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Influence of Estrogen Deficiency and Replacement on T-Cell Populations in Rat Lymphoid Tissues and Organs

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Estrogen deficiency following ovariectomy or menopause results in bone loss. Although evidence strongly suggests that the immune system is involved in the pathogenesis of estrogen-deficient osteoporosis, it is not clear what role, if any, the T-lymphocyte plays in this process. Therefore, we examined the distribution of T-cell subsets in lymphoid organs and tissues, under varying estrogenic states in the rat. Six-month-old female Sprague-Dawley rats, ovariectomized (Ovx) and sham-operated, were randomized 5 d post-surgery into six groups to receive the following treatments: (A) sham/placebo; (B) sham/low-dose E_2; (C) sham/high-dose E_2; (D) Ovx/placebo; (E) Ovx/low-dose E_2; (F) Ovx/high-dose E_2. Half of the treated rats (groups A–F) were sacrificed on d 14; the remainder on d 28. Following euthanasia, mononuclear cells were isolated from the thymus, peripheral blood, spleen, lymph node and bone marrow, and were labeled for flow cytometric analysis using mouse anti-rat monoclonal antibodies directed against CD5, CD4, and CD8 antigenic markers. In the thymus, ovariectomy caused a dramatic increase and E_2 treatment caused a dose-dependent decrease in weight that was proportional to the number of thymocytes. In the bone marrow, ovariectomy caused a significant reduction in the percentage of all T-cell subsets examined and this effect persisted throughout the duration of the study. Estrogen replacement therapy at the low-dose reversed the effects of ovariectomy and high-dose E_2 treatment caused an increase in T-cell subsets in both the sham and Ovx groups, an effect that was more pronounced at d 14 compared with d 28. Although the percentages of some T-cell subsets in the other lymphoid organs/tissues were altered by ovariectomy or E_2 treatment at d 0 and 14, all these changes had normalized by d 28 except for CD5 and CD4 cells in peripheral blood. In summary, with the exception of T-lymphocytes in the bone marrow, the effects of varying estrogenic states on T-cells were variable and transient. The influence of estrogen status on bone marrow T-lymphocytes suggests that these cells may play a role in mediating the effects of estrogen on bone turnover and warrant additional studies focusing on the functional role of T-cells in the bone marrow compartment.

Key Words: Estrogen deficiency; estrogen replacement; T-lymphocytes; ovariectomy; lymphoid tissue.

Introduction

Postmenopausal osteoporosis is a common metabolic bone disorder occurring after natural or surgical menopause and is characterized by reduced bone mass associated with an increased risk of spontaneous fractures. It is widely accepted that a decrease in circulating estrogen following menopause is responsible for mediating the events that ultimately result in bone loss. Estrogen deficiency following loss of gonadal function causes an increase in osteoblast and osteoclast function, with the increase in bone resorption exceeding the increase in bone formation, thereby causing a net loss of bone *(1)*. Studies have demonstrated that estrogen replacement therapy decreases the rate of bone loss and occurrence of fractures in postmenopausal women *(2)*. However, the cellular and molecular mechanisms responsible for estrogen-deficient bone loss have yet to be determined.

Numerous immune modulators (e.g., cytokines) have been shown to have complex, overlapping effects on bone mineral metabolism *(3)*. Recent clinical and experimental observations suggest a possible role for the immune system and its soluble mediators in the pathogenesis of estrogen-deficient osteopenia. Estrogen deficiency is associated with an increase in the production of interleukin-1 (IL-1) *(4,5)* and tumor necrosis factor-α from peripheral blood mono-

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cytes and bone marrow macrophages and increased production of IL-6 by osteoblasts and bone marrow stromal cells *(6–11)*. The net effect of an increase in these cytokines on bone is to stimulate osteoclast development and function. Although T-lymphocytes have been shown to modulate the production of bone-resorbing cytokines, it is unclear what role the T-lymphocyte plays in mediating the effects of the immune system on bone in response to estrogen deficiency.

It is well established that sex steroid hormones modulate thymic size in experimental animals. During pregnancy, the increase in circulating sex steroids induces a transient involution of the thymus with suppression of the immune response *(12,13)*. By contrast, ovariectomy results in hyperplasia of the reticular-endothelial system, including the thymus, although it is not clear whether there is a corresponding increase in T-lymphocytes *(13)*. Although an increase in the blood $CD4^+$:$CD8^+$ ratio has been reported in some postmenopausal women with osteoporosis *(14)*, this finding is not consistent in all patients. A more recent study in rats demonstrated that the percentage of $CD4^+$ and $CD8^+$ T-cells in peripheral blood was unaffected by ovariectomy throughout the 12-wk study period except for a transient increase in $CD4^+$ cells at 1 wk postovariectomy *(15)*.

The T-lymphocyte has been shown to play a critical role in the development of cyclosporin A–induced osteopenia in the rat *(16)*. However, the role of the T-lymphocyte in estrogen-deplete osteopenia remains unclear. In a previous study, we demonstrated that the bone loss was similar following ovariectomy in Rowett homozygous (rnu/rnu) athymic nude rats compared with heterozygous (rnu/+) euthymic rats *(17)*. Despite the fact that the athymic rats had decreased numbers of T-lymphocytes, they were not devoid of lymphocytes bearing T-cell markers. The well-documented effects of estrogen on the thymus combined with the undefined role of T-lymphocytes in estrogen-deficient bone loss prompted us to examine comprehensively the effects of ovariectomy and/or estrogen treatment on the distribution of T-cell subsets in peripheral blood and other lymphoid organs/tissues, including the thymus, lymph node, spleen, and bone marrow. Bone marrow was included because its cells and their products can directly influence bone cell development and function.

Results

The body weights of animals in all groups demonstrated similar gains over the 28-d period of the experiment. There were trends toward higher weights in the ovariectomized (Ovx)-placebo group and lower weights in the high-dose E_2 groups when compared with the sham-operated placebo group, but none of the differences were statistically significant (data not shown). Serum E_2 levels, measured in all animals at d 14 of the experiment, were significantly reduced in Ovx-placebo animals compared to the sham-placebo group (Fig. 1). Circulating levels of E_2 remained within the normal physiological range in the sham-operated group treated with low-dose E_2. This dose is commonly referred to as the physiological dose by the manufacturer of the E_2 pellets used in this study. However, treatment of Ovx rats with this same dose resulted in significant elevation of serum E_2 levels when compared with the sham-operated group (Fig. 1). High-dose E_2 treatment resulted in significant increases in both the sham-operated and ovariectomy groups when compared with their respective placebo controls (Fig. 1), but again the Ovx rats had significantly higher levels compared with the sham-operated rats.

Estrogen deficiency (Ovx) resulted in a dramatic increase in the size of the thymus (Fig. 2). E_2 treatment caused a dose-dependent decrease in thymic weight in both the sham and ovariectomy groups when compared with their respective placebo controls (Fig. 2). Low-dose E_2 replacement in Ovx animals resulted in a reduction in thymic weight compared with Ovx placebo animals, to a size comparable to that in the sham-placebo group. The number of thymocytes harvested was directly proportional to the thymic weights. The average number of thymocytes in sham-placebo animals was $87.7 \pm 13.9 \times 10^6$ cells; it increased up to an average of $223.8 \pm 51.5 \times 10^6$ ($p < 0.05$) in the Ovx-placebo group and decreased to $28.4 \pm 10.4 \times 10^6$ ($p < 0.01$) and $18.7 \pm 4.0 \times 10^6$ ($p < 0.001$) in the sham-low-dose and sham-high-dose groups, respectively. There was a slight decrease in the percentage of $CD5^+$ and $CD4^+CD8^+$ double-positive lymphocytes in the thymus at d 0, which normalized by d 14 (Table 1).

Analysis of T-cell subsets in spleen, lymph node, and peripheral blood revealed significant reductions in some of the T-cell subsets in the Ovx vs sham-operated rats at d 0 (5 d postsurgery) of the experiment (Table 1). Except for the spleen, all these differences had normalized by d 14 of the experiment. The percentage of T-cells expressing $CD5^+$, $CD4^+$, or $CD8^+$ were similar between Ovx-placebo and sham-placebo groups; only in the spleen, were $CD5^+$ and $CD8^+$ cell subsets decreased in the Ovx group. At d 14, there were also some differences as a result of E_2 treatment (Table 1). For example, both low- and high-dose E_2 treatment caused significant increases in splenic $CD5^+$ lymphocytes compared with the placebo group in Ovx rats (Table 1). However, by d 28, all differences resulting from either ovariectomy or E_2 treatment had normalized except in the peripheral blood, where $CD4^+$ lymphocytes were increased in Ovx-placebo compared to sham-placebo rats, and high-dose E_2 caused an increase in $CD5^+$ lymphocytes compared to placebo and low-dose E_2 treatment in the Ovx rats. The CD4:CD8 ratios were not significantly different as a result of ovariectomy or E_2 treatment at any time point.

The most significant observations were made in the bone marrow compartment. Although the number of mature

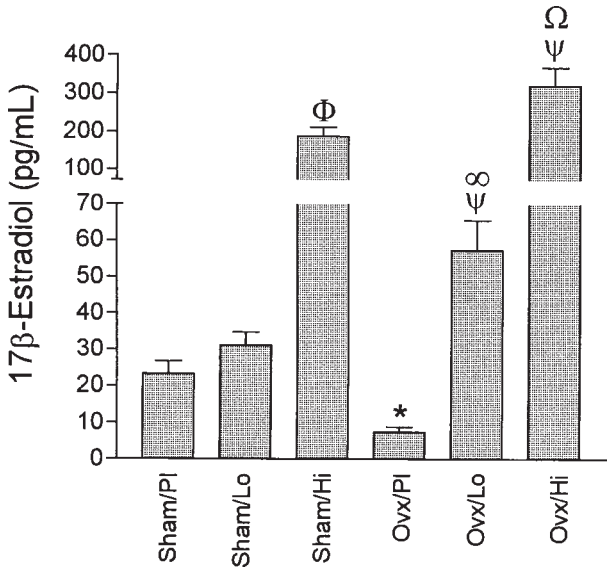

Fig. 1. Serum 17β-estradiol levels were measured in all animals on d 14 following implantation of E_2 pellets. Ovx/Pl is significantly lower compared with Sham/Pl ($^{*}p < 0.001$); Ovx/Lo and Ovx/Hi significantly increased compared with Sham/Lo ($^{y}p < 0.05$) and Sham/Hi ($^{y}p < 0.05$), respectively. Sham/Hi significantly increased compared with Sham/Pl and Sham/Lo ($^{F}p < 0.001$). Ovx/Hi significantly increased when compared to Ovx/Pl and Ovx/Lo ($^{W}p < 0.001$). Ovx/Lo significantly higher compared to Ovx/Pl ($^{\bullet}p < 0.001$). Data are represented as the mean ± SEM.

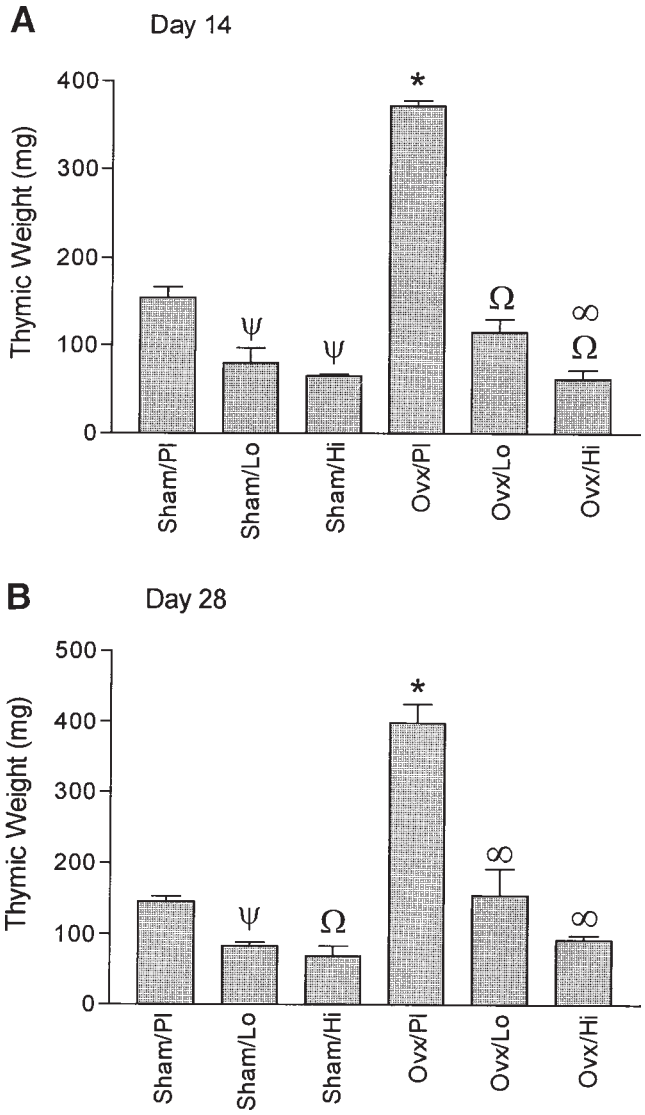

Fig. 2. Thymic weight at 14 and 28 d following E_2 treatment. **(A)** Thymic weights at d 14 of experiment. Thymic size in Ovx/Pl significantly increased compared to Sham/Pl ($^{*}p < 0.001$). Thymic weight in Sham/Lo and Sham/Hi significantly decreased compared with Sham/Pl ($^{y}p < 0.01$). Ovx/Lo and Ovx/Hi thymic weights significantly decreased compared with Ovx/Pl ($^{W}p < 0.001$); Ovx/Hi significantly decreased compared to Ovx/Lo ($^{\bullet}p < 0.01$). Data are represented as the mean ± SEM. **(B)** Thymic weights at d 28 of the experiment. Thymic weight in Ovx/Pl significantly increased compared with Sham/Pl ($^{*}p < 0.001$). Sham/Lo and Sham/Hi significantly decreased compared with Sham/Pl ($^{y}p < 0.01$ and $^{W}p < 0.001$, respectively). Ovx/Lo and Ovx/Hi significantly decreased compared with Ovx/Pl ($^{\bullet}p < 0.001$). Data are represented as the mean ± SEM.

T-cells in the bone marrow is relatively low, these cells are believed to play an important role in the local regulation of bone turnover. The general observation was that ovariectomy caused a decrease and E_2 treatment caused a dose-dependent increase in the T-cell subsets examined (Figs. 3–5). There was a consistent reduction in the percentage of $CD5^+$ (Fig. 3), $CD4^+$ (Fig. 4) and $CD8^+$ (Fig. 5) lymphocytes in Ovx vs sham-operated rats at d 0, and these reductions persisted for the duration of the experiment (d 14 and 28). E_2 treatment caused a dose-dependent increase in the percentage of $CD5^+$, $CD4^+$, and $CD8^+$ T-lymphocytes in both the sham-operated and Ovx rats when compared to their respective placebo controls, and this E_2-mediated effect was more pronounced at d 14 compared to d 28 (Figs. 3–5).

At d 14, low-dose E_2 replacement in Ovx rats restored the percentage of each T-lymphocyte subset to levels that were similar to the sham-placebo group; high-dose E_2 replacement caused an even greater increase (Figs. 3B, 4B, and 5B). This same effect was also observed in sham-operated rats in which E_2 treatment caused a dose-dependent increase in the percentage of T-lymphocytes expressing $CD5^+$, $CD4^+$, and $CD8^+$. At d 28, the response to E_2 treatment was somewhat diminished compared to d 14. Although low-dose E_2 replacement in Ovx rats increased T-lymphocyte subsets compared to the placebo control, high-dose E_2 replacement was necessary to bring the levels up to those observed in sham-operated placebo controls (Figs. 3C, 4C, and 5C). Among the sham-operated rats at d 28, there was also a blunted response to E_2 compared to d 14. The low-dose E_2 treatment had little effect on $CD4^+$ lymphocytes, and there was an unexpected reduction in the percentage of $CD5^+$ and $CD8^+$ lymphocytes (Figs. 3C, 4C,

Table 1
T-Cell Subsets in Lymphoid Organs and Blood

Factor	Day of experiment	Spleen	Lymph node	Blood	Thymus
Ovx	0	$\downarrow CD5^+$; $\downarrow CD8^{+b}$	$\downarrow CD4^+$	$\downarrow CD5^+$; $\downarrow CD4^+$	$\downarrow CD5^+$; $\downarrow CD4^+8^+$
	14	$\downarrow CD5^+$; $\downarrow CD8^{+b}$			$\downarrow CD5^{+f}$; $\downarrow CD4^+8^{+g}$
	28			$\uparrow CD4^{+b}$	
Low E_2	14	$\uparrow CD5^{+c}$			$\downarrow CD4^+8^{+h}$
	28				
High E_2	14	$\uparrow CD5^{+d}$		$\downarrow CD4^{+d}$; $\uparrow CD8^{+d,e}$	
	28			$\uparrow CD5^{+d,e}$; $\uparrow CD4^{+b}$	

[a]Only changes in T-cell subsets that were statistically significant are shown (↑, increased; ↓, decreased; $p < 0.05$). At d 0 (5 d postsurgery), Ovx group was compared to sham-operated group. All other comparisons at d 14 and 28 are as given in footnotes b–h (*see* Materials and Methods for abbreviations). Blank spaces indicate that there was no change in any T-cell subset.
[b]Comparison of Sham/Pl vs Ovx/Pl.
[c]Comparison of Ovx/Pl vs Ovx/Lo.
[d]Comparison of Ovx/Pl vs Ovx/Hi.
[e]Comparison of Ovx/Lo vs Ovx/Hi.
[f]Comparison of Sham/Pl vs Sham/Hi.
[g]Comparison of Sham/Lo vs Sham/Hi.
[h]Comparison of Sham/Pl vs Sham/Hi.

and 5C). The higher dose of E_2 did significantly increase the percentage of $CD4^+$ cells, but had no effect on $CD5^+$ or $CD8^+$ cells compared to sham-placebo rats (Figs. 3C, 4C, and 5C).

Discussion

Alterations in T-lymphocytes have been implicated in the pathogenesis of estrogen-deficient bone loss that occurs following loss of gonadal function *(18)*. In the present study, we examined the effect of ovariectomy and E_2 replacement on T-cell subpopulations in different lymphoid organs and tissues and peripheral blood. Surgical removal of the ovaries in rodents is a widely used model system to study estrogen-deficient osteopenia *(17)*. Following successful ovariectomy of the rats in this study, serum E_2 levels were significantly reduced when compared with sham-operated rats. Circulating E_2 levels remained within the normal physiological range *(19)* in sham-operated rats treated with low-dose E_2, but were significantly elevated in similarly treated Ovx rats. High-dose E_2 treatment resulted in significantly elevated levels of serum E_2 in both Ovx and sham-operated groups when compared to their respective placebo groups. The reason for the intriguing observation that E_2 replacement produced significantly higher circulating levels in Ovx compared to sham-operated rats is unclear. It is possible that altered estrogen metabolism in Ovx rats may account for the higher circulating levels, but additional studies are necessary to explore this hypothesis. Although a standard endocrine approach was employed in the present study, it is also important to point out that the E_2 replacement regimen does not replicate the cyclic changes in estrogen that occur in the intact animal.

It is well documented that ovariectomy causes an increase in body weight and E_2 treatment causes a reduction in body weight *(20–24)*. In the present study, these same trends were observed. This effect cannot be explained by differences in food intake because it is not prevented by pair feeding with controls *(24)*, indicating that other factors account for the postovariectomy weight gain. Perhaps the influence of E_2 on basal metabolic rate could account for estrogen-dependent alterations in body weight and lipid metabolism.

Precursor T-cells derived from the bone marrow differentiate (via positive or negative selection) in the thymus from immature double-positive ($CD4^+8^+$) cells and give rise to mature single-positive $CD4^+$ (helper/inducer) or $CD8^+$ (suppresser/cytotoxic) cells *(25–27)*. Estrogen is believed to act via receptors in the thymic reticular stroma to modulate the growth and differentiation of T-lymphocytes *(28–30)*. We observed that ovariectomy induced thymic hyperplasia with an increase in the number of lymphocytes whereas E_2 treatment, especially at the high dose, induced thymic atrophy with an associated decrease in the number of lymphocytes. These findings are consistent with previously reported observations *(28,29,31–34)*. Although we observed changes in the percentage of $CD4^+8^+$ and $CD5^+$ T-cells induced by either ovariectomy or E_2 treatment at d 0 and 14, respectively, by d 28 there were no differences between any of the groups of ovariectomy or E_2 treatment.

The most obvious effects of ovariectomy and E_2 replacement on T-cell subpopulations were observed in bone marrow which serves as the skeletal microenvironment and regulates bone cell development and function. Bone marrow T-cells have been documented to be immunocompetent *(35)* and are believed to play a role in the local regula-

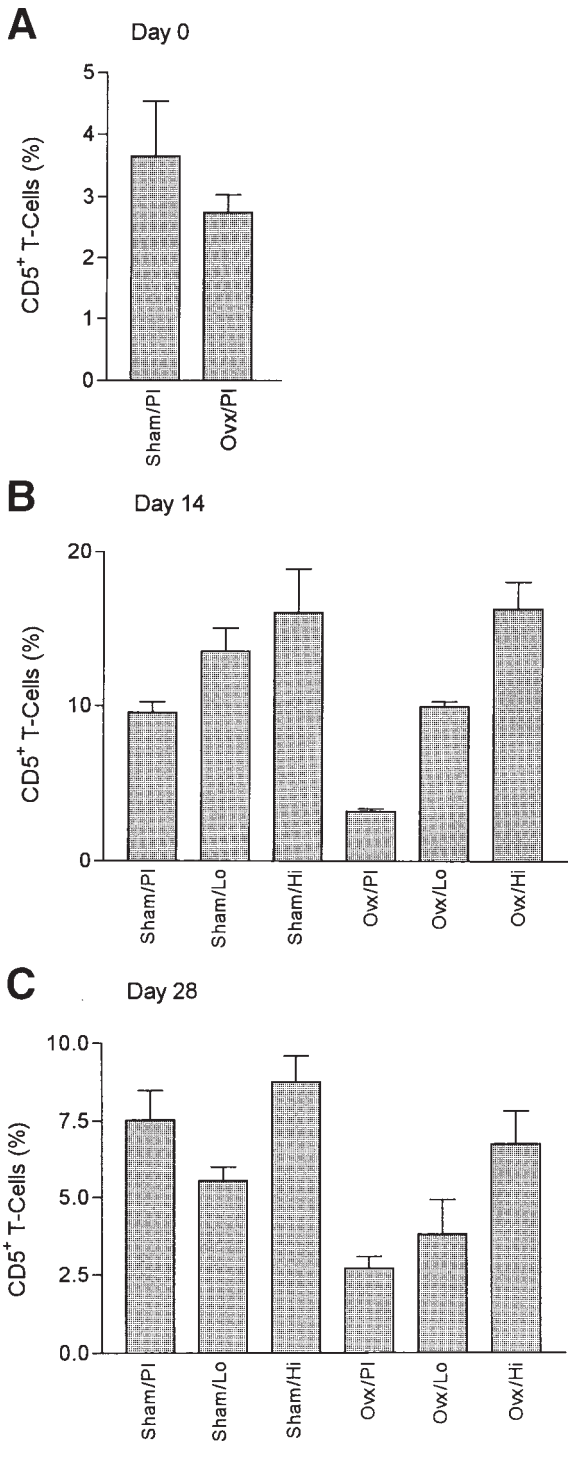

Fig. 3. Percentage of $CD5^+$ T-Cell subsets in bone marrow from sham-operated and Ovx rats **(A)** The percentage of $CD5^+$ T-cells was reduced in Ovx/Pl compared with Sham/Pl group at d 0. **(B)** At d 14, percentage of $CD5^+$ T-cells in Ovx/Pl group is significantly decreased compared with Sham/Pl ($^*p < 0.001$). Percentage of $CD5^+$ cells in Ovx/Lo and Ovx/Hi groups are significantly higher compared with Ovx/Pl ($^\gamma p < 0.01$ and $^W p < 0.001$, respectively); Ovx/Hi is also significantly higher compared to Ovx/Lo ($^\bullet p < 0.01$). **(C)** At d 28, percentage of $CD5^+$ T-cells in Ovx/Pl group is significantly lower compared with Sham/Pl ($^*p < 0.001$). Sham/Hi is significantly higher compared to Sham/Lo group ($^\gamma p < 0.05$). Ovx/Hi is significantly higher when compared to Ovx/Pl group ($^W p < 0.05$). Data are represented as the mean ± SEM.

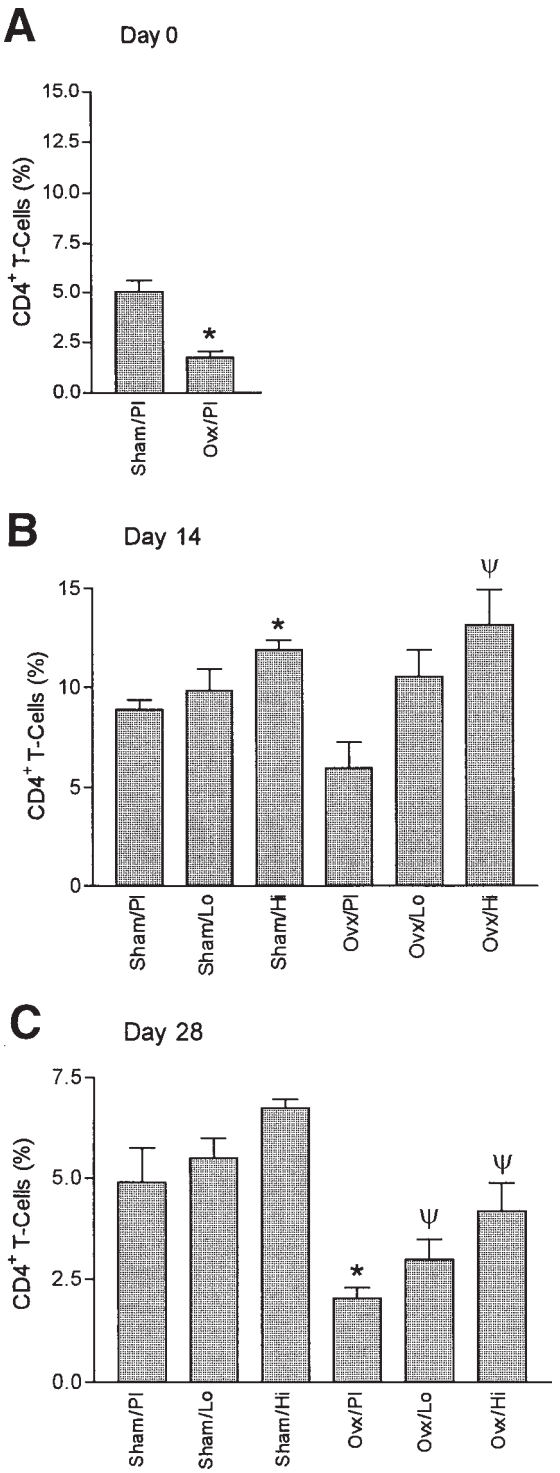

Fig. 4. $CD4^+$ T-Cell subsets in bone marrow from sham-operated and Ovx **(A)** At d 0, percentage of $CD4^+$ T-cells in Ovx/Pl group is significantly decreased compared with Sham/Pl group ($^*p < 0.001$). **(B)** At d 14, percentage of $CD4^+$ T-cells in Sham/Hi group is significantly increased compared with Sham/Pl ($^*p < 0.05$). The percentage of $CD4^+$ cells in Ovx/Hi group is significantly increased compared with Ovx/Pl ($^\gamma p < 0.05$). **(C)** At d 28, percentage of $CD4^+$ T-cells in Ovx/Pl group is significantly decreased compared with Sham/Pl ($^*p < 0.01$). Ovx/Lo and Ovx/Hi are significantly decreased when compared with Sham/Lo and Sham/Hi, respectively ($^\gamma p < 0.01$). Data are represented as the mean ± SEM.

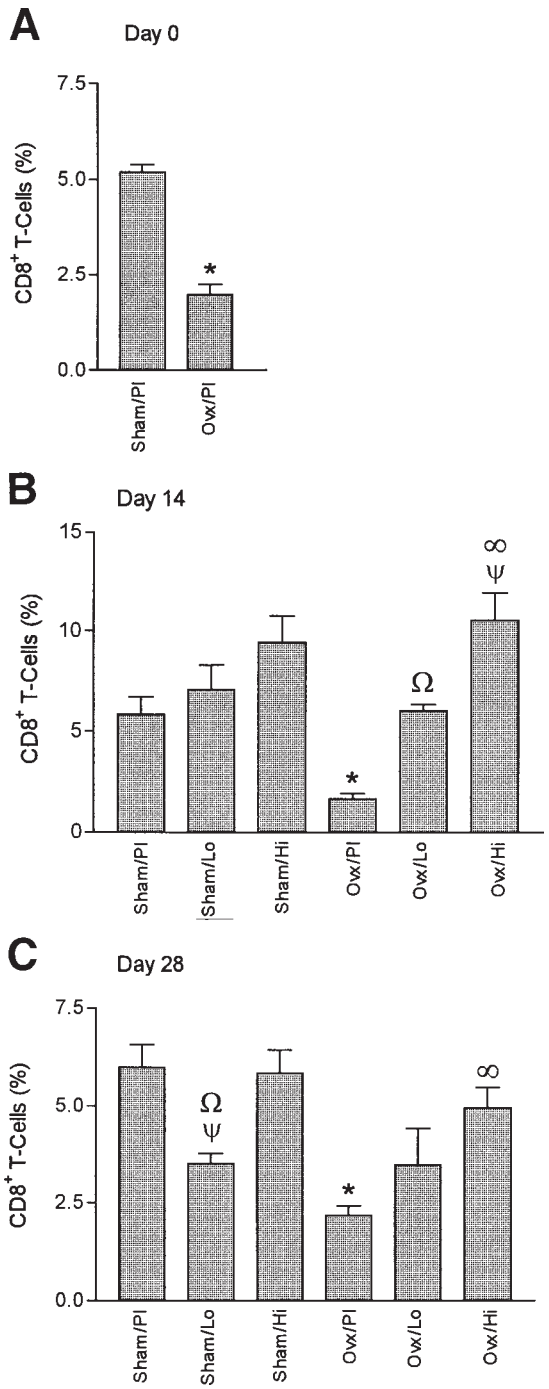

Fig. 5. Percentage of $CD8^+$ T-Cell subsets in bone marrow from sham-operated and Ovx rats. **(A)** At d 0, percentage of $CD8^+$ T-cells in Ovx/Pl group is significantly lower compared with Sham/Pl ($^*p < 0.001$). **(B)** At d 14, percentage of $CD8^+$ T-cells in Ovx/Pl group is significantly lower compared with Sham/Pl ($^*p < 0.01$). $CD8^+$ T-Cells in Ovx/Hi and Ovx/Lo groups are significantly higher when compared to Ovx/Pl ($^yp < 0.001$ and $^Wp < 0.01$, respectively); Ovx/Hi is significantly higher compared with Ovx/Lo ($^\bullet p < 0.01$). **(C)** At d 28, percentage of $CD8^+$ T-cells in Ovx/Pl group is significantly lower compared with Sham/Pl ($^*p < 0.001$). Percentage of $CD8^+$ cells in Sham/Lo group is significantly lower than Sham/Pl and Sham/Hi ($^yp < 0.05$ and $^Wp < 0.01$, respectively). Ovx/Hi is significantly higher compared with Ovx/Pl ($^\bullet p < 0.05$). Data are represented as the mean ± SEM.

tion of bone metabolism and hematopoiesis. In the present study, the data showed that ovariectomy produced a significant decrease in the percentages of all T-cell subsets ($CD5^+$, $CD4^+$, and $CD8^+$) in the bone marrow that persisted until the end of the study (d 28). Low-dose E_2 replacement in Ovx rats prevented this decrease in T-cell subsets, and high-dose E_2 treatment caused an increase in the percentage of T-cell subsets in both the Ovx and sham-operated groups.

The mechanism whereby estrogen modulates T-cell subpopulations in the bone marrow is not known. There is evidence suggesting that estrogen suppresses the increase in osteoclastic resorptive activity induced by parathyroid hormone or ovariectomy through an effect on hematopoietic progenitor cells of the osteoclast lineage *(36)*. A recent study *(37)* showed that depletion of mouse bone marrow $CD8^+$ T-cells resulted in a significant increase in the number of osteoclasts in vitro and hypothesized that $CD8^+$ T-cells regulate osteoclast differentiation and proliferation. They suggested that $CD8^+$ T-lymphocytes produce a factor that suppresses osteoclast differentiation either directly or indirectly by modulating cytokines involved in this process. This hypothesis is supported by other case studies in which decreased $CD8^+$ lymphocytes have been associated with disease states accompanied by increased bone resorption, such as histiocytosis X *(38)* and in patients with significant alveolar bone loss *(39)*. Futhermore, imbalances of bone marow T-lymphocyte subsets have been documented in patients with multiple myeloma *(40)*. These previous observations coupled with our findings suggest that the reduction of bone marrow T-lymphocytes following ovariectomy may play a crucial role in mediating the effects of estrogen deficiency on bone resorption by stimulating osteoclast development and function. Additional studies are required to validate T-cell involvement in estrogen-deficient bone loss and to elucidate the mechanism responsible for this paracrine effect.

Analyses of T-cell subsets ($CD5^+$, $CD4^+$, and $CD8^+$) in the spleen, lymph node, and peripheral blood revealed that ovariectomy significantly reduced the percentage of some T-cell subsets when compared to sham-operated rats at 5 d postsurgery. E_2 treatment also caused some changes in T-cell subpopulations at d 14. However, most of these changes were transient and had normalized by d 28 of the experiment, with the exception of peripheral blood $CD4^+$ cells, which were increased, and $CD5^+$ cells in the peripheral blood, which were increased by high-dose E_2 treatment. A recent study reported that the percentage of peripheral blood $CD5^+$, $CD4^+$, and $CD8^+$ lymphocytes in Ovx rats remained unchanged throughout the 12-wk study period, except for a transient increase in $CD4^+$ T-cells in Ovx compared with sham-operated rats at 1 wk postsurgery *(15)*. Although there are differences between our findings and those of the former study, the conclusion drawn from both is similar: namely that consistent changes in T-lymphocyte

subpopulations in peripheral blood, spleen, and lymph node are not demonstrable in Ovx rats.

In summary, the present study documents significant changes in T-cell subsets in the bone marrow that persisted for the duration of the study (28 d). Changes in T-cell subsets in other lymphoid organs and tissues were largely transient; most had normalized by the end of the study. The thymic hyperplasia induced by ovariectomy or thymic atrophy induced by high-dose E_2 treatment was associated with an increase or decrease in the number of lymphocytes, respectively. The significant and persistent changes in bone marrow T-lymphocytes, especially $CD8^+$ cells, as previously suggested by John et al. *(37)*, may play an important role in osteoclastogenesis. Additional studies are necessary to determine whether or not the estrogen-dependent changes in bone marrow T-cell subpopulations observed have an effect on osteoclast differentiation and function.

Materials and Methods

Animals

Fifty-eight, 6 mo-old female Sprague-Dawley rats weighing approx 240 g were purchased from Taconic (Germantown, NY). Twenty-nine rats were Ovx and the reminder were sham-operated. All animals were housed under similar conditions in a 12-h light/12-h dark cycle and maintained on a diet containing 0.75% calcium, 0.85% phosphorus, and 1045 IU/kg of vitamin D_3. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, and animals were used according to principles established in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals (41)*.

Experimental Procedures

The subcutaneous implantation of E_2 pellets (Innovative Research of America, Sarasota, FL) (d 0 of the experiment) was performed 5 d after ovariectomy or sham surgery to ensure that all animals were rehydrated and acclimated to the operation. Two doses of E_2 were administered: a low (0.5 mg) dose and a high (5.0 mg) dose. The manufacturer considers the low dose to be physiological because normal female rats treated with this dose maintain circulating E_2 levels within the normal, physiological range *(19)*. Ten animals (5 Ovx and 5 sham-operated) were sacrificed on d 0 for T-cell subset analyses and used as a baseline control. The remaining 48 rats were divided into 6 groups of 8 rats each treated with placebo, low-, or high-dose E_2 pellets, for 14 or 28 d (4 rats per treatment period) according to the following protocol:

- Group A. Sham-operated rats received placebo pellets (Sham/Pl).
- Group B. Sham-operated rats received low-dose (0.5 mg) E_2 pellets (Sham/Lo).
- Group C. Sham-operated rats received high-dose (5.0 mg) E_2 pellets (Sham/Hi).
- Group D. Ovx rats received placebo pellets (Ovx/Pl).
- Group E. Ovx rats received low-dose (0.5 mg) E_2 pel lets (Ovx/Lo).
- Group F. Ovx rats received high-dose (5.0 mg) E_2 pellets (Ovx/Hi).

Serum E_2 Measurement

Animals were anesthetized intramuscularly with ketamine hydrochloride (10 mg/100 g of body weight) in combination with acepromazine maleate (1 mg/kg of body weight) (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA). Blood was collected from all animals at d 14 of the experiment via the retro-orbital vein. Serum samples were stored at –70°C until assayed for E_2 using a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA) according to the manufacturer's protocol. The detection limit of the assay was 1.4 pg/mL. The intraassay coefficient of variation was 10%.

Lymphocyte Isolation

Blood was obtained by cardiac puncture from anesthetized rats using heparinized syringes. An equal volume of phosphate-buffered saline (PBS) was added to each sample, and lymphocytes were isolated using Ficoll-Hypaque as described previously *(18)*. Spleen, thymus, and abdominal lymph nodes were harvested and single-cell suspensions prepared by gently teasing the tissue apart in a small volume (4–6 mL) of RPMI-1640 (Gibco, Grand Island, NY) using stainless steel rakes. Because the thymus and lymph node cell suspensions were primarily composed of lymphocytes, they were not further separated using Ficoll-Hypaque. However, splenocytes were subjected to Ficoll-Hypaque separation to isolate lymphocytes. Bone marrow was flushed from both tibiae with PBS, and red blood cells were lysed using an ammonium chloride (0.8%)–potassium bicarbonate buffer. Following appropriate lymphocyte isolation, all cell suspensions were washed three times in PBS prior to immunofluorescent labeling.

Flow Cytometry Analyses

Three fluorochrome-conjugated mouse anti-rat monoclonal antibodies were used, including CD5-fluorescein isothiocyanate (FITC) (pan-T marker), CD4-FITC (T helper/inducer), and CD8-PE (T cytotoxic/suppresser) (Harlan Bioproducts, Indianapolis, IN). Cells 2×10^6 from each tissue were incubated with 10 μg of CD5-FITC or CD4-FITC. The CD4-labeled cells were washed and incubated with CD8-PE for double-labeling using a modified procedure previously described *(18)*. All incubations were performed with the appropriate antibody in 200 μL of PBS supplemented with 0.5% bovine serum albumin for 1 h at 4°C. Background fluorescence was determined using FITC- or PE-conjugated nonimmune IgG. Following single or double labeling, cells were washed twice in PBS, fixed in 4% paraformaldehyde for 7 min at 4°C, washed again, and resuspended in α-modified Eagle's medium supplemented

with 10% fetal calf serum (Gibco). From each sample, 10,000 cells were analyzed by flow cytometry, and the percentages of labeled $CD5^+$, $CD4^+$, $CD8^+$, and $CD4^+8^+$ double-positive cells were determined.

Statistical Analyses

Data were analyzed using the two-tailed unpaired *t*-test for the comparison of Sham/Pl vs Ovx/Pl, Sham/Lo vs Ovx/Lo, and Sham/Hi vs Ovx/Hi. Single-factor analysis of variance was used to evaluate the effect of E_2 on sham-operated or Ovx groups; in the presence of a significant group effect, a post-hoc Newman-Keuls multiple comparison test was performed to compare individual pairs of means. Data were considered significantly different when the probability was <0.05.

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