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The surface antigen CD45R identifies a population of estrogen-regulated murine marrow cells that contain osteoclast precursors

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Abstract

We examined the osteoclastogenic potential of murine bone marrow cells that were fractionated according to their expression of the surface antigen CD45R. Osteoclast-like cells (OCL) with many authentic osteoclast characteristics readily formed in purified $CD45R^+$ murine bone marrow cell cultures after treatment with receptor activator of nuclear factor κB ligand (RANKL) and M-CSF. Ovariectomy (Ovx) caused a 1.5- to 2-fold increase in OCL number in unfractionated and $CD45R^+$ murine bone marrow cell cultures without affecting OCL formation in $CD45R^-$ marrow cells. Limiting dilution assays confirmed that Ovx caused an increase in osteoclast precursor cell number in $CD45R^+$ but not $CD45R^-$ cells. Mice deficient in the type 1 IL-1 receptor (IL-1R1 KO) do not lose bone mass after Ovx. We found that unfractionated, $CD45R^+$, and $CD45R^-$ bone marrow cells from IL-1R1 KO mice showed no increase in OCL formation in vitro after Ovx. In both the wild-type (WT) and the IL-1R1 KO mice Ovx was associated with a 2-fold increase in pre-B-lymphocytes. About 1.3–3.5% of murine marrow cells expressed surface RANK (the receptor for RANKL) while about 11.9–15% of murine bone marrow cells expressed c-Fms (the receptor for M-CSF). There was little effect of Ovx on cells expressing either RANK or c-Fms. These results demonstrate that CD45R expression identifies a subset of murine bone marrow cells whose ability to form OCL in vivo is regulated by estrogen in WT but not IL-1R1 KO cells. The effects of estrogen on bone mass may be related to these responses.

Keywords: B-lymphocytes; Bone marrow; Bone resorption; Flow cytometry; Ovariectomy; Type 1 IL-1 receptor knockout mice

Introduction

The osteoclast is a terminally differentiated multinuclear cell with the unique ability to resorb bone [1]. It is of hematopoietic origin [2] but its exact relationship to other hematopoietic lineages is uncertain. Osteoclasts morphologically resemble macrophages [3], but unlike macrophages express membrane receptors for calcitonin, form a ruffled border, and resorb bone [4].

The mononuclear precursor cell that differentiates into the osteoclast circulates in the monocyte population [5]. A number of cytokines enhance osteoclast precursor cell development or differentiation. GM-CSF appears to induce the differentiation of multipotential progenitor cells toward the osteoclast phenotype [6]. Mice lacking M-CSF fail to form osteoclasts early in their development [7], demonstrating that this cytokine is involved in normal osteoclast differentiation. Mature osteoclasts also express c-Fms, the receptor for M-CSF, on their cell membrane [8] and respond to M-CSF by decreasing their apoptosis rate [9].

Recently, a new cytokine system was shown to be essential for osteoclast formation [10,11]. Receptor activator of nuclear factor κB ligand (RANKL), first identified as a

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product of activated T-lymphocytes [12,13], regulates osteoclast formation [11]. It is produced by stromal and osteoblastic cells in response to stimulators of resorption [14] and interacts with its receptor, RANK, on the cell membrane of osteoclast precursors to activate their differentiation into mature osteoclasts [12,15]. RANKL can also bind to osteoprotegerin (OPG), a soluble "decoy" receptor [16], which inhibits RANKL/RANK interactions. OPG is produced widely and a number of stimulators of resorption regulate its production [14,17].

Estrogen is another important regulator of osteoclast formation both in vivo and in vitro [18,19]. It is also a negative regulator of B-lymphopoiesis in mice [20,21]. The number of osteoclasts in bone as well as B-lymphocyte precursor cells in marrow increases in mice after ovariectomy (Ovx) [18,22]. Conversely, estrogen treatment of Ovx mice decreases osteoclast formation rates and B-lymphopoiesis [18,23]. In addition, pregnancy in mice is associated with a marked inhibition of B-lymphopoiesis through an estrogen-mediated mechanism [20,21].

Previous studies in murine models have demonstrated that cells expressing the antigen CD45R (B220) (a Ly5 gene product that is present on essentially all murine B-lymphocyte lineage cells [24]) contain a population of precursors that can differentiate into osteoclast-like (OCL) cells in vitro [25,26]. In addition, ovariectomy was found to increase the number of OCL that formed when CD45R-expressing cells were cultured with ST2 cells and treated with 1,25-$(OH)_2$ vitamin D_3 [26]. This result implied that ovariectomy increased the number of $CD45R^+$ osteoclast precursor cells in bone marrow.

In the current studies we examined whether ovariectomy altered the ability of RANKL and M-CSF to stimulate OCL formation in cultures of murine bone marrow cells that expressed or did not express CD45R. Cells were derived from either wild-type (WT) or type 1 IL-1 receptor deficient (IL-1R1 KO) mice because previous experiments have demonstrated that IL-1R1 KO mice fail to lose bone mass after ovariectomy [27] and we postulated that the lack of a response to ovariectomy in this model involved changes in osteoclast precursor cells.

Methods

Isolation of cells from bone marrow

Mice were either strain C57BL/6 (WT) or type IL-1R1 KO mice in a mixed C57BL/6 × 129Sv background. Control mice for the IL-1R1 KO mice were derived from WT siblings and were of similar background. At 8–12 weeks of age, mice were sacrificed and their long bones (tibia, femur, and humerus) dissected free of connective tissue. The ends of the bones were removed, and αMEM was flushed through the marrow cavity by a 25-gauge needle attached to a syringe. The medium and accompanying cells were collected in a sterile tube and the bone spicules were allowed to settle. The medium and suspended cells were transferred to a new tube and centrifuged for 5 min at 4°C to isolate bone marrow cells. These were washed in fresh medium and resuspended in 5 ml of Tris ammonium chloride (TAC, 13 mM Tris, pH 7.2, 13.5 mM ammonium chloride) at 4°C for 5 min to lyse red cells. The animal care committee of the University of Connecticut Health Center approved all animal protocols.

Antibodies

A rat monoclonal FITC-conjugated antibody to CD45R, as well as its isotype negative control (rat $IgG_{2\kappa}$) and monoclonal antibodies to HSA, CD24, and IgM, was obtained commercially (BD PharMingen, San Diego, CA). A rat monoclonal anti-murine-RANK antibody was a gift from Dr. William Dougall of Immunex Corp. Seattle, WA. It was used in flow cytometry studies after conjugation with allophycocyanin (Prozyme, San Leandro, CA). Its isotype control (rat IgG_{2a}) was obtained commercially (BD PharMingen). The AFS98 rat hybridoma, which produced a monoclonal antibody to murine c-Fms, was obtained from Dr. Shin-Ichi Nishikawa, Kyoto University Medical School, Kyoto, Japan, and grown in culture [28]. Anti-c-Fms antibody was purified from the conditioned medium by affinity chromatography and used in flow cytometry after addition of a PE-conjugated anti rat monoclonal antibody (BD PharMingen) as previously described [22,25]. Its isotype control (rat IgG_{2a}) was obtained commercially (BD PharMingen). To block nonspecific binding of immunoglobulins to the FcγIII and FcγII receptors (CD16/CD32), and to reduce the background staining, the cells were incubated with Fc Block (BD PharMingen) or incubated with excess of rat IgG for at least 15 min prior to staining with specific antibodies. This step was omitted when secondary anti-rat antibodies were used for detection.

Immunophenotyping of cells for sorting and analysis

Labeling of cells for cytofluorometric analysis or cell sorting was performed by standard staining procedures in 1X HBSS (Gibco, Invitrogen Corp., Carlsbad, CA) containing 0.01 M Hepes (pH 7.4) and supplemented with 2% FCS. All staining was done on ice, to prevent quenching of signals. Appropriate dilutions of directly conjugated antibodies were sequentially added to the cell preparation of interest. We analyzed B-lymphocyte lineage cells based on surface expression of CD45R antigen. Mature B-cells were identified as also expressing surface IgM. Immature B-cells were designated by their expression of heat stable antigen (HSA, CD24) as either pro-B-cells (HSA^{low}) or pre-B-cells (HSA^{high}). Dead cells were excluded by their ability to incorporate propidium iodide. Analyses were done on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) with argon and helium–neon lasers with at least

5×10^4 events examined for each sample and sorts were performed on a FACS Vantage SE cell sorter (BD Biosciences) equipped with three lasers and seven-parameter sorting capability.

Cell cultures

Mouse bone marrow cells were cultured in αMEM supplemented with 10% heat inactivated FCS for 6–7 days. Cultures were fed every 3 days with fresh medium. Cells were cultured with recombinant mouse (rm)M-CSF (30 ng/ml) (R&D Systems, Minneapolis, MN) and rmRANKL (30 ng/ml) (Immunex Corp.), added at day 0 and with each medium change. In all experiments, unstimulated cultures contained fewer than 10 OCL per well.

OCL quantitation

Cells were cultured in 96-well culture plates ($0.8–1.0 \times 10^5$ cells per well in 200 μl medium). In some experiments cells were cultured with RANKL and M-CSF for 6 days and the number of OCL that formed in the wells was quantitated. In other experiments cells were first incubated with M-CSF (30 ng/ml) for 7 days to expand the osteoclast precursor cell population and then treated for another 7 days with RANKL and M-CSF (both at 30 ng/ml) to induce OCL formation in the cultures. At the conclusion of an experiment, cells were fixed with 2.5% glutaraldehyde in PBS for 30 min at room temperature and then stained for tartrate-resistant acid phosphatase (TRAP). Enzyme histochemistry for TRAP was performed using a commercial kit (Sigma–Aldrich Corp., St. Louis, MO). TRAP-positive multinucleated giant cells with more than four nuclei per cell were considered OCL and were counted using an inverted microscope at 100× magnification. In all experiments at least 4 wells per treatment group were evaluated.

To assess the differentiation potential of osteoclast precursors within the unfractionated, $CD45R^+$, and $CD45^-$ bone marrow cell populations, we used a limiting dilution assay. Cells were plated at four decreasing dilutions in 20 wells per dilution of a 384-well plate (Nalge Nunc, Naperville, IL). The cells were cultured in αMEM supplemented with 10% heat-inactivated FCS for 8 days, stimulated with M-CSF and RANKL (at 30 ng/ml for both), and medium was changed every 3 days. They were then stained for TRAP. The number of wells without OCL was determined for each dilution of cells, and their 37.5% detection failure was calculated, which represents the limiting number for detection, i.e., an approximation of the number of OCL precursors within a cell population [29,30].

The frequency of osteoclast precursors in the bone marrow cell populations was calculated according to the formula $1/\text{frequency} = N/\{\ln[T/(T\text{-}P)]\}$ where N is the number of cells seeded in a well, T is the number of wells per group (20 for all experiments), and P is the number of osteoclast-positive wells [26,29,30].

In some experiments radiolabeled [^{125}I]-salmon calcitonin (sCT; Amersham, Piscataway, NJ) was incubated with or without excess cold sCT (10^{-7} M, 100-fold excess). Cells were washed and developed by autoradiography to demonstrate the presence of calcitonin receptors on cells. Briefly, cells were plated on slide flasks (Lab Tek, Nalge Nunc, 2×10^6 cells/cm^2) and incubated at the end of culture with radiolabeled [^{125}I]-sCT (0.04 μCi, 100,000 cpm/ml) in the absence or presence of cold sCT (10^{-7} M; Bachem, Torrence, CA) at room temperature for 2 h. They were then washed twice with PBS to remove nonspecific radioactivity, fixed with 2.5% glutaraldehyde in PBS, and TRAP-stained. Slides were dipped in LM-1 photographic emulsion (1:1 dilution with 1.7% glycerol; Amersham, Arlington Heights, IL) for autoradiography and then developed.

Bone resorption and pit formation

Bone resorption was assayed by measuring the ability of cultured bone marrow cells to form resorption pits on the surface of devitalized bovine cortical bone slices ($4.4 \times 4.4 \times 0.2$ mm) as previously described [31]. Briefly, isolated bone marrow cells were cultured in wells containing devitalized bovine bone slices with RANKL and M-CSF (both at 30 ng/ml) at 37°C in αMEM and 10% HIFCS. After the culture, cells were fixed with 2.5% glutaraldehyde in PBS for 30 min and stained for TRAP, followed by staining with 1% toluidine blue in 1% borax to observe resorption pits.

Statistics

All statistical analyses except for differences in osteoclast precursor frequency were performed by one-way ANOVA and the Bonferroni post hoc test when ANOVA showed significant differences ($P < 0.05$). Differences in osteoclast precursor frequency between cells from sham and ovariectomized mice were analyzed by t test. All experiments were repeated at least twice.

Results

In initial experiments, $CD45R^+$ cells from C57BL/6 mouse bone marrow were isolated by FACS to high purity (typically >94–98%) and cultured with M-CSF and RANKL (30 ng/ml for both) for 6–7 days at a plating density of 10^5 cells per well of a 96-well plate. At the conclusion of experiments, cells were stained for TRAP. Mono and multinucleated $TRAP^+$ cells formed in these cultures (typically 100–200 $TRAP^+$ multinucleated cells per well of a 96-well plate) (data not shown). However, to increase the number of OCL in the cultures so that they could be further characterized, $CD45R^+$ cells were first cultured with M-CSF (30 ng/ml) alone for 7 days to increase the total cell number. Cultures were then treated with both RANKL and M-CSF (both at 30 ng/ml) for an additional 7

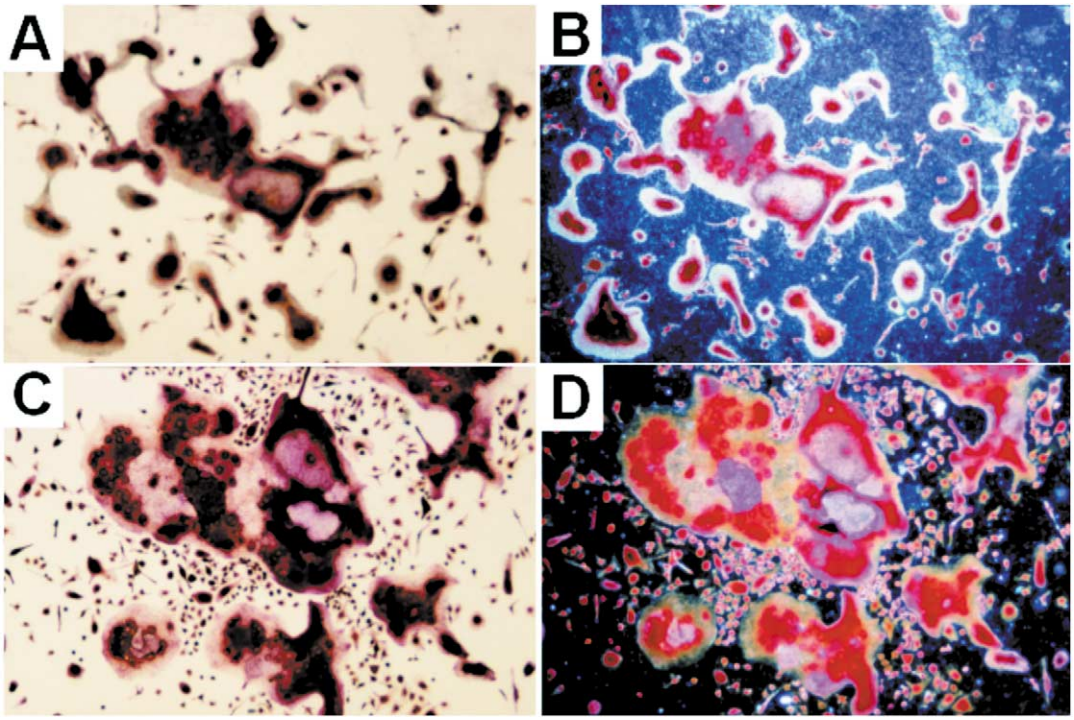
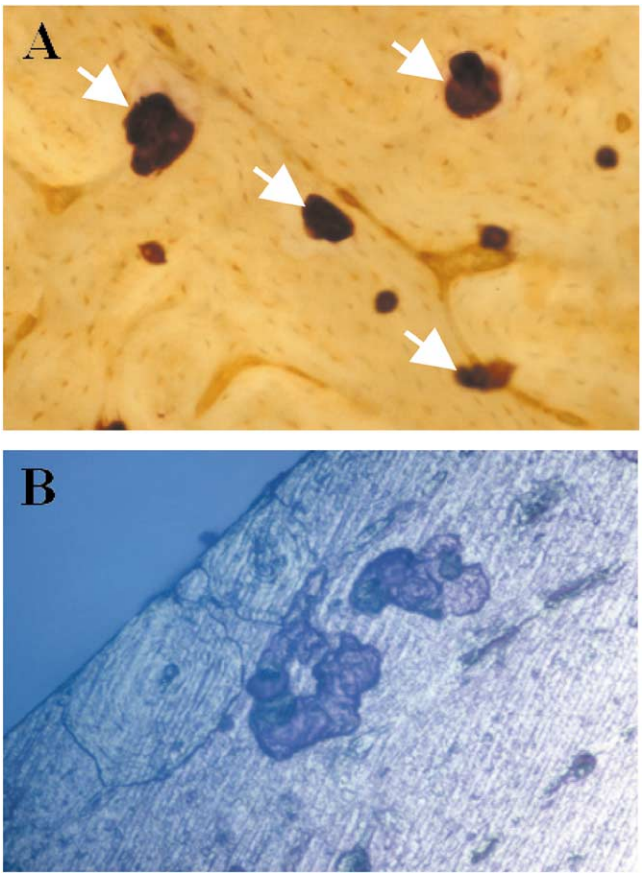


Fig. 1. Expression of calcitonin receptors on OCL formed in CD45R$^+$ bone marrow cultures. CD45R$^+$ bone marrow cells were isolated by FACS and cultured for 1 week (10^5 cells per well of a 8-well slide chamber) with M-CSF (30 ng/ml) and then an additional week with M-CSF + RANKL (both at 30 ng/ml). At the conclusion of the experiment, cells were incubated with and without radiolabeled calcitonin (CT) (A and B) or with 100-fold excess cold CT (C and D). Cells were stained for TRAP and then developed for autoradiography. Images A and C are brightfield. Images B and D are darkfield. Silver grains, indicating radiolabeled CT binding, appear as black dots in brightfield images and white dots in darkfield images. Note the white reticular pattern in B overlying OCL, which is not present in D demonstrating specific CT binding to OCL.



days to generate OCL. An increased number of OCL formed using this protocol. To confirm the osteoclastic nature of the OCL that formed in 2-week cultures, cells were incubated at the conclusion of experiments with [^{125}I]sCT and with or without a 100-fold excess of unlabeled sCT. The ability of cells to specifically bind calcitonin is a characteristic of mature osteoclasts and demonstrates the presence of membrane calcitonin receptors. We found that greater than 98% of the multinucleated TRAP$^+$ OCL in the 2-week cultures expressed high levels of calcitonin receptors as demonstrated by specific [^{125}I]sCT binding (Fig. 1). Similarly, we also found that greater than 98% of the OCL in 7-day cultures of CD45R$^+$ bone marrow cells stimulated with M-CSF and RANKL expressed high levels of calcitonin receptors (data not shown).

We also characterized the ability of the OCL that formed

Fig. 2. OCL from CD45R$^+$ bone marrow cell cultures resorb bone. CD45R$^+$ bone marrow cells were isolated by FACS and cultured for 1 week (10^5 cells per well of a 96-well plate) with M-CSF (30 ng/ml) and then an additional week with M-CSF + RANKL (both at 30 ng/ml) on bovine cortical bone slices. Bone slices were then stained for TRAP and photographed (A). Note the multiple, large TRAP$^+$ OCL in A as identified by arrows. Bone slices were then sonicated to remove adherent cells, stained with toluidine blue, and photographed by indirect light to demonstrate resorption lacunae (B). Note the characteristic multiple overlapping resorption tracts produced by the OCL in B.

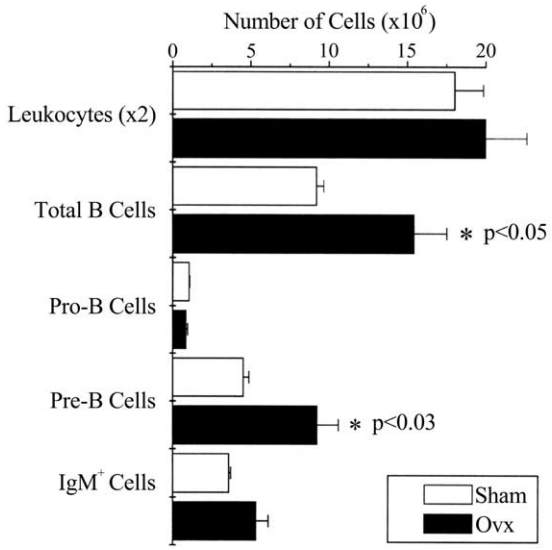


Fig. 3. Ovariectomy increases total B-lymphocytes and pre-B-lymphocytes in bone marrow. Eight-week-old female C57BL/6 mice were either sham-operated (Sham) or ovariectomized (Ovx) and then sacrificed at 12 weeks of age. Bone marrow cells were removed and analyzed for total and B-lymphocyte subgroups by flow cytometry as outlined under Methods. *Significant difference between Sham and Ovx groups as indicated.

from $CD45R^+$ cells in 2-week cultures to resorb bone. $CD45R^+$ bone marrow cells were isolated by FACS to high purity and incubated with M-CSF for 7 days and then with RANKL and M-CSF for an additional 7 days in wells containing devitalized bovine cortical bone slices. $TRAP^+$ OCL in these cultures produced numerous characteristic resorption lacunae on the bone slices (Fig. 2).

We examined the effects of Ovx-induced estrogen withdrawal on the number of OCL that formed in 7-day cultures of $CD45R^+$ bone marrow cells. Murine marrow cells were examined 3–4 weeks after either sham operation (Sham) or Ovx. We have previously demonstrated that changes in trabecular bone mass occurred at this time [32]. It is well established that the number of B-lymphocyte precursor cells in murine bone marrow increases after Ovx [22]. In agreement, we found that Ovx had only a small effect on the total leukocyte number in the marrow of mice but did increase the total B-lymphocyte number in marrow by approximately 1.5-fold (Fig. 3). This result occurred predominantly because Ovx caused an approximately 2-fold increase in the number of $CD45R^+$ HSA^{low} IgM^- pre-B-lymphocytes in the marrow.

We also examined whether Ovx altered the number of OCL which formed in 7-day cultures of $CD45R^+$ bone marrow cells that were treated with RANKL and M-CSF. In these experiments marrow cells were incubated either unfractionated or separated into $CD45R^+$ and $CD45R^-$ cells. OCL formation in marrow from Ovx mice was increased approximately 1.5 to 2-fold in both unfractionated and $CD45R^+$ marrow cells compared to similar cultures from Sham mice (Fig. 4). Significantly, there was no effect of Ovx on the number of OCL that formed in cultures of $CD45R^-$ bone marrow cells. These results implied that the effect of Ovx to increase the number of osteoclast precursor cells in bone marrow cultures occurred predominantly in the $CD45R^+$ cells. To further examine this issue, we measured osteoclast precursor frequency in murine bone marrow using a limiting dilution assay. This assay has been used previously to determine the relative number of hematopoietic progenitor cells in a population [26,29,30]. In this assay the number of wells which are free of OCL (of 20 plated) is determined for each serial dilution of cells. Populations of murine marrow cells containing fewer osteoclast precursors produce more OCL-free wells for any given plating density at the limits of cell dilution. Using this assay, we confirmed

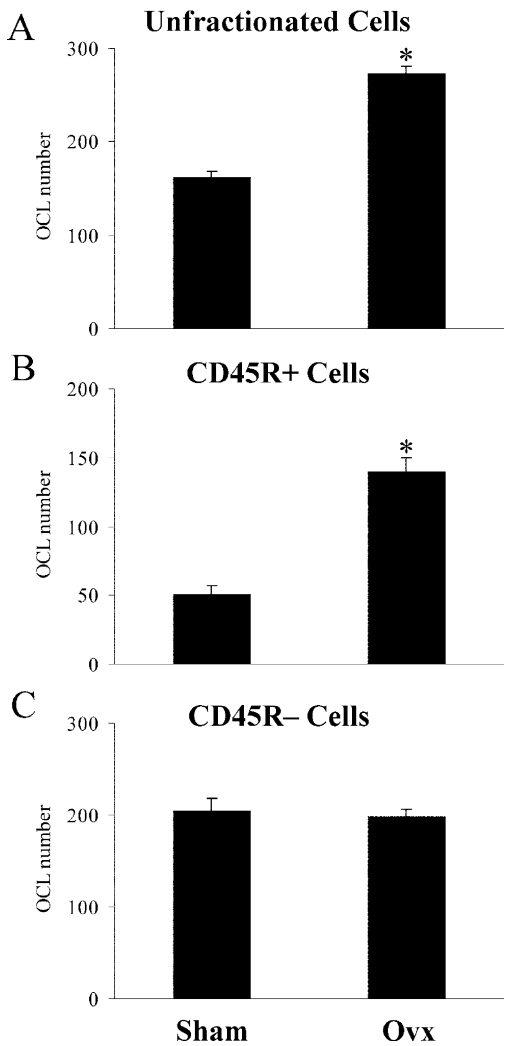


Fig. 4. Ovariectomy increases the number of OCL that form in cultured unfractionated and $CD45R^+$ bone marrow cells. Eight-week-old female C57BL/6 mice were either sham-operated (sham) or ovariectomized (Ovx) and then sacrificed at 11 weeks of age. Bone marrow cells were removed, cultured either unfractionated (A) or fractionated into either $CD45R^+$ (B) or $CD45R^-$ (C) cells by FACS, and treated with M-CSF + RANKL (both at 30 ng/ml) for 6 days. OCL were identified as $TRAP^+$ multinucleated cells. *Significant difference between sham and Ovx groups, $P < 0.01$.

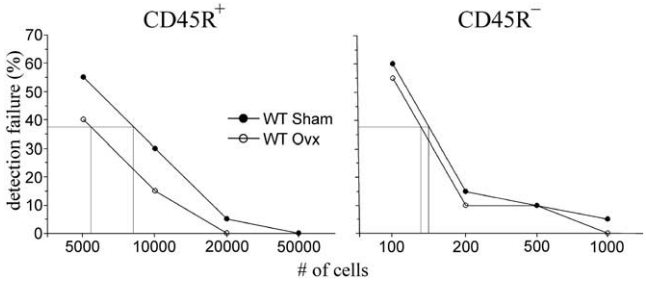


Fig. 5. Ovariectomy increases the number of OCL precursor cells in cultured $CD45R^+$ but not in $CD45R^-$ bone marrow cells as determined by limiting dilution assay. Eight-week-old female C57BL/6 mice were either sham-operated (Sham) or ovariectomized (Ovx) and then sacrificed at 11 weeks of age. Bone marrow cells were removed, fractionated into either $CD45R^+$ or $CD45R^-$ cells by FACS, and cultured with M-CSF + RANKL (both at 30 ng/ml) for 6 days. OCL were identified as $TRAP^+$ multinucleated cells. Cells were plated at four decreasing dilutions in 20 wells per dilution of a 384-well plate. OCL were identified as $TRAP^+$ multinucleated cells.

an appoximate 1.5-fold greater number of osteoclast precursor cells in the $CD45R^+$ population of marrow cells from Ovx C57BL/6 mice compared to $CD45R^+$ cultures from Sham mice (compare the dilution of cells in which 37.5% of the wells contain OCL, Fig. 5). To confirm this finding we calculated the frequency of osteoclast precursors in $CD45R^+$ cells and found Ovx to increase this value by 60 $\pm$ 6% ($P < 0.01$) compared to the results in Sham cells. We also found that there was little change in osteoclast precursor frequency in $CD45R^-$ population from these same mice after Ovx. The $CD45R^-$ populations contained a roughly 50 to 100-fold higher concentration of osteoclast precursor cells than did the $CD45R^+$ populations (Fig. 5) since a much higher number of $CD45R^+$ cells had to be added to wells to produce results similar to that seen with $CD45R^-$ cells.

IL-1R1 KO mice do not lose bone mass after Ovx [27]. Therefore, we examined the ability of Ovx to alter B-lymphocyte subsets and OCL formation rates in unfractionated, $CD45R^+$, and $CD45R^-$ bone marrow cell cultures from WT and IL-1R1 KO mice. In these experiments WT and IL-1R1 KO mice were both in a C57BL/6 $\times$ 129Sv background that was originally derived from heterogeneous (IL-1R1 +/−) siblings. As we saw with WT mice in a pure C57BL/6 background, Ovx induced a 1.5-fold increase in total B-lymphocytes and a 2-fold increase in pre-B-lymphocytes in the marrow of both WT and IL-1R1 KO mice in a C57BL/6 $\times$ 129Sv background (data not shown). However, in contrast to the 1.5 to 2-fold increase in OCL formation we found in unfractionated and $CD45R^+$ bone marrow cells from WT mice in either background (Figs. 4 and 6), there was no effect of Ovx on the number of OCL that formed in unfractionated, $CD45R^+$, or $CD45R^-$ cells from IL-1R1 KO mice (Fig. 6).

Using flow cytometry, we examined the number of cells in bone marrow from WT or IL-1R1 KO mice that expressed CD45R, RANK, or c-Fms (Fig. 7). We found that in Sham and Ovx mice only 1.3$\pm$0.6–3.5$\pm$0.5% of bonemarrow cells expressed RANK. About 1/3 to 1/2 of Sham or Ovx bone marrow $RANK^+$ cells were also $CD45R^+$. Because of the small number of $RANK^+$ cells in murine bone marrow, it was difficult to make conclusions about the effects of Ovx on this population.

c-Fms was expressed by 14.3 $\pm$ 0.7 and 12.7 $\pm$ 0.8% of murine bone marrow cells from Sham WT and Sham IL-1R1 KO mice, respectively, and 33.2 $\pm$ 2.1% of these cells were also $CD45R^+$. The percentage of all cells expressing c-Fms did not change markedly with Ovx (11.6 $\pm$ 0.6 and 12.1 $\pm$ 0.4%, respectively, for WT and IL-1R1 KO). In WT or IL-1R1 KO Ovx mice 42 $\pm$ 2% of $c\text{-}Fms^+$ marrow cells were also $CD45R^+$. Hence, there was little effect of Ovx on $c\text{-}Fms^+$ cells in either WT or IL-1R1 KO mice.

Discussion

Our results demonstrate that a highly enriched population of $CD45R^+$ cells is capable of differentiating into OCL with many characteristics of authentic osteoclasts. These

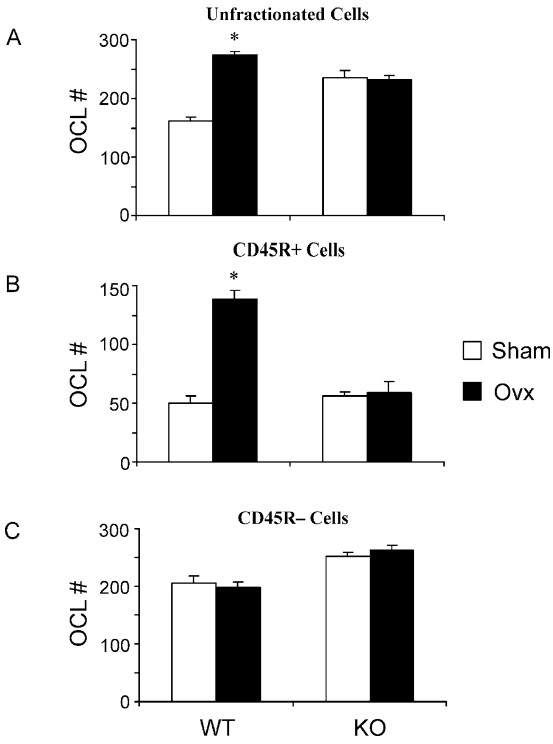


Fig. 6. Ovariectomy does not increase the number of OCL that form in cultured unfractionated, $CD45R^+$, or $CD45R^-$ bone marrow cells from IL-1R1 KO mice. Eight-week-old female C57BL/6 $\times$ 129Sv wild-type (WT) or IL-1R1 KO (KO) mice were either sham-operated (Sham) or ovariectomized (Ovx) and then sacrificed at 11 weeks of age. Bone marrow cells were removed, cultured either unfractionated (A) or fractionated into either $CD45R^+$ (B) or $CD45R^-$ (C) cells by FACS, and treated with M-CSF + RANKL (both at 30 ng/ml) for 6 days. OCL were identified as $TRAP^+$ multinucleated cells. *Significant difference between Sham and Ovx groups, $P < 0.01$.

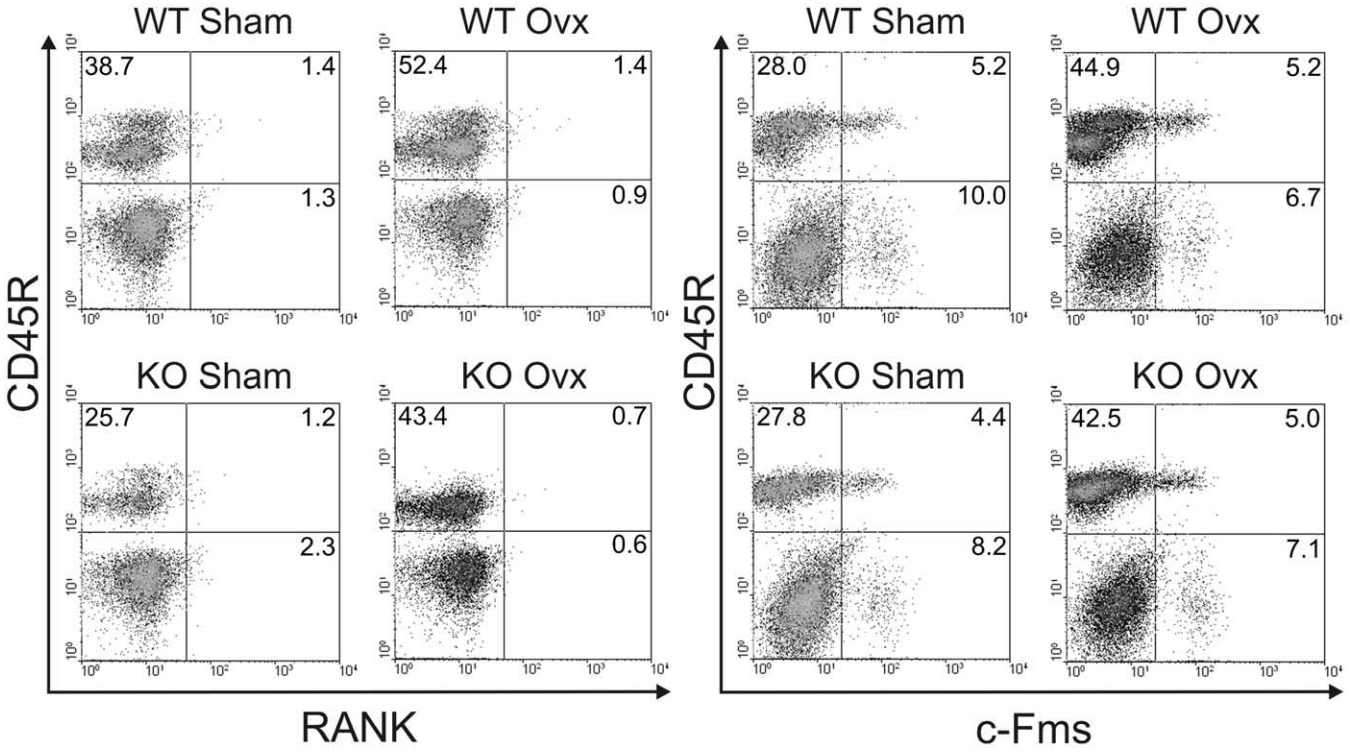


Fig. 7. Expression of CD45R, RANK, and c-Fms in bone marrow cells from sham and Ovx wild-type and IL-1R1 KO mice. Eight-week-old female C57BL/6 × 129Sv wild-type (WT) or IL-1R1 KO (KO) mice were either sham-operated (Sham) or ovariectomized (Ovx) and then sacrificed at 11 weeks of age. Bone marrow cells were immediately collected, stained for the indicated antigens, and analyzed by flow cytometry, after gating out dead cells stained with propidium iodide. Numbers in each corner indicate the relative percentage of cells for the fraction represented by that quadrant.

include multinucleation, calcitonin receptor expression, TRAP production, and the ability to resorb bone. In addition, we found that, like pre-B-lymphocytes and CFU-GM, the number of OCL that form in the cultures of $CD45R^+$ bone marrow cells is increased in bone marrow cell cultures from Ovx animals. In support of our findings another group recently described a $CD45R^+$ osteoclast precursor cell in murine bone marrow [25]. In addition, it was shown that Ovx increased OCL formation in mixed cultures of $CD45R^+$ bone marrow cells and ST2 mesenchymal stromal cells that were treated with vitamin D [26].

However, we also found that highly enriched $CD45R^-$ murine bone marrow cell cultures do not demonstrate an increase in OCL number after Ovx. Furthermore, we found that OCL formation in vitro was not increased by estrogen withdrawal in unfractionated, $CD45R^+$, or $CD45R^-$ bone marrow cells from IL-1R1 KO mice, which do not lose bone mass after estrogen withdrawal. Hence, our data demonstrate that the increase in OCL formation, which occurs in unfractionated murine bone marrow cell cultures after Ovx, is only reproduced in the $CD45R^+$ subpopulation when bone marrow is fractionated into $CD45R^+$ and $CD45R^-$ cells. In addition, our finding that Ovx did not increase OCL formation in cultured marrow cells from IL-1R1 KO mice suggests that increases in OCL formation in either unfractionated or $CD45R^+$ bone marrow cells predict whether bone loss will occur in mouse models of estrogen withdrawal.

The existence of a common lymphoid/myeloid precursor cell in marrow has been demonstrated previously [33,34]. This cell has the capacity to differentiate into either B-lymphocytes or monocytes and the decision to enter either lineage appears to be under the control of cytokines [34]. Expression of CD45R is present on early B-lymphocyte lineage cells, which do not yet express CD19 but do express membrane c-kit and Flt3/Flk-2 [16] or CD24 [35]. Hence, the expression of CD45R on cells from murine marrow identifies multiple B-lymphocyte developmental stages including some very immature cells, which may have the capacity to differentiate into other lineages.

Recently, it was demonstrated that B-lineage commitment depends on the orderly expression of three transcription factors: the basic helix–loop–helix proteins E2A and early B-cell factor (EBF) and the transcription factor Pax5 [36]. In mice deletion of any of these genes causes the complete absence of B-lymphopoiesis [23,37]. E2A and EBF are essential for early B-cell differentiation and their loss blocks lymphopoiesis before immunoglobulin rearrangement has occurred. In contrast, in Pax5 −/− mice early B-lymphocyte development is arrested at the pre-B-lymphocyte stage [38]. Interestingly, expression of Pax5 by retroviral transduction in Pax5 −/− pre-B cells downregu-

lated expression of myeloid genes like c-Fms [38]. Hence, expression of Pax5 appears to be a critical step in the decision of multilineage precursor cells to commit to the B-lymphocyte lineage. In support of this hypothesis, it was shown that pre-B-lymphocytes from Pax5 −/− mice have the capacity to develop into a variety of myeloid lineages including osteoclasts [1]. These results support the hypothesis that some $CD45R^+$ precursor cells in WT mice have the capacity to differentiate into multiple lineages including osteoclasts.

Regulation of osteoclast precursor cell abundance with Ovx as manifested by an increase in CFU-GM [18] and pre-B-lymphocytes [22] is well established. Our findings that the number of OCL that formed in cultures from $CD45R^+$ cells increased proportionally to that of pre-B-lymphocytes in marrow implies that these events are related. Significantly, it was previously demonstrated that Ovx increased B-lymphopoiesis in rat bone marrow [39]. Together, these results suggest that estrogen withdrawal increases the abundance of a common lymphoid/osteoclast precursor cell in marrow without altering the abundance of precursors of other lineages. In support of this hypothesis, we failed to see an effect of Ovx on OCL formation in cultures of $CD45R^-$ cells, which likely contain cells committed to the myeloid and granulocyte lineages.

It is unlikely that the cells within the $CD45R^+$ population, which differentiate into OCL, solely represent contaminating $CD45R^-$ cells since we failed to demonstrate increases in OCL formation with Ovx in $CD45R^+$ cells from IL-1R1 KO mice. The IL-IR1 KO mouse does not lose bone mass after Ovx [27]. However, this mouse does increase $CD45R^+$ pre-B-lymphocytes in its marrow similar to the increase that occurs in cells from WT mice. If contaminating $CD45R^-$ cells within the $CD45R^+$ populations were the sole cause of the OCL that formed in these cultures, this contamination should have been equivalent in cells from WT and ILR1 KO mice. As a consequence, OCL formation should have increased equally after Ovx in cultured $CD45R^+$ cells from both WT and IL-1R1 KO mice. The fact that it did not argues that there are fundamental differences in the osteoclastogenic potential after Ovx of $CD45R^+$ cells from WT and IL-1R1 KO mice and contamination of these populations with $CD45R^-$ cells does not appear to account for these differences.

Our results showing 50- to 100-fold fewer osteoclast precursor cells in the $CD45R^+$ populations than in the $CD45R^-$ population is in disagreement with previous data [26] which found an equal number of osteoclast precursors in both the $CD45R^+$ and the $CD45R^-$ population. The reason for this discrepancy is unknown but may reflect differences in the methodology used to identify osteoclast precursor cells. The finding that a greater number of OCL formed in unfractionated and $CD45R^+$ populations of WT marrow cells after Ovx than in cells from WT Sham mice argues that changes in the $CD45R^+$ population influence the ability of the $CD45R^-$ population to differentiate into osteoclasts by some unknown pathway. This could occur through an amplification process whereby a small population of $CD45R^+$ cells that are estrogen responsive influences the osteoclastogenic potential of the much larger $CD45R^-$ osteoclast precursor population. The recent finding that $CD45R^+$ cells express RANKL [25] may be a mechanism for this effect.

The exact pathway by which estrogen regulates the number of lymphoid or osteoclast progenitor cells in bone marrow is unknown. Both B-lymphocytes and osteoclasts develop through an interaction of hematopoietic precursors and stromal cells [1,32]. The effects of estrogen on B-lymphopoiesis have been implicated to occur through responses of stromal cells [40] and direct effects on B-lineage precursor cells [41]. Similarly, regulation of osteoclast formation by estrogen may be mediated by either stromal cells [42] or direct actions of estrogen on osteoclast precursor cells [19]. In addition, M-CSF expression can also be influenced by estrogen [43] and this response may be involved in regulating the number of osteoclast precursors in bone marrow.

It is likely that IL-1 responsiveness is necessary for estrogen to increase the osteoclastogenic potential of $CD45R^+$ murine bone marrow cells since there was no effect of Ovx on OCL formation in cultured bone marrow cells from IL-1R1 KO mice. This result occurred even though Ovx increased $CD45R^+$ cells in bone marrow. Hence, it appears that the role of IL-1 in estrogen-mediated effects on $CD45R^+$ cells is to increase their ability to form osteoclasts by possibly enhancing their commitment toward the osteoclast lineage.

Since increases in osteoclast precursor cell abundance in marrow have been linked to the development of estrogen-withdrawal-induced osteoporosis [43], it is likely that alterations of the osteoclastic potential of $CD45R^+$ cell populations in bone marrow are involved in the increased osteoclastic activity that occurs under this condition.

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References

[1] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289: 1504–8.
[2] Walker DG. Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. Science 1975;190: 784–5.

[3] Holtrop ME, King GJ. The ultrastructure of the osteoclast and its functional implications. Clin Orthop 1977;123:177–96.
[4] Chambers TJ, Horton MA. Failure of cells of the mononuclear phagocyte series to resorb bone. Calcif Tissue Int 1984;36:556–8.
[5] Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA. The human osteoclast precursor circulates in the monocyte fraction. Endocrinology 1996;137:4058–60.
[6] Menaa C, Kurihara N, Roodman GD. CFU-GM-derived cells form osteoclasts at a very high efficiency. Biochem Biophys Res Commun 2000;267:943–6.
[7] Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nisikawa S. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 1990;345:442–4.
[8] Hofstetter W, Wetterwald A, Cecchini MC, Felix R, Fleisch H, Mueller C. Detection of transcripts for the receptor for macrophage colony-stimulating factor, c-*fms*, in murine osteoclasts. Proc Natl Acad Sci USA 1992;89:9637–41.
[9] Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ. Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. Am J Pathol 2000;157:435–48.
[10] Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76.
[11] Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998;95:3597–602.
[12] Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390:175–9.
[13] Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS III, Frankel WN, Lee SY, Choi Y. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272:25190–4.
[14] Lee SK, Lorenzo JA. Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. Endocrinology 1999;140:3552–61.
[15] Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 1999;96:3540–5.
[16] Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997;89:309–19.
[17] Nagai M, Sato N. Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation. Biochem Biophys Res Commun 1999;257:719–23.
[18] Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 1992;257:88–91.
[19] Shevde NK, Bendixen AC, Dienger KM, Pike JW. Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. Proc Natl Acad Sci USA 2000;97:7829–34.
[20] Medina KL, Smithson G, Kincade PW. Suppression of B lymphopoiesis during normal pregnancy. J Exp Med 1993;178:1507–15.
[21] Kincade PW, Medina KL, Smithson G. Sex hormones as negative regulators of lymphopoiesis. Immunol Rev 1994;137:119–34.
[22] Masuzawa T, Miyaura C, Onoe Y, Kusano K, Ohta H, Nozawa S, Suda T. Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. J Clin Invest 1994;94:1090–7.
[23] Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. Nature 1995;376:263–7.
[24] Coffman RL, Weissman IL. B220: a B cell-specific member of the T200 glycoprotein family. Nature 1981;19:289:681–3.
[25] Manabe N, Kawaguchi H, Chikuda H, Miyaura C, Inada M, Nagai R, Nabeshima Y, Nakamura K, Sinclair AM, Scheuermann RH, Kuro-o M. Connection between B lymphocyte and osteoclast differentiation pathways. J Immunol 2001;167:2625–31.
[26] Sato T, Shibata T, Ikeda K, Watanabe K. Generation of bone-resorbing osteoclasts from $B220^{+}$ cells: its role in accelerated osteoclastogenesis due to estrogen deficiency. J Bone Miner Res 2001;16:2215–21.
[27] Lorenzo JA, Naprta A, Rao Y, Alander C, Glaccum M, Widmer M, Gronowicz G, Kalinowski J, Pilbeam CC. Mice lacking the type I interleukin-1 receptor do not lose bone mass after ovariectomy. Endocrinology 1998;139:3022–5.
[28] Sudo T, Nishikawa S, Ogawa M, Kataoka H, Ohno N, Izawa A, Hayashi S, Nishikawa S. Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. Oncogene 1995;11:2469–76.
[29] Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J Immunol 1981;126:1614–9.
[30] Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. III. Validity tests for the single-hit Poisson model. J Immunol Methods 1984;72:29–40.
[31] Hurley MM, Lee SK, Raisz LG, Bernecker P, Lorenzo J. Basic fibroblast growth factor induces osteoclast formation in murine bone marrow cultures. Bone 1998;22:309–16.
[32] Melchers F, Rolink A. B-lymphocyte development and biology. In: Paul WE, editor. Fundamental immunology. Philadelphia: Lippincott–Raven; 1999, p. 183–224.
[33] Hirayama F, Shih JP, Awgulewitsch A, Warr GW, Clark SC, Ogawa M. Clonal proliferation of murine lymphohemopoietic progenitors in culture. Proc Natl Acad Sci USA 1992;89:5907–11.
[34] Hirayama F, Clark SC, Ogawa M. Negative regulation of early B lymphopoiesis by interleukin 3 and interleukin 1 alpha. Proc Natl Acad Sci USA 1994;91:469–73.
[35] Li YS, Wasserman R, Hayakawa K, Hardy RR. Identification of the earliest B lineage stage in mouse bone marrow. Immunity 1996;5:527–35.
[36] Busslinger M, Nutt SL, Rolink AG. Lineage commitment in lymphopoiesis. Curr Opin Immunol 2000;12:151–8.
[37] Zhuang Y, Soriano P, Weintraub H. The helix–loop–helix gene E2A is required for B cell formation. Cell 1994;79:875–84.
[38] Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature 1999;401:556–62.
[39] Erben RG, Raith S, Eberle J, Stangassinger M. Ovariectomy augments B lymphopoiesis and generation of monocyte–macrophage precursors in rat bone marrow. Am J Physiol 1998;274:E476–83.

[40] Smithson G, Medina K, Ponting I, Kincade PW. Estrogen suppresses stromal cell-dependent lymphopoiesis in culture. J Immunol 1995; 155:3409–17.
[41] Medina KL, Strasser A, Kincade PW. Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors. Blood 2000;95:2059–67.
[42] Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R. Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1- and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. J Biol Chem 1996;271:28890–7.
[43] Srivastava S, Weitzmann MN, Kimble RB, Rizzo M, Zahner M, Milbrandt J, Ross FP, Pacifici R. Estrogen blocks M-CSF gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. J Clin Invest 1998;102: 1850–9.