scintillation spectroscopy. Macrophage CIITA mRNA levels were measured by real-time PCR as described in ref. 5.

Statistical Analysis. To determine statistical significance, one-way ANOVA with the Tukey–Kramer multiple comparisons post test was performed by using INSTAT 3.0B for Macintosh (GraphPad, San Diego). $P < 0.05$ was considered statistically significant.

Results

ovx Induces Increased IL-7 Synthesis and T Cell TNF-α Production. IL-7 is central to ovx-induced bone loss (11), but the sources of the cytokine are unknown. In E replete mice, IL-7 mRNA levels were approximately seven times higher in the thymus than in the spleen and BM, and ovx caused a further 2- to 3-fold increase in each of these organs (Fig. 1*a*). Thus, the thymus, spleen, and BM are all lymphoid sources of IL-7 in ovx mice.

To investigate whether IL-7 mediates the expansion of the T cell pool by which ovx causes bone loss (4), 12-week-old mice were sham-operated and left untreated or ovx and treated with either the anti-IL-7 Ab M25 (IL-7 Ab) or isotype-matched IRR Ab for 4 weeks, as described in ref. 11. In the BM, ovx led to an ≈2-fold increase in both the number of $CD4^+$ T cells (Fig. 1*b*) and the population of TNF-α-secreting $CD4^+$ T cells (Fig. 1*c*). Such changes were completely prevented by IL-7 Ab treatment, demonstrating that ovx increases TNF-α producing T cells by means of an IL-7-dependent mechanism.

ovx Expands HSCs, Common Lymphoid Precursors, and Early T Cell Populations Through IL-7. The mechanism by which E deficiency drives the expansion of the pool of TNF-α-secreting T cells, critical to bone destruction in ovx, is largely unknown. To further investigate this issue, we examined the effects of ovx on the earliest stages of T cell development. HSCs ($Lin^-Sca\text{-}1^{high}c\text{-}Kit^{high}$IL-7R$\alpha^-$), which include the earliest T cell precursors, and CLP cells ($Lin^-Sca\text{-}1^{lo}c\text{-}Kit^{lo}$IL-7R$\alpha^+$), which represent the earliest B cell precursors (21), were analyzed in the BM. The data show an ≈3-fold up-regulation of HSCs (Fig. 1*d*) and an increase in CLP cells (Fig. 1*e*) after ovx, which were prevented in part by the anti-IL-7 Ab. The CLP cell data are consistent with the previous demonstration that E is a negative regulator of IL-7-responsive B cell progenitors (25). However, the HSC findings demonstrate that ovx up-regulates the earliest stages of T cell development within the BM and the critical role of IL-7 in this process.

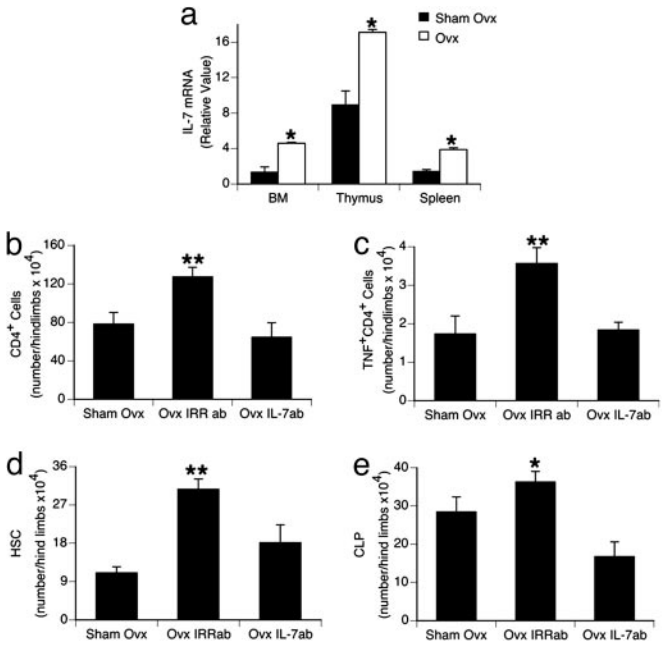

Fig. 1. ovx induces increases in IL-7, T cells, and stem cells. (*a*) Real-time PCR of the relative quantity of IL-7 in the BM, thymus, and spleen after ovx. All samples were performed in triplicate, were normalized to actin as an input control, and are relative to sham controls. (*b* and *c*) Number of BM $CD4^+$ T cells (*b*) and number of TNF-α^+CD4^+ T cells (*c*) in the BM as measured by intracellular cytokine staining. Data (mean ± SEM) are representative of at least three independent experiments with four to six mice per group. *, $P < 0.05$; **, $P < 0.001$ compared with all other groups. To determine HSC and CLP cell levels, BM cells were gated on Lin^- cells (B220, CD11b, GR-1, Ly76, CD3, and THY1), stained for c-Kit, IL-7 Rα, and Sca-1, and analyzed by FACS. (*d* and *e*) HSCs defined as Lin^-IL-7Rα^-c-$Kit^+Sca\text{-}1^+$ (*d*) and CLP cells defined as Lin^-IL-7Rα^+c-$Kit^-Sca\text{-}1^-$ (*e*). Data (mean ± SEM) are representative of at least two independent experiments with four to six mice per group. **, $P < 0.001$ compared with all other groups; *, $P < 0.05$ compared with IL-7 Ab.

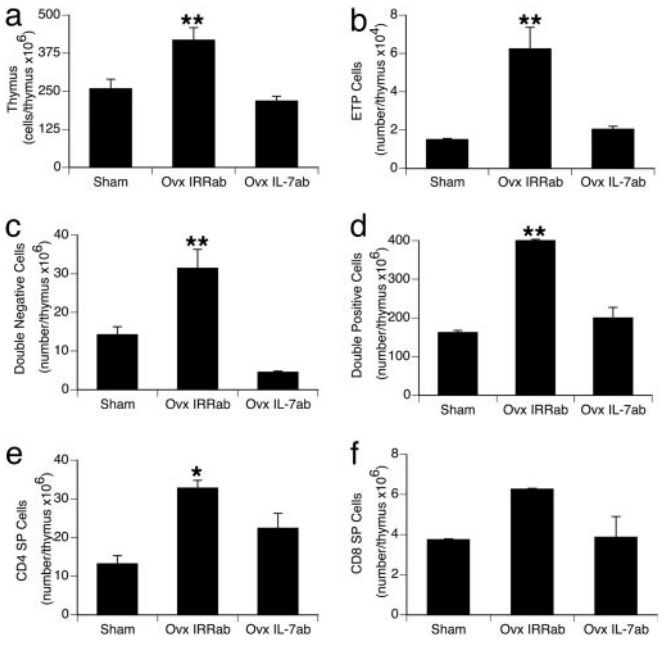

Fig. 2. IL-7 regulates early T cell populations in the thymus. (*a*) Total number of thymocytes. (*b*) Total number of ETP cells (Lin^-c-Kit^+IL-7Ra$^-CD44^+CD25^-$). (*c*) Total number of double-negative T cells ($Lin^-CD4^-CD8^-$). (*d–f*) Thymocytes were gated on Lin^- cells and stained with CD4 and CD8 to determine $CD4^+CD8^+$ double-positive (*d*), $CD4^+CD8^-$ SP (*e*), and $CD4^-CD8^+$ SP (*f*) populations. Data (mean ± SEM) are representative of at least three independent experiments with four to six mice per group. **, $P < 0.005$ compared with all other groups; *, $P < 0.05$ compared with sham.

T cells originate in the thymus from BM precursors present in the HSC pool. Thymic-derived signals direct the migration and the seeding of these BM precursors into the thymus at distinct temporal windows (26). The earliest identifiable intrathymic T cell precursor is the ETP cell (Lin-c-Kit^{high}IL-$7R^{high}CD44^+CD25^-$) (21, 22). We found that ovx causes an ≈1.5-fold increase in the total cellularity of the thymus (Fig. 2*a*), an ≈3-fold increase in ETP cells (Fig. 2*b*), and an ≈2- to 4-fold increase in the total pool of double-negative cells ($CD3^+CD4^-CD8^-$) (Fig. 2*c*), $CD4^+CD8^+$ double-positive cells (Fig. 2*d*), and CD4 single-positive (SP) cells, but CD8 SP T cell populations did not reach significance (Fig. 2 *e* and *f*). The ovx-induced changes in the number of all of these cell populations did not occur in ovx mice treated with anti-IL-7 Ab, thus demonstrating that ovx leads to an increase in thymocyte populations through an IL-7-dependent mechanism.

IL-7 Ab Prevents the Increase in both Thymic Output and T Cell Peripheral Expansion and Activation Induced by ovx. To determine whether E deficiency enhances thymic output, TREC assays were performed on cells harvested from the thymus after ovx. TRECs are stable extrachromosomal pieces of DNA generated during T cell receptor alpha gene rearrangement. Because TREC DNA does not replicate as the T cell divides, as T cells become activated and proliferate, the amount of TREC DNA is diluted compared with naïve RTEs (23, 27). Fig. 3*a* shows a 3-fold ovx-induced decrease in the number of TRECs measured in thymic tissue that was completely prevented by anti-IL-7 Ab.

A reduction in thymic TREC DNA may be due to dilution of TRECs within the thymus as a result of elevated T cell proliferation and expansion, or it may be a consequence of increased export of naïve T cells from the thymus. To differentiate between these two mechanisms, we measured SP T cell proliferation in the thymus by *in vivo* BrdUrd incorporation. ovx induced (Fig. 3*b*) an increase in

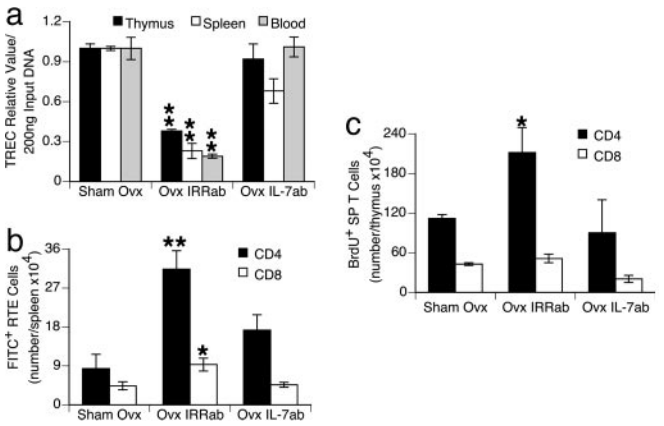

Fig. 3. ovx induces an IL-7-dependent increase in thymic export and dilution of TREC. (*a*) Real-time PCR of $TREC^+$ DNA in the thymus, spleen, and whole blood after ovx. All values are relative to sham controls. Data (mean ± SEM) are representative of at least three independent experiments. (*b*) The total number of proliferating ($BrdUrd^+$) CD4 and CD8 SP T cells in the thymus after ovx. (*c*). Intrathymic FITC injection after ovx or sham operation. Forty-eight hours after injection, thymus and spleen were harvested, and percentage of FITC was measured by FACS. Naïve CD4 RTEs and naïve CD8 RTEs were defined as $FITC^+$ and $CD44^-$. The percentage of FITC dye incorporated into the thymus was used to adjust for variability among mice. Data (mean ± SEM) are representative of two independent experiments with five mice per group. *, $P < 0.05$; **, $P < 0.001$ compared with all other groups.

CD4 SP T cell proliferation within the thymus, indicating that the reduction of thymic TRECs is due in part to increased SP T cell proliferation. To determine whether increased export from the thymus was also contributing to the reduction of TRECs, we directly injected FITC into the thymus of mice from all groups. Forty-eight hours after intrathymic FITC injection, there was an ≈3-fold increase in the number of $FITC^+$ $CD4^+CD44^-$ RTEs in the spleens of ovx mice treated with IRR Ab but not with anti-IL-7 Ab (Fig. 3*c*). Thus, the reduction in thymic TREC observed in ovx mice is due to both increased preexport expansion and increased thymic export, and down-regulation of IL-7 *in vivo* prevents these actions.

ovx also caused a 3-fold decrease in TREC levels in spleen and whole-blood samples from ovx mice that was prevented by anti-IL-7 Ab (Fig. 3*a*). A decreased value of TREC in the periphery may reflect either blunted export of naïve T cells from the thymus or dilution of TREC secondary to increased RTE proliferation (peripheral expansion). Because ovx leads to increased export of naïve T cells from the thymus (Fig. 3*c*), the data indicate that ovx stimulates naïve RTE proliferation through IL-7. An analysis of either naïve (RTEs and older peripheral naïve T cells) or activated/memory T cells by *in vivo* BrdUrd labeling revealed that, in both the spleen and the BM of ovx mice, there was an increase in proliferation of naïve ($CD44^-$) and memory ($CD44^+$) CD4 T cells that was prevented by *in vivo* IL-7 neutralization (Table 1, which is published as supporting information on the PNAS web site). Thus, the reduction in peripheral TRECs observed in ovx mice is the result of an IL-7-mediated increase in naïve T cell proliferation that subsequently leads to a dilution in TREC values. The data also show that in addition to regulating naïve T cell proliferation, ovx up-regulates activated/memory T cell proliferation in a manner that is at least partially dependent on IL-7.

Furthermore, the ovx-induced increase in T cell proliferation correlates with T cell activation, as assessed by the expression of the early T cell activation marker CD69. ovx increased by 1.5- to 2-fold the number of activated T cells in the spleen and BM, and IL-7 neutralization prevented this phenomenon (Table 1). Together, these data demonstrate that, through IL-7, ovx induces increased export of T cells from the thymus, increased proliferation of naïve and memory T cells, and increased T cell activation after ovx.

IL-7 Neutralization Prevents the Increase in Macrophage Antigen Presentation Induced by ovx Through Modulation of CIITA Expression. ovx up-regulates T cell proliferation through stimulation of macrophage antigen presentation (5, 28). In the BM, this stimulation is due to the ability of ovx to increase the level of IFN-γ, which in turn induces the expression of CIITA (5), the master regulator of MHC class II antigen presentation (29). Because T cell activation and proliferation are regulated by IL-7, ovx could regulate T function through an effect of IL-7 on the IFN-γ/CIITA/antigen presentation pathway. Indeed, ovx caused an ≈2-fold increase in the number of $CD4^+$ cells producing IFN-γ in the BM (Fig. 4*a*), a 3-fold increase in macrophage CIITA expression (Fig. 4*b*), and a 10-fold up-regulation of macrophage antigen presentation (Fig. 4*c*). All of these phenomena were completely prevented by treatment with anti-IL-7 Ab. Thus, ovx enhances activation-induced T cell activation, proliferation, and the resulting peripheral expansion of T cells by increasing macrophage antigen presentation through a multistep, IL-7-dependent mechanism.

Thymic-Derived T Cells Are Responsible for ≈50% of the ovx-Induced Bone Loss. To determine the relative contribution of thymic T cell export versus the expansion of T cells in the periphery to the bone loss induced by ovx, mice were THX or subjected to control surgery (sham THX) and, 1 week later, underwent either ovx or sham ovx. ovx mice were treated with neutralizing IL-7 Ab or IRR Ab for 4 weeks. Bone loss was assessed by sequential *in vivo* measurement of BMD by DEXA (dual-energy x-ray absorptiometry), a highly precise and sensitive technique using the method described in ref. 11. ovx in THX mice led to approximately half of the bone loss seen in sham THX mice (Fig. 5*a*), demonstrating an essential role for both RTEs and proliferating peripheral T cells in ovx-induced bone loss. Furthermore, *in vivo* IL-7 neutralization prevented bone loss in both euthymic and THX mice, demonstrating that neutralizing IL-7 is sufficient to prevent both the thymic-dependent and -independent mechanisms of E deficiency-induced bone loss.

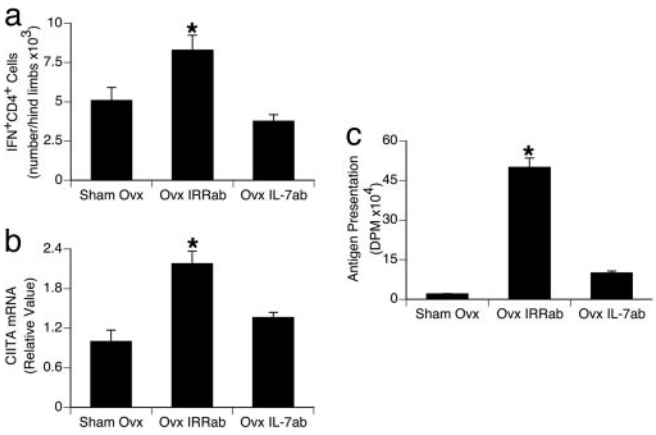

Fig. 4. ovx-induced increases in IFN-γ production and antigen presentation are blocked by anti-IL-7 Ab. (*a*) The total number of IFN-γ^+ CD4 T cells in BM was measured by intracellular cytokine staining. (*b*) Real-time RT-PCR measurement of CIITA mRNA in $CD11b^+$ BM macrophages. (*c*) Antigen-presenting cell assay of $CD11b^+$ BM macrophages as assessed by incorporation of [^{3}H]thymidine by target T cells. Data (mean ± SEM) are representative of at least three independent experiments. *, $P < 0.05$ compared with all other groups.

In response to ovx, THX mice demonstrated a significant increase in the number of activated ($CD69^+$) T cells (Table 1) as well as the number of TNF-α-producing T cells (Fig. 5*b*), which was prevented with anti-IL-7 Ab. However, the increase in T cell activation and cytokine production observed in the BM of THX/IRR Ab mice was only 50% of the level observed in sham THX/IRR Ab mice, consistent with the reduced bone loss observed in these mice. The THX/IRR Ab mice also demonstrated an IL-7-dependent increase in proliferation of naïve CD4 T cells in the spleen and BM, although it was at a reduced level compared with euthymic mice. We also observed an ovx-induced increase in proliferation of memory CD4 cells in the athymic ovx mice at comparable levels to the euthymic mice, thus confirming that the expansion of the memory T cell pool remains responsive to ovx in the absence of the thymus (Table 1). Together, these results demonstrate that both newly generated naïve T cells and the peripheral T cells derived from peripheral expansion contribute to the increase in T cell populations after ovx. Furthermore, in the absence of the thymus, peripheral T cells are activated, expand, and produce TNF-α in an IL-7-dependent manner.

Discussion

Although E withdrawal is known to up-regulate IL-7 levels in the BM (11), to our knowledge, this report is the first to demonstrate an increase in thymic levels of IL-7 in response to ovx. Unexpectedly, we also found that ovx leads to an expansion of the T cell pool by increasing both thymic output and peripheral expansion of T cells. Both phenomena are the end result of multiple effects of ovx, including stimulation of HSCs in the BM, thymocyte expansion, antigen presentation, and T cell activation. Restoration of physiologic IL-7 levels prevents all of these changes. Thus, up-regulation of T cell mass and the resulting bone loss induced by ovx are likely to be the end result of multiple effects of stimulated IL-7 production. However, these changes could also be explained by a few key upstream effects of IL-7, such as stimulation of thymopoiesis and T

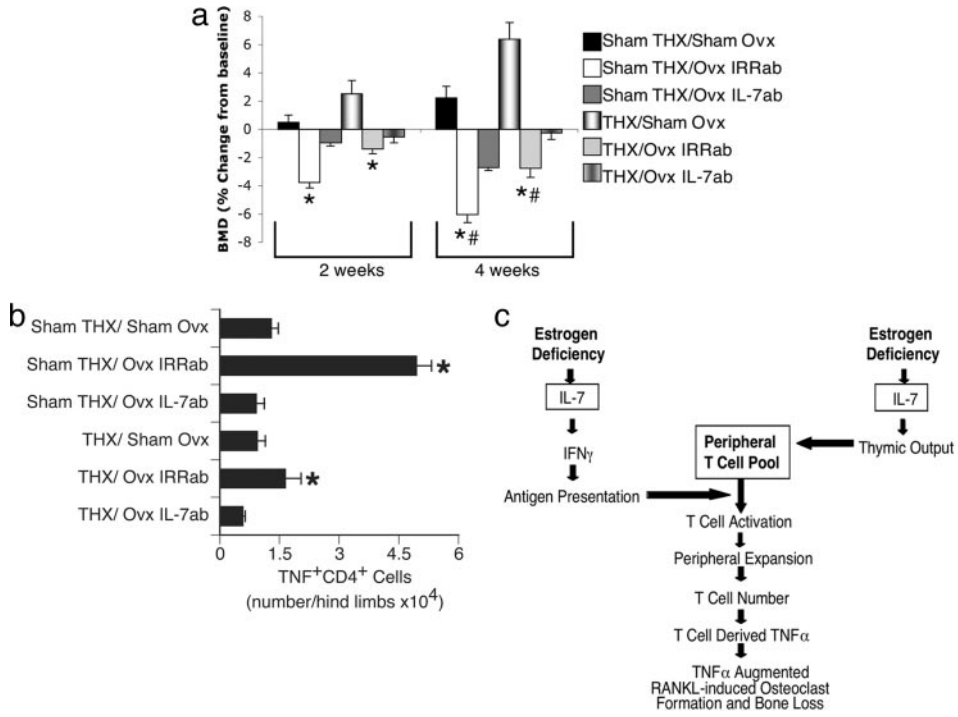

Fig. 5. ovx induces bone loss and TNF-α production in THX mice. (*a*) Mice were either THX or sham THX and, 1 week later, were sham ovx, ovx and treated with IRR Ab, or ovx and treated with anti-IL-7 Ab. Total body BMD was measured at baseline, 2 and 4 weeks after ovx. All data are expressed as percentage change in BMD from baseline. Data (mean ± SEM) are representative of two independent experiments with six to eight mice per group. There was no significant difference between sham THX/sham ovx and THX/sham ovx mice. *, $P < 0.001$ compared with corresponding sham ovx and IL-7 Ab groups; #, $P < 0.01$, comparing sham THX/IRR Ab and THX/IRR Ab. (*b*) The number of TNF-α^+ CD4$^+$ T cells in the BM was measured by intracellular cytokine staining. *, $P < 0.01$; **, $P < 0.001$ compared with all other groups. (*c*) Proposed mechanism of E regulation of T cell TNF-α production. E deficiency leads to increased production of IL-7 in the BM and the thymus. IL-7 stimulation of the thymus augments thymopoiesis and thymic output, leading to an increase in the size of the peripheral T cell pool. IL-7 stimulates the production of IFN-γ, which acts on macrophages to up-regulate CIITA and MHC class II antigen presentation. Together with an IL-7-dependent stimulation of the basal level of T cell activation, increased antigen presentation leads to increased T cell proliferation and increased T cell numbers. An increase in the number of TNF-α-producing T cells thus leads to an augmentation of RANKL (receptor activator of NF-κB ligand)-induced osteoclast formation and bone loss.

cell IFN-γ production. Such effects could trigger the additional downstream modifications observed herein.

A significant finding of this study is the IL-7-dependent increase in HSCs in response to ovx. Because HSCs do not express IL-7R (22), the expansion of HSCs observed in ovx mice is likely not the direct result of IL-7 on HSCs. This HSC expansion may be the result of an indirect self-renewal feedback loop triggered to replenish the stem cell populations that cycle through the thymus at an enhanced rate under the control of E deficiency and IL-7. In fact, recent studies have demonstrated a central role for the thymus in regulating the formation and release of early progenitors from the BM and their seeding of the thymus (26).

An unknown issue addressed herein is that of the effects of ovx on thymic T cell output and its impact on the increase in BM T cells observed in ovx mice. Our findings demonstrate that ovx mediates the expansion of thymic T cells and increases the export of naïve T cells from the thymus. Furthermore, the data suggest that T cell renewal and expansion are regulated differently in the presence or absence of physiologic E levels. In fact, whereas T cell mass is mainly regulated by peripheral expansion in physiologic conditions, thymic output becomes more relevant in E deficiency due to increased levels of IL-7. Not only does this cytokine induce a state of stimulated peripheral expansion (30), but it also abrogates a major compensatory mechanism, the capacity of thymic emigrants to suppress such expansion (12). As a result, the ovx-induced stimulation of thymic output is accompanied by an inappropriate peripheral expansion, and the combination of these events results in an enlarged T cell pool.

Our demonstration that the thymus plays a previously unrecognized causal effect in ovx-induced bone loss is not in conflict with the known effects of aging on thymic function. By middle age, most thymic parenchymal tissue is replaced by fat, and in both mice and humans, fewer T cells are produced and exported to secondary lymphoid organs as aging progresses. However, lymphocytic thymic tissue has been documented in adults up to 107 years of age (31), and recent evidence has shown that functional thymic tissue continues to contribute naïve T cells in humans until at least the sixth decade of life (32). Furthermore, mature human subjects treated with autologous BM transplants develop thymic hypertrophy and a marked resurgence of thymic T cell output, which contributes to the restoration of a wide T cell repertoire (33).

Although a role for increased thymic output in postmenopausal bone loss in humans awaits demonstration, increased thymic output could be particularly relevant for the bone loss in young women undergoing surgical menopause (34) or for the rapid bone loss characteristic of women in their first 5–7 years after natural menopause (35). Indeed, an age-related decrease in thymic T cell output could mitigate the stimulatory effects of sex steroid deprivation and explain why the rate of bone loss in postmenopausal women diminishes as aging progresses (35).

Because IL-7 is essential for baseline lymphopoiesis, in this study, anti-IL-7 Ab was titrated so as to reduce the levels of IL-7 to the sham range without causing an absolute deficiency in this cytokine. Evidence that M25 treatment does not completely silence IL-7 includes direct measurements of IL-7 levels (data not shown) and the finding that, in response to M25 treatment, all of the measured

indices decreased to, but not below, the level observed in sham controls. This strategy led us to demonstrate that IL-7 is a critical cytokine that regulates downstream immune functions relevant for bone involution (Fig. 5*c*) and that ovx stimulates thymic T cell output through IL-7. This factor is known to increase thymopoiesis and thymic T cell output and to reverse, in part, age-dependent thymic involution (36, 37). Genetic and *in vivo* neutralization studies have, in fact, shown that silencing of IL-7 or its receptor causes profound defects in thymic cellularity (38–40) with a block in thymocyte development (20). In a manner similar to IL-7, ovx leads to increased generation and maturation of thymocytes and enhanced thymic output. Attesting to the potency of the regulatory effects of sex steroids, ovx has been shown to restore thymic function in aged mice and rats (41), whereas E treatment induces thymic atrophy (42, 43). Furthermore, studies in rodents and humans have demonstrated that the loss of a repressive effect of sex steroids may explain a slower involution of the thymus in female aging mice than in male aging mice (44) and why aged women have increased numbers of circulating RTEs compared with aged men (45). Although the opposing effects of E and IL-7 on thymic function have long been recognized, to our knowledge, our study is the first to suggest a link between these mechanisms and to demonstrate that ovx stimulates thymic activity through IL-7. In addition, we demonstrate that induced thymic enlargement is not merely an artifact of E deficiency but significantly contributes to the T cell pool responsible for ovx-induced bone loss.

As disclosed by this study, another mechanism by which ovx increases the number of T cells and promotes bone loss is stimulating T cell peripheral expansion through an IL-7-dependent mechanism. IL-7 promotes both antigen-independent (46) and antigen-dependent (15) proliferation of naïve T cells. In addition, IL-7 decreases the threshold of T cell activation in response to weak or tolerogenic antigens and facilitates memory T cell generation (13). Accordingly, we found that IL-7 Ab prevents the ovx-induced increase in the peripheral expansion of naïve and memory T cells.

The ability of IL-7 to increase T cell reactivity to antigens offers additional insight on the central role of IL-7 for ovx-induced bone loss. ovx leads to increased production of IFN-γ and to stimulated antigen presentation by macrophages (5). The current investigation shows that IL-7 neutralization prevents ovx from increasing T cell production of IFN-γ and antigen presentation. These effects, along with the ability of IL-7 to lower the T cell activation threshold, indicate that ovx regulates antigen presentation through multiple effects of IL-7 on both T cells and macrophages.

Another mechanism by which ovx stimulates IFN-γ production is the blunting TGF-β levels in the BM (6). Because the production of TGF-β and IL-7 is under reciprocal control (47, 48), the evidence suggests that a complex cytokine cascade regulated by E accounts for the increase in IFN-γ-driven antigen presentation observed in estroprevic mice. Furthermore, E is known to directly stimulate TGF-β gene expression (49) and indirectly repress IL-7 (11). The human and murine IL-7 promoters do not contain binding regions for nuclear proteins known to mediate a repressive effect of E. Thus, it is conceivable that E represses IL-7 indirectly by stimulating TGF-β gene expression.

In summary, we have found that IL-7 is a key upstream cytokine, and potential therapeutic target, through which E deficiency regulates immune functions that are critical for bone homeostasis.

This work was supported in part by National Institutes of Health (NIH) Grants DK55746 and AR49659. M.N.W. was supported in part by NIH Grant DK067389 and by the National Osteoporosis Foundation. M.R.R. was supported by NIH/National Institute of Diabetes and Digestive and Kidney Diseases Grant T32 DK007298.

1. Pfeilschifter, J., Koditz, R., Pfohl, M. & Schatz, H. (2002) *Endocr. Rev.* **23,** 90–119.
2. Nanes, M. S. (2003) *Gene* **321,** 1–15.
3. Cenci, S., Weitzmann, M. N., Roggia, C., Namba, N., Novack, D., Woodring, J. & Pacifici, R. (2000) *J. Clin. Invest.* **106,** 1229–1237.
4. Roggia, C., Gao, Y., Cenci, S., Weitzmann, M. N., Toraldo, G., Isaia, G. & Pacifici, R. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 13960–13965.
5. Cenci, S., Toraldo, G., Weitzmann, M. N., Roggia, C., Gao, Y., Qian, W. P., Sierra, O. & Pacifici, R. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 10405–10410.
6. Gao, Y., Qian, W. P., Dark, K., Toraldo, G., Lin, A. S., Guldberg, R. E., Flavell, R. A., Weitzmann, M. N. & Pacifici, R. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 16618–16623.
7. Finkelman, R. D., Bell, N. H., Strong, D. D., Demers, L. M. & Baylink, D. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 12190–12193.
8. Gorelik, L. & Flavell, R. A. (2002) *Nat. Rev. Immunol.* **2,** 46–53.
9. Weitzmann, M. N., Cenci, S., Rifas, L., Brown, C. & Pacifici, R. (2000) *Blood* **96,** 1873–1878.
10. Toraldo, G., Roggia, C., Qian, W. P., Pacifici, R. & Weitzmann, M. N. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 125–130.
11. Weitzmann, M. N., Roggia, C., Toraldo, G., Weitzmann, L. & Pacifici, R. (2002) *J. Clin. Invest.* **110,** 1643–1650.
12. Mackall, C. L., Fry, T. J., Bare, C., Morgan, P., Galbraith, A. & Gress, R. E. (2001) *Blood* **97,** 1491–1497.
13. Fry, T. J. & Mackall, C. L. (2002) *Blood* **99,** 3892–3904.
14. Jaleco, S., Swainson, L., Dardalhon, V., Burjanadze, M., Kinet, S. & Taylor, N. (2003) *J. Immunol.* **171,** 61–68.
15. Tan, J. T., Dudl, E., LeRoy, E., Murray, R., Sprent, J., Weinberg, K. I. & Surh, C. D. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 8732–8737.
16. Kondrack, R. M., Harbertson, J., Tan, J. T., McBreen, M. E., Surh, C. D. & Bradley, L. M. (2003) *J. Exp. Med.* **198,** 1797–1806.
17. Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J. & Surh, C. D. (2002) *J. Exp. Med.* **195,** 1523–1532.
18. Borger, P., Kauffman, H. F., Postma, D. S. & Vellenga, E. (1996) *J. Immunol.* **156,** 1333–1338.
19. Cenci, S., Weitzmann, M. N., Gentile, M. A., Aisa, M. C. & Pacifici, R. (2000) *J. Clin. Invest.* **105,** 1279–1287.
20. Grabstein, K. H., Waldschmidt, T. J., Finkelman, F. D., Hess, B. W., Alpert, A. R., Boiani, N. E., Namen, A. E. & Morrissey, P. J. (1993) *J. Exp. Med.* **178,** 257–264.
21. Bhandoola, A., Sambandam, A., Allman, D., Meraz, A. & Schwarz, B. (2003) *J. Immunol.* **171,** 5653–5658.
22. Allman, D., Sambandam, A., Kim, S., Miller, J. P., Pagan, A., Well, D., Meraz, A. & Bhandoola, A. (2003) *Nat. Immunol.* **4,** 168–174.
23. Sempowski, G. D., Gooding, M. E., Liao, H. X., Le, P. T. & Haynes, B. F. (2002) *Mol. Immunol.* **38,** 841–848.
24. Scollay, R. G., Butcher, E. C. & Weissman, I. L. (1980) *Eur. J. Immunol.* **10,** 210–218.
25. Kouro, T., Medina, K. L., Oritani, K. & Kincade, P. W. (2001) *Blood* **97,** 2708–2715.
26. Donskoy, E., Foss, D. & Goldschneider, I. (2003) *J. Immunol.* **171,** 3568–3575.
27. Hazenberg, M. D., Verschuren, M. C., Hamann, D., Miedema, F. & van Dongen, J. J. (2001) *J. Mol. Med.* **79,** 631–640.
28. Wira, C. R., Rossoll, R. M. & Kaushic, C. (2000) *Endocrinology* **141,** 2877–2885.
29. Reith, W. & Mach, B. (2001) *Annu. Rev. Immunol.* **19,** 331–373.
30. Fry, T. J. & Mackall, C. L. (2001) *Trends Immunol.* **22,** 564–571.
31. Steinmann, G. G., Klaus, B. & Muller-Hermelink, H. K. (1985) *Scand. J. Immunol.* **22,** 563–575.
32. Jamieson, B. D., Douek, D. C., Killian, S., Hultin, L. E., Scripture-Adams, D. D., Giorgi, J. V., Marelli, D., Koup, R. A. & Zack, J. A. (1999) *Immunity* **10,** 569–575.
33. Hakim, F. T., Memon, S. A., Cepeda, R., Jones, E. C., Chow, C. K., Kasten-Sportes, C., Odom, J., Vance, B. A., Christensen, B. L., Mackall, C. L. & Gress, R. E. (2005) *J. Clin. Invest.* **115,** 930–939.
34. Hreshchyshyn, M. M., Hopkins, A., Zylstra, S. & Anbar, M. (1988) *Obstet. Gynecol.* **72,** 631–638.
35. Riggs, B. L., Khosla, S. & Melton, L. J., III (2002) *Endocr. Rev.* **23,** 279–302.
36. Aspinall, R. & Andrew, D. (2001) *Cell Immunol.* **212,** 150–157.
37. Andrew, D. & Aspinall, R. (2002) *Exp. Gerontol.* **37,** 455–463.
38. Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M. & Yoshida, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9125–9129.
39. Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., *et al.* (1994) *J. Exp. Med.* **180,** 1955–1960.
40. von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. & Murray, R. (1995) *J. Exp. Med.* **181,** 1519–1526.
41. Utsuyama, M. & Hirokawa, K. (1989) *Mech. Ageing Dev.* **47,** 175–185.
42. Okasha, S. A., Ryu, S., Do, Y., McKallip, R. J., Nagarkatti, M. & Nagarkatti, P. S. (2001) *Toxicology* **163,** 49–62.
43. Silverstone, A. E., Frazier, D. E., Jr., Fiore, N. C., Soults, J. A. & Gasiewicz, T. A. (1994) *Toxicol. Appl. Pharmacol.* **126,** 248–259.
44. Aspinall, R. & Andrew, D. (2001) *Dev. Immunol.* **8,** 95–106.
45. Pido-Lopez, J., Imami, N. & Aspinall, R. (2001) *Clin. Exp. Immunol.* **125,** 409–413.
46. Brenchley, J. M., Douek, D. C., Ambrozak, D. R., Chatterji, M., Betts, M. R., Davis, L. S. & Koup, R. A. (2002) *Clin. Exp. Immunol.* **130,** 432–440.
47. Tang, J., Nuccie, B. L., Ritterman, I., Liesveld, J. L., Abboud, C. N. & Ryan, D. H. (1997) *J. Immunol.* **159,** 117–125.
48. Dubinett, S. M., Huang, M., Dhanani, S., Wang, J. & Beroiza, T. (1993) *J. Immunol.* **151,** 6670–6680.