Ovariectomy augments B lymphopoiesis and generation of monocyte-macrophage precursors in rat bone marrow

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Ovariectomy augments B lymphopoiesis and generation of monocyte-macrophage precursors in rat bone marrow. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E476–E483, 1998.—To investigate the effects of estrogen depletion on hematopoiesis and bone turnover, female rats were either ovariectomized (OVX) or sham operated and killed at 1, 2, 3, and 4 wk postsurgery. Flow cytometric analysis of bone marrow cells (BMC) revealed that, in close temporal association with the rise in bone turnover as measured by bone histomorphometry, the number of Thy 1.1¹ and KIB1R¹ BMC increased two- to threefold in OVX rats relative to sham controls. The Thy 1.1¹ BMC were further characterized as Thy 1.1¹/KIB1R¹ and Thy 1.1¹/HIS24 double-positive cells of the B cell lineage. A transient rise in ED8⁺ myeloid BMC expressing a lysosomal antigen specific for the monocyte-macrophage and osteoclast lineage coincided with the upregulation of osteoclast numbers in OVX rats at 2 wk postsurgery, but the number of ED8⁺ myelomonocytic BMC remained unchanged. Administration of estradiol prevented the rise in Thy 1.1⁺, KIB1R⁺, and ED1⁺ BMC in OVX animals. Our study indicates that ovariectomy upregulates B lymphopoiesis in rat bone marrow and increases myeloid cell differentiation into the monocyte-macrophage and possibly also the osteoclast lineage.

ONE OF THE MOST IMPORTANT factors in the etiology of postmenopausal osteoporosis is estrogen deficiency due to ovarian insufficiency after menopause (27). It is firmly established, in both humans (11, 33) and animal models of estrogen deficiency (37), that estrogen withdrawal results in an increase in bone turnover, especially an increase in osteoclast activity, with subsequent bone loss. The mechanisms involved in the development of estrogen deficiency-induced bone loss and especially the target cells of estrogen in bone, however, are still controversial. In the past few years, there has been accumulating evidence that estrogen deficiency has pleiotropic effects on bone marrow cells (BMC) of different lineages. The study by Jilka et al. (16) in ovariectomized (OVX) mice suggested that estrogen deficiency not only upregulates the number of granulocyte-macrophage (GM) colony-forming units (CFU) and osteoclastogenesis in bone marrow but also increases the number of multipotent CFU and the number of erythroid burst-forming units. Osteoclasts are very likely derived from myeloid precursor cells of the GM lineage (reviewed in Ref. 28). Moreover, it has been shown that estrogen withdrawal increases B lymphopoiesis in mouse bone marrow in vivo (23). Utilizing a coculture system of murine BMC and a bone marrow-derived stromal cell line, the same study suggested that the effects of estrogen deficiency on lymphopoiesis are mediated through stromal cells. It remains unclear, however, whether the changes in B lymphopoiesis observed in OVX mice are causally linked to the postovariectomy rise in bone turnover. Furthermore, it is not known whether there is a temporal association between the changes in bone metabolism and the changes in BMC subpopulations after the loss of ovarian function.

In contrast to other species including mice, the changes in bone turnover and bone structure induced by ovariectomy have been very well characterized in rats (18, 37, 38). One of the reasons for the lack of information with respect to the temporal sequence of bone changes in OVX mice may be the low amount of cancellous bone present in bones of the axial and appendicular skeleton, a fact that complicates histomorphometric analysis of murine cancellous bone. However, although OVX rats are the best characterized animal model of estrogen deficiency-induced bone loss, little is known about the effects of estrogen depletion on hematopoiesis in the rat. Using a Coulter counter technique, the study by Kalu et al. (19) indicated that ovariectomy increased the number of mononuclear cells in the thymus, spleen, and bone marrow in the rat. Recently, Shvede and Pike (30) showed that ovariectomy of rats led to an increase in CFU-GM in ex vivo cultures of rat BMC and to an increase in Thy 1.1⁺-positive BMC, which was interpreted as an increase in the early hematopoietic stem-progenitor cell population. Thy 1.1 is a surface antigen that is expressed on B cell precursors, immature B cells, hematopoietic stem cells, thymocytes, and neuronal cells in the rat (3, 10, 13, 14, 36).

In the present study, we sought to investigate further the effects of estrogen depletion on hematopoiesis in the rat by use of bone marrow flow cytometry to analyze and quantify changes in BMC subpopulations. Furthermore, we intended to temporally correlate the flow cytometric findings with the changes in bone turnover as measured by bone histomorphometry. The current experiment showed that, similar to the findings in OVX mice (23), acute estrogen withdrawal upregulates the number of B lineage cells in rat bone marrow. Moreover, relative to sham and estrogen-treated OVX rats, we found a transient rise in myeloid BMC expressing a lysosomal antigen specific for cells of the monocyte-macrophage and osteoclast lineage in estrogen-deficient OVX rats, which temporally coincided with the upregulation of osteoclast numbers in OVX rats at 2 wk postsurgery and may be indicative of an increased...
differentiation of myeloid cells into the osteoclast lineage under the situation of acute estrogen depletion.

**MATERIALS AND METHODS**

Animal procedures. All animal procedures were approved by the local government authorities. Female 3-month-old Fischer rats (Charles River, Sulzfeld, Germany) weighing 150–160 g were either OVX or sham operated. During surgery, some rats received subcutaneously implanted 60-day slow-release pellets containing either vehicle (Veh) or 0.05 mg estradiol/pellet (Innovative Research of America, Sarasota, FL). The animals were kept in pairs at 24°C with a 12:12-h light-dark cycle and were allowed free access to a pelleted standard rat laboratory diet (Altromin, Lage, Germany) and tap water. Groups of sham, OVX, and estradiol-supplemented OVX rats (n = 6–8 each) were killed 1, 2, 3, and 4 wk post-surgery by an ether overdose. Blood was taken at 3 wk post-variaectomy before animals were killed. Immediately after they were killed, the proximal 1 cm of the right tibiae was used for BMC preparation and subsequent FACS analysis, whereas the left proximal tibiae were used for bone histomorphometry. The right uterine horn was removed in each animal, rinsed in physiological saline, blotted dry, and weighed. To avoid an increase in background fluorescence for FACS analysis, the rats were not injected with calcium-seeking fluorochromes for the histomorphometric evaluation of bone formation.

Bone histology. At necropsy, the left proximal tibiae were defleshed and fixed immediately in 40% ethanol at 4°C for 48 h. After fixation, the bones were embedded undecalcified in methylmethacrylate, as described previously (29). Undecalcified sections (5 µm thick) were prepared in the midsagittal plane of the tibiae with a HM 360 microtome (Microm, Walldorf, Germany) and stained with von Kossa-toluidine blue (9) and with toluidine blue at acid pH (2).

Bone histomorphometry. All histomorphometric measurements were performed on the cancellous bone of the proximal tibial metaphysis. The area within 1 mm from the growth plate was excluded from the measurements. The measurement of cancellous bone area was made with an automatic image analysis system (Vidas, C. Zeiss, Oberkochen, Germany) connected to a Zeiss stereomicroscope (C. Zeiss) via a TV camera (Bosch, Stuttgart, Germany) on sections stained with von Kossa-toluidine blue. The average measuring area was 10–12 mm² in each section. Cancellous bone area was calculated as a percentage of tissue area. Histomorphometric measurements of cellular parameters were made using a semiautomatic system (Videoplan, Zeiss) and a Zeiss Axioskop microscope with a drawing attachment. In the centrally located cancellous bone of the proximal tibial metaphysis, ~5 mm² of tissue area were evaluated in each section, corresponding to ~20–40 mm of trabecular bone surface. The following primary parameters were determined at ×200 on sections stained with toluidine blue: bone perimeter, osteoid perimeter, osteocyte perimeter, and the number of osteoclasts. Osteoid perimeter and osteoblast perimeter were expressed as a percentage of bone perimeter. Osteoclasts were defined as large, irregularly shaped cells with a foamy, slightly metachromatic cytoplasm containing one or more nuclei and located within Howship's lacunae. Osteoclast numbers were expressed using the mineralized bone perimeter as referent.

Single-cell suspensions of BMC. BMC were harvested by repeatedly flushing the interior of the right proximal tibia with 10 ml of cold phosphate-buffered saline (PBS) containing 100IU heparin/ml (Liquemin, Hoffmann-La Roche, Grenzach, Germany). The cells were dispersed by repeated pipetting, and a single-cell suspension was achieved by expelling the cells through a 23-gauge syringe needle. The viability of the cells was determined by trypan blue exclusion. The percentage of viable cells was >95% in all samples. By use of a Sysmex CC 130 micro-cell counter (Toa Medical Electronics, Hamburg, Germany), the number of nucleated cells in all BMC suspensions was evaluated and subsequently adjusted to 10^7 cells/ml.

Permeabilization of cells for intracytoplasmic immunofluorescent staining. Single-cell suspensions of BMC were fixed in PBS with 1% paraformaldehyde (PFA) for 15 min at room temperature. After being washed in PBS, cells were permeabilized (1) by resuspension in PBS containing 2% fetal calf serum (FCS, Flow, Meckenheim, Germany), 0.02% NaN₃ (PBSN), and 0.5% saponin (Sigma, Deisenhofen, Germany). Alternatively, BMC were permeabilized by suspension in PBSN containing 0.3% saponin, without prior fixation with PFA (15).

Single immunofluorescent labeling of surface antigens on BMC. For single labeling of surface antigens, native BMC suspensions were incubated with mouse immunoglobulin (Ig) G1 anti-rat Thy 1.1 (MRC-OX-7), pan B-cells (KiB1R), and CD11b (ED8) for 30 min on ice. Nonimmune mouse IgG1 (MOPC-21, Sigma) was used as a negative control. With the exception of KiB1R (Bionmedicals, Augst, Switzerland) and F11 (see below), all monoclonal antibodies (mAbs) were purchased from Serotec (Oxford, UK). After being washed twice with PBSN, the cells were incubated with rat-adsorbed, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Sigma) for 30 min on ice to reveal bound mouse anti-rat mAbs. Cells were washed twice with PBSN, resuspended in PBS with 1% PFA, and subsequently used for FACS analysis.

We also tried to stain BMC with mAb F11 (Dianova, Hamburg, Germany), a more specific marker of the osteoclast lineage, directed against rat CD61, the β₃-integrin chain, expressed by osteoclasts (αβ₃-vitronectin receptor) as well as by megakaryocytes and thrombocytes (4). However, we were able to harvest only extremely small numbers of osteoclast-like BMC coexpressing αβ₃ and tartrate-resistant acid phosphatase enzyme activity as determined in cytoplasmic preparations. This was probably due to the fact that adherence to the bone surface may be a prerequisite of terminal osteoclast differentiation in vivo, so that committed osteoclast precursors cannot be flushed out of whole bones without proteolytic pretreatment.

Double immunofluorescent labeling of surface antigens on lymphoid BMC. For double labeling of surface antigens on lymphoid BMC, cell suspensions were incubated with mouse IgG₁ anti-iat-rat pan-B-cells (KiB1R), CD45RA (MRC-OX-33), and CD45R (HIS24) for 30 min on ice. Nonimmune mouse IgG₁ (MOPC-21) was used as a negative control. After being washed twice with PBSN, the cells were resuspended with rat-adsorbed, FITC-labeled goat anti-mouse IgG antibody (Sigma) for 30 min on ice. Thereafter, cells were washed twice with PBSN, the cells were incubated with rat-adsorbed, FITC-labeled goat anti-mouse IgG antibody (Sigma) for 30 min on ice to reveal bound mouse anti-rat mAbs. Cells were washed twice with PBSN, resuspended in PBS with 1% PFA, and subsequently used for FACS analysis.

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PE-labeled nonimmune IgG1 (Serotec) were used as negative controls. Cells were washed twice with PBSN, resuspended in PBS with 1% PFA, and subsequently used for FACS analysis.

Immunofluorescence staining of intracellular antigens in BMC. BMC permeabilized by PFA-saponin treatment were incubated for 15 min at room temperature with a mouse IgG1 mAb (ED1) directed against an intracellular antigen present in all cells of the rat mononuclear phagocytic system. Nonimmune mouse IgG1 (MOPC-21) was used as a control. After the cells were washed three times with PBSN containing 0.1% saponin, cells were incubated for 30 min on ice with rat-adsorbed, FITC-labeled goat anti-mouse IgG antibody (Sigma) in PBSN containing 0.5% saponin. Subsequently, the cells were washed twice with PBSN containing 0.1% saponin before FACS analysis.

Studies in our laboratory have shown that, in contrast to binding of mAb ED1 to its intracellular antigen, binding of the mAb MARM-4 directed against the rat IgM µ-chain is greatly diminished in PFA-fixed cells. Therefore, for the demonstration of intracytoplasmic µ-chain in permeabilized BMC, cells were first incubated with PE-labeled anti-rat Thy 1.1 mAb on ice. After being washed twice with PBSN, cells were permeabilized with 0.3% saponin (without prior PFA fixation) and incubated with FITC-labeled mAb against rat IgM µ-chain (MARM-4) for 30 min on ice. Cells were washed twice with PBSN containing 0.1% saponin and analyzed by flow cytometry. FITC- and PE-labeled nonimmune IgG1 (Serotec) were used as negative controls.

Immunofluorescence staining of B lymphocytes in peripheral blood. To determine the effects of ovariectomy on the percentage of B cells in peripheral blood, 100 µl of whole blood were incubated with FITC-labeled mAbs KIB1R and MRC-OX-33. Control samples were incubated with an isotype-specific FITC-labeled nonimmune mAb (Serotec). Thereafter, erythrocytes were hemolyzed by addition of FACS lysing solution (Becton-Dickinson, Heidelberg, Germany). Stained leukocytes were centrifuged, washed twice with PBS, fixed in PBSN with 1% PFA, and analyzed on the flow cytometer.

FACS analysis. All flow cytometric analyses were performed on a FACSscan flow cytometer using FACSscan and Consort 30 software (Becton Dickinson).

Statistical analysis. Statistics were computed using SPSS for Windows 6.1 (SPSS, Chicago, IL) and Instat 2.0 (GraphPad, San Diego, CA). Statistical comparisons between two groups were performed using a two-sided t-test. Statistical comparisons between three groups were made using one-way analysis of variance (ANOVA). When the ANOVA performed over all groups indicated a significant (P < 0.05) difference among the groups, statistical differences between two groups were subsequently evaluated with the Student-Newman-Keuls multiple comparison test. P < 0.05 was considered significant. The data are presented as means ± SE.

RESULTS

Effects of ovariectomy and estrogen supplementation on body and uterine weight. Compared with vehicle-treated sham rats and estradiol-treated OVX rats, vehicle-treated OVX rats had a higher body weight at 2 and 3 wk postovariectomy (data not shown). The uterine weight in OVX + Veh rats was significantly decreased from 1 wk postsurgery throughout the study (Fig. 1). Treatment of OVX rats with subcutaneously implanted 60-day slow-release pellets containing 0.05 mg estradiol per pellet increased uterine weight beyond that observed in the sham + Veh group, suggesting that this treatment regimen resulted in supraphysiological levels of estradiol in OVX rats. Furthermore, the increase in uterine weight in estradiol-treated OVX animals relative to sham controls was 82% at 1 wk postovariectomy and declined to 31% at 4 wk postovariectomy. Thus the kinetics of estradiol release from the slow-release pellets may have been nonlinear.

Bone histomorphometry. To be able to compare the time course of the effects of estrogen withdrawal on bone turnover with the time course of the changes in BMC subpopulations, we performed sequential bone histomorphometry on the proximal tibial metaphysis of vehicle-treated sham and vehicle-treated or estradiol-treated OVX rats. The results are shown in Fig. 2. Presumably as a consequence of the surgical procedures, cancellous bone mass tended to be lower in all groups of rats at 1 wk postovariectomy compared with baseline values. Ovariectomy induced high turnover osteopenia in OVX rats. The reduction in tibial cancellous bone mass in OVX + Veh animals relative to sham + Veh rats reached statistical significance at 3 wk postovariectomy (Fig. 2A). Compared with vehicle-treated sham controls, the increase in both osteoclast number and osteoblast perimeter in OVX + Veh rats became evident at 2 wk postovariectomy (Fig. 2B and C). Treatment of OVX rats with estradiol suppressed osteoclast number and osteoblast perimeter to levels below that observed in sham + Veh rats and increased tibial cancellous bone mass beyond the sham + Veh group (Fig. 2). In agreement with the data on uterine weight, these findings further suggest that estradiol blood levels in OVX rats that had received 60-day slow-release pellets containing 0.05 mg estradiol per pellet were supraphysiological. Interestingly, however, there was a time lag of ∼1 wk between the suppressive effects of estradiol on osteoclast number and osteoblast perimeter (Fig. 2B, 2C, and 2D), suggesting that osteoclastic bone resorption responded earlier to estradiol treatment than osteoblastic bone formation.
Flow cytometric analysis of BMC and peripheral blood lymphocytes. To examine the effects of estrogen withdrawal on hematopoiesis, we sequentially analyzed bone marrow of the same animals as used for assessment of bone turnover by flow cytometry. For the mAbs MRC-OX-7 and KiB1R reacting mainly with lymphoid cells, the number of positive BMC was expressed as a percentage of all cells in a lymphoid cell gate (Fig. 3). The number of BMC positive for the mAbs ED1 and ED8 was expressed as a percentage of all cells in a myeloid cell gate (Fig. 3). Ovariectomy led to a transient increase in myeloid cells intracellularly expressing the monocyte-macrophage antigen reacting with mAb ED1 at 2 wk postovariectomy (Fig. 4A). In contrast, the percentage of ED8+ myeloid cells expressing CD11b, an integrin present on most of the cells of the myelomonocytic lineage, remained unaffected by ovariectomy or estrogen supplementation (data not shown). Compared with baseline levels, the number of Thy 1.1+ BMC tended to be higher and the number of cells positive for the B cell marker KiB1R tended to be lower at 1 wk postsurgery in all groups of animals (Fig. 4, B and C), a finding that may be explained as a reaction to abdominal surgery. In close temporal association with the increase in bone turnover in OVX rats, the numbers of Thy 1.1+ and KiB1R+ BMC were increased about two- to threefold in OVX + Veh animals relative to sham + Veh controls from 2 to 4 wk postovariectomy (Fig. 4, B and C). Treatment of OVX rats with estradiol prevented the rise in ED1+, Thy 1.1+, and KiB1R+ BMC observed in vehicle-treated OVX animals. In estradiol-treated OVX rats, KiB1R+ cells were significantly decreased at 2 wk postovariectomy, and Thy 1.1+ cells were significantly decreased at 4 wk postovariectomy relative to sham + Veh controls. The data presented in Fig. 4 were confirmed in several independent experiments.

The finding that the rise in Thy 1.1+ and KiB1R+ BMC in OVX animals occurred at the same time and with similar magnitude already suggested that the increase in Thy 1.1+ BMC in OVX rats was due to an increase in B lineage cells. However, it remained to be shown that the Thy 1.1+ BMC were actually cells of the B lymphocyte lineage. We therefore used two-color FACS analysis of BMC in sham and OVX rats at 3 wk postovariectomy.

**Fig. 2.** Histomorphometric parameters measured in cancellous bone of proximal tibial metaphysis in Veh-treated sham, Veh-treated OVX rats, and E2-treated OVX rats. Bone area (A), osteoclast number (B), and osteoblast perimeter (C) are plotted on time postovariectomy. Each data point is mean ± SE of 6 animals. Significance of differences between groups was evaluated by ANOVA followed by Student-Newman-Keuls multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001 vs. OVX + Veh. #P < 0.05; ##P < 0.01; ###P < 0.001 vs. sham + Veh.

**Fig. 3.** Typical light scattering characteristics of a bone marrow cell (BMC) suspension harvested from proximal tibia of a 3-mo-old female Fischer rat. In dot plot of sideward scatter on forward scatter shown, region (gate) R1 contains greatest number of lymphoid cells but also other cell types such as erythroid precursor cells. Almost all Thy 1.1+ cells are located in lymphoid cell gate R1. Region R2 encompasses most of myeloid cells, which are larger cells and have more intracellular granules relative to cells in region R1. About 60% of all cells in myeloid cell gate R2 are positive for monoclonal antibody (mAb) ED8 recognizing type 3 complement receptor shared by myelomonocytic cells. Almost all BMC positive for mAb ED1 directed against an intracellular antigen specific for cells of monocyte-macrophage lineage are found within region R2.
Figure 4. Flow cytometric analysis of BMC harvested from proximal tibiae of Veh-treated sham, Veh-treated OVX, and E2-treated OVX rats. Percentages of all cells in a myeloid cell gate positive for ED1 (A) and of all cells in a lymphoid cell gate positive for Thy 1.1 (B) and KiB1R (C) are plotted as a function of time postovariectomy. Each data point is mean ± SE of 6 animals. Significance of differences between groups was evaluated by ANOVA followed by Student-Newman-Keuls multiple comparison test. **P < 0.01; ***P < 0.001 vs. OVX + Veh; #P < 0.05; ##P < 0.01, vs. sham + Veh.

Postovariectomy to clarify the nature of the Thy 1.1+ BMC found to increase postovariectomy. In the experiment shown in Table 1 and Fig. 5, the number of Thy 1.1+ BMC in a lymphoid cell gate was increased about twofold (21.6 ± 1.1 vs. 11.1 ± 1.0%, P < 0.001) in OVX rats compared with sham controls. It is evident from Table 1 and Fig. 5 that the greatest number of Thy 1.1+ cells (~90%) in both sham and OVX animals coexpressed the B lymphocyte antigens reacting with mAbs KiB1R and HIS24 (CD45R). Hematopoietic stem cells positive for Thy 1.1 but negative for B lineage markers KiB1R and CD45R formed a very small percentage of BMC in both sham and OVX rats (0.5–1% of cells in a lymphoid cell gate; data not shown). Most of the Thy 1.1+ cells (~80%) in sham and OVX rats were also found to coexpress the surface antigen CD45RA reacting with the mAb MRC-OX-33. In both sham and OVX rats, about one-third of the Thy 1.1+ BMC expressed surface (Fig. 5) or cytoplasmic IgM μ chain (Table 1). Moreover, the percentages of Thy 1.1+ BMC positive for KiB1R, CD45R, or CD45RA were not different in sham and OVX animals (data not shown). The only statistically significant change in the coexpression of B lymphocyte differentiation antigens in Thy 1.1+ BMC of OVX rats relative to sham controls was a moderately increased percentage of cells positive for cytoplasmic IgM (Table 1). These findings suggest that ovariectomy induced a broad increase in B lineage cells of various differentiation stages, i.e., a general upregulation of B lymphopoiesis in rat bone marrow at 3 wk postovariectomy. In peripheral blood lymphocytes, ovariectomy led to a nonsignificant trend toward increased numbers of KiB1R+ (22.8 ± 1.7 vs. 21.0 ± 1.8%) and CD45RA+ (25.3 ± 1.5 vs. 22.9 ± 1.9%) B lymphocytes in OVX rats relative to sham controls (data not shown).

**DISCUSSION**

The results of the current experiment have shown that acute estrogen withdrawal in OVX rats resulted in a severalfold increase in Thy 1.1+ BMC that followed a similar time course as the upregulation in bone turnover observed in OVX animals. As mentioned in RESULTS, Thy 1.1+ is expressed on B cell precursors, immature B cells, and hematopoietic stem cells in bone marrow in the rat (3, 10, 13, 14, 36). In close temporal association with the rise in Thy 1.1+ cells, BMC positive for the B lymphocyte marker KiB1R were increased in OVX rats from 2 to 4 wk postovariectomy in the current study. Shevde and Pike (30) have recently also reported an increase in Thy 1.1+ cells in bone marrow of OVX rats. These authors interpreted this finding as an increase in the hematopoietic stem-progenitor cell population. However, the results of the present study have clearly shown that the greatest number of Thy 1.1+ cells in OVX and sham rats were B lineage cells as characterized by the colocalization of Thy 1.1 and B lymphocyte surface antigens recognized by the mAbs KiB1R and HIS24. It is known from previous studies that CD45R, the antigen reacting with mAb HIS24, is found on most of the B cells in bone marrow, including

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Values are means ± SE; n = 5–8 each for sham and ovariectomized (OVX) groups. Cells were double stained with monoclonal antibody (mAb) MRC-0X-7 directed against Thy 1.1 antigen and mAbs specific for a variety of B lymphocyte antigens. Values are given as percentage of all Thy 1.1+ BMC in a lymphoid cell gate expressing surface immunoglobulin (IgM (sIgM)), cytoplasmic IgM (cIgM), or antigens reacting with mAbs KiB1R, HIS24, and MRC-OX-33. Statistical comparisons between groups were performed using a 2-sided t-test.
early B cell progenitors (7, 21). Furthermore, in close agreement with our investigation, McCarthy et al. (24) reported that almost all Thy 1.1$^+$ cells in rat bone marrow are B lymphopoietic cells because they also express the high-molecular-weight form of leukocyte common antigen found on pre-B cells (recognized by mAb MRC-OX-22). The same study has demonstrated that hematopoietic stem cells able to rescue lethally
irradiated rats have the phenotype Thy1.1

Thy 1.1+ /MRC-OX-22 - and form only a very small percentage of BMC (0.34%).

Thus the results of our study indicate that, similar to the findings in OVX mice (23), ovariectomy upregulated the number of Thy 1.1 - B lineage cells in rat bone marrow. Supplementation of OVX rats with estradiol prevented the rise in both Thy 1.1 - and KiB1R + BMC in the present study. Furthermore, it has been shown by Medina et al. (25) that B lymphopoiesis is suppressed in bone marrow of pregnant mice and that administration of estrogen to intact female mice selectively suppressed B lymphocyte precursors in bone marrow. Taken together, all these findings strongly support the notion that estrogen is an important regulator of B lymphopoiesis in bone marrow of at least rats and mice.

The percentage of Thy 1.1 + BMC expressing CD45R, CD45RA, surface IgM µ-chain or the antigen recognized by the mAb KiB1R was not different in sham and OVX animals in the present investigation. We observed only a small increase in the percentage of Thy 1.1 + BMC expressing cytoplasmic IgM µ-chain in OVX relative to sham rats, which can be interpreted as a rise in pre-B cells (7, 21). However, a major shift in the expression of B cell differentiation antigens was not demonstrable in Thy 1.1 + BMC of OVX rats, despite an about two- to threefold increase in the number of Thy 1.1 + cells in OVX animals. This suggests that estrogen deficiency did not selectively expand a B cell subpopulation at a certain differentiation stage but, rather, caused a more general upregulation of B lymphopoiesis in rat bone marrow. In line with this notion, we observed a tendency for increased numbers of B lymphocytes in the peripheral blood of OVX rats 3 wk postsurgery.

Estrogen deficiency in OVX rats induced a transient upregulation of ED1 + myeloid BMC in the current study, whereas the number of ED8 + cells remained unchanged. This finding suggests that acute estrogen withdrawal increased differentiation of myeloid cells into the monocyte-macrophage lineage. The antigen recognized by mAb ED8 is a subunit (CD11b) of the type 3 complement receptor, which is a common marker of myelomonocytic cells (monocytes, macrophages, and granulocytes) (6). The mAb ED1 recognizes a mainly intracellularly localized 97-kDa protein present in almost all cells of the rat mononuclear phagocyte system (5). The antigen reacting with mAb ED1 is also abundantly present in mature rat osteoclasts (8, 22, 31). The mAb ED1 binds a protein that is mainly located on lysosomal membranes and is probably homologous to human CD68 (5). In frozen sections of bone tissue, human osteoclasts were also shown to stain with a monoclonal antibody directed against human CD68 (17).

In the study by Masuzawa et al. (23), an increase in F4/80 + macrophages could not be demonstrated in OVX mice. However, in contrast to the antigen recognized by the mAb ED1 used in the present experiment, the antigen reacting with the mAb F4/80 is lost during differentiation of murine osteoclast precursor cells (34). Interestingly, the rise in ED1 + BMC temporally coincided with the upregulation in osteoclast numbers in OVX rats at 2 wk postsurgery. Thus the increase in ED1 + myeloid cells in bone marrow of OVX rats at 2 wk postovariectomy may be interpreted as an increase of myeloid cell differentiation into the osteoclast lineage. In line with this interpretation, earlier experimental findings have suggested that commitment to the monocyte-macrophage lineage precedes commitment to the osteoclast lineage (34, 35). This notion has been strongly supported by a recent study in which injection of a genetically tagged monocytic cell line into the bloodstream of chicken embryos gave rise to tagged osteoclasts, suggesting that osteoclast divergence takes place after the monocyte stage (32). If this hypothesis is correct, however, it remains unclear why estrogen treatment of OVX rats did not suppress the number of ED1 + BMC relative to sham controls despite the fact that osteoclast numbers were distinctly lower in estradiol-treated OVX animals compared with sham rats over the whole experiment. Moreover, ED1 + BMC were elevated only at 2 wk postovariectomy, whereas osteoclast numbers remained higher in OVX relative to sham rats from 2 wk postovariectomy until the end of the study. It is likely, however, that under physiological circumstances the number of myeloid precursors needed for osteoclast recruitment is much lower than that required for production of circulating monocytes. Thus a rise in ED1 + BMC due to increased osteoclast recruitment may only be evident in the situation of an acute upregulation of osteoclast recruitment. The recent finding that in cocultures of murine fetal long bones and BMC the osteoclastic potential of BMC is only transiently increased in OVX mice (26) lends further support to this hypothesis. Moreover, estrogen may regulate the life span of mature osteoclasts through the induction of apoptosis (12, 20). Thus estrogen may influence osteoclast numbers in cancellous bone by additional effects on mature osteoclasts that are independent of changes in the generation of osteoclast precursors from bone marrow.

It remains to be demonstrated, however, that the transient increase in myeloid cell differentiation into the monocyte-macrophage lineage observed in OVX animals is actually due to increased osteoclast recruitment under the situation of acute estrogen withdrawal. The availability of early markers specific for the osteoclast lineage will be of crucial importance for the further clarification of this question. Moreover, although this study has shown a temporal association between the increase in B lymphopoiesis and the upregulation in bone turnover in OVX rats, future experiments will have to delineate whether these findings are causally linked effects or independent phenomena.

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