The Journal of Immunology

Connection Between B Lymphocyte and Osteoclast Differentiation Pathways

Noriyo Manabe, Hiroshi Kawaguchi, Hirotaka Chikuda, Chisato Miyaura, Masaki Inada, Ryozo Nagai, Yo-ichi Nabeshima, Kozo Nakamura, Angus M. Sinclair, Richard H. Scheuermann and Makoto Kuro-o

J Immunol 2001; 167:2625-2631; ;
doi: 10.4049/jimmunol.167.5.2625
http://www.jimmunol.org/content/167/5/2625

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Connection Between B Lymphocyte and Osteoclast Differentiation Pathways[1]

Noriyo Manabe,* Hiroshi Kawaguchi,* Hirotaka Chikuda,* Chisato Miyaura,‡ Masaki Inada,‡ Ryozo Nagai,† Yo-ichi Nabeshima,§¶ Kozo Nakamura,* Angus M. Sinclair,‖ Richard H. Scheuermann,‖ and Makoto Kuro-o[2]‖

Osteoclasts differentiate from the hemopoietic monocyte/macrophage cell lineage in bone marrow through cell-cell interactions between osteoclast progenitors and stromal/osteoblastic cells. Here we show another osteoclast differentiation pathway closely connected with B lymphocyte differentiation. Recently the TNF family molecule osteoclast differentiation factor/receptor activator of NF-κB ligand (ODF/RANKL) was identified as a key membrane-associated factor regulating osteoclast differentiation. We demonstrate that B-lymphoid lineage cells are a major source of endogenous ODF/RANKL in bone marrow and support osteoclast differentiation in vitro. In addition, B-lymphoid lineage cells in earlier developmental stages may hold a potential to differentiate into osteoclasts when stimulated with M-CSF and soluble ODF/RANKL in vitro. B-lymphoid lineage cells may participate in osteoclastogenesis in two ways: they 1) express ODF/RANKL to support osteoclast differentiation, and 2) serve themselves as osteoclast progenitors. Consistent with these observations in vitro, a decrease in osteoclasts is associated with a decrease in B-lymphoid cells in *klotho* mutant mice (*KL*$^{-/-}$), a mouse model for human aging that exhibits reduced turnover during bone metabolism, rather than a decrease in the differentiation potential of osteoclast progenitors. Taken together, B-lymphoid lineage cells may affect the pathophysiology of bone disorders through regulating osteoclastogenesis. ***The Journal of Immunology*, 2001, 167: 2625–2631.**

A multipotent hemopoietic stem cell undergoes lineage commitment and subsequent sequential differentiation along each lineage pathway, giving rise to all types of blood cells. The stem cells committed to the myeloid lineage (common myeloid progenitors) differentiate into granulocytes, monocyte/macrophages, and dendritic cells. The common lymphoid progenitors differentiate into NK, B, and T lymphocytes.

Osteoclasts, multinucleated cells primarily responsible for bone resorption, have been shown to differentiate from the monocyte/macrophage lineage (1–4). Mature osteoclasts can be formed in vitro from bone marrow cells in the presence of two cytokines, M-CSF and osteoclast differentiation factor/receptor activator of NF-κB ligand (ODF/RANKL)[3] (5–9). Osteoblastic cells and stromal cell lines such as ST2 also support osteoclast formation from bone marrow cells in vitro. In addition to the in vitro studies, recent experiments using knockout mice demonstrated that disruption of either *m-csf* or *odf/rankl* gene causes increased bone mass (osteopetrosis) due to a complete lack of osteoclasts (10, 11). Thus, M-CSF and ODF/RANKL are regarded as key regulators of osteoclastogenesis. However, the osteoclast defect in *m-csf* mutant mice can be restored by overexpression of anti-apoptotic protein Bcl-2 in monocyte lineage cells (12), suggesting that M-CSF is essential for the survival but not the differentiation of osteoclast progenitors.

The monocyte/macrophage lineage cells that give rise to osteoclasts and lymphoid lineage cells are thought to be distantly related. However, evidence is accumulating in support of a close relationship between macrophage and B-lymphoid cell differentiation pathways. There are numerous reports on B cell lymphomas that acquired macrophage-like phenotypes, including characteristic morphological changes and expression of mature macrophage surface markers (see Ref. 13 for review). In addition, a subset of normal B cells (CD5$^+$ B cells) is known to have properties of both B cells and macrophages (13, 14). CD5$^+$ B cells are adherent and highly phagocytic, and they coexpress surface markers characteristic of macrophages (F4/80 and Mac-1) and B cells (B220, IgM, and IgD). Recently, it was reported that Pax5-deficient pro-B cells have the ability to take various differentiation pathways, including the monocyte/macrophage lineage, and can give rise to osteoclasts in vitro (15). These findings are challenging current ideas of lineage commitment in B-lymphoid lineage cells.

Departments of *Orthopaedic Surgery and †Cardiovascular Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan; ‡Department of Biochemistry, Tokyo University of Pharmacy and Life Science, Tokyo, Japan; §Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; ¶Core Research for Evolutional Science and Technology, Research Development Corporation of Japan, Tokyo, Japan; and ‖Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390

Received for publication September 21, 2000. Accepted for publication July 12, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Japanese Ministry of Education, Science, Sports and Culture Grants 12137201 and 12307031, the Japan Space Forum (to H.K.), a Bristol-Myers Squibb/Zimmer Unrestricted Research Grant (to K.N.), Southwestern Medical Foundation at the University of Texas Southwestern Medical Center, Pew Scholars Program in the Biomedical Science, Donald W. Reynolds Cardiovascular Clinical Research Center (Dallas, Texas), Tanabe Medical Frontier Conference, and Eisai Research Fund (to M.K.).

[2] Address correspondence and reprint requests to Dr. Makoto Kuro-o, Department of Pathology, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-9072. E-mail address: kuroo.makoto@pathology.swmed.edu

[3] Abbreviations used in this paper: ODF/RANKL, osteoclast differentiation factor/receptor activator of NF-κB ligand; *KL*, *klotho*; TRAP, tartrate-resistant acid phosphatase; PBS$^-$, Ca^{2+}- and Mg^{2+}-free PBS.

0022-1767/01/$02.00

We recently established a mouse model for human aging termed *klotho* (16). The *klotho* mouse was serendipitously generated by insertional mutation of a transgene in a transgenic mouse, which disrupted the *klotho* gene encoding a novel single-pass membrane protein. A defect in *klotho* gene expression leads to a syndrome closely resembling human aging including a shortened lifespan, decreased spontaneous activity, infertility, skin atrophy, arteriosclerosis, premature thymic involution, pulmonary emphysema, and osteopenia, among others. Osteopenia observed in *klotho* mutant mice ($KL^{-/-}$ mice) is accompanied by low turnover during bone metabolism, in which the decrease in bone formation exceeds the decrease in bone resorption, resulting in a net bone loss (17). The number of osteoclasts was significantly decreased in $KL^{-/-}$ mice. In addition, osteoclast formation from bone marrow cells of $KL^{-/-}$ mice in vitro was significantly reduced (17). The pathophysiology of the bone in $KL^{-/-}$ mice recapitulates senile osteoporosis in humans rather than postmenopausal osteoporosis, which is primarily caused by increased bone resorption accompanied by an increase in the number of osteoclasts.

In an effort to find out the cellular mechanism by which $KL^{-/-}$ mice develop osteopenia resembling senile osteoporosis, we found two novel roles that B-lymphoid lineage cells appear to play in osteoclastogenesis. In the present study, we report that B-lymphoid lineage cells express abundant ODF/RANKL and have the ability to support osteoclast differentiation. In addition, we show that a subset of normal bone marrow cells that express markers of B-lymphoid lineage cells in early developmental stages can serve as osteoclast progenitors and are able to give rise to osteoclasts in vitro. We also show that $KL^{-/-}$ mice have significantly reduced numbers of B-lymphoid lineage cells in the bone marrow, which could cause a decrease in osteoclasts in $KL^{-/-}$ mice due to the simultaneous reduction in cells expressing ODF/RANKL and in cells serving as osteoclast progenitors. These findings provide new insights into current concepts of osteoclast differentiation and molecular mechanisms of bone disorders such as osteoporosis.

Materials and Methods

Animals

Wild-type ($KL^{+/+}$) and $KL^{-/-}$ mice were generated by mating of heterozygous *klotho* mutant mice ($KL^{+/-}$). The genetic background of the original *klotho* mouse was a mixture of C57BL/6J and C3H/J (16).

Histomorphometric analysis of the bone

All histological analyses were conducted using 8-wk-old $KL^{+/+}$ and $KL^{-/-}$ littermates. Tibiae were excised, fixed with 100% ethanol, embedded in methyl methacrylate, and sectioned in 6-μm slices for Villanueva-Goldner staining and tartrate-resistant acid phosphatase (TRAP) staining. TRAP staining was conducted at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate (Sigma, St. Louis, MO) in *N,N*-dimethyl formamide as a substrate. The specimens were subjected to histomorphometric analyses under a light microscope with a micrometer using an image analyzer (System Supply, Nagano, Japan). Parameters for bone resorption were measured in a trabecular bone area (1.2 mm in length from 0.1 mm below the growth plate) at the proximal metaphysis of the tibiae. The number and function of osteoclasts were determined by counting the number of TRAP-positive cells and by measuring the percentage of bone surface area eroded by osteoclasts with the image analyzer.

Cell preparation

Bone marrow cells were prepared from mouse tibiae by flushing out the bone marrow with Ca^{2+}- and Mg^{2+}-free PBS (PBS^-). The bone marrow cells were resuspended in 2 ml of ammonium chloride-Tris buffer to lyse red blood cells. The cell suspension was washed with PBS^- three times and resuspended in 1 ml of PBS^- containing 1% BSA. Spleen cell suspensions were prepared in the same way. PBMC were separated using Ficoll-Conray solution for mouse lymphocytes.

Flow cytometric analysis

Cells (1×10^6) were incubated for 30 min on ice with various combinations of FITC-conjugated anti-B220/CD45R (RA3-6B2; BD PharMingen, San Diego, CA), Gr-1 (RB6-8C5; BD PharMingen), CD3 (17A2; BD PharMingen), or F4/80 (MCA497F; Serotec, Oxford, U.K.), and PE-conjugated TER-119 (TER-119; BD PharMingen) or IgM-μ-chain Abs (Tago Scientific, Burlingame, CA), washed twice with PBS^-, and resuspended in PBS^- containing 1% BSA. The cells labeled with fluorescent dyes were analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Mountain View, CA). For two-color analysis of ODF/RANKL and B220, cells were incubated for 30 min on ice with FITC-conjugated anti-B220 and an affinity-purified goat polyclonal anti-ODF/RANKL Ab (C-20: sc-7627; Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide mapping at the carboxyl terminus of human ODF/RANKL and shown to react with mouse, rat, and human ODF/RANKL. After washing with PBS^-, cells were incubated with biotin-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and washed twice with PBS^-. The cells were then incubated for 10 min at room temperature with streptavidin-PE conjugate (BD PharMingen), and washed twice with PBS^-. As negative controls, goat IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated rat IgG2a, κ (BD PharMingen) were used instead of anti-ODF/RANKL Ab and FITC-conjugated anti-B220 (isotype: rat IgG2a, κ), respectively. Two-color FACS analysis with PE-labeled anti-ODF Ab vs FITC-conjugated rat IgG2aκ and PE-labeled goat IgG vs FITC-conjugated anti-B220 Ab was used to set the appropriate levels of compensation.

Osteoclast formation in vitro

Bone marrow cells were collected from tibiae and femora of 6-wk-old $KL^{+/+}$ or $KL^{-/-}$ mice. The bone marrow cells were cultured in αMEM containing 10% FBS. Osteoclast differentiation was induced for 8 days either with 1,25$(OH)_2$ vitamin D_3 (10^{-8} M) and PGE_2 (10^{-6} M) or with ODF/RANKL (100 ng/ml; PeproTech, Rocky Hill, NJ) and M-CSF (recombinant human M-CSF, 20 ng/ml; Genzyme, Cambridge, MA). TRAP-positive multinucleated cells with three or more nuclei were counted as osteoclastic cells. Genomic DNA from osteoclasts was prepared as follows. First, the plates were washed five times with PBS^- to remove nonadherent cells that failed to differentiate into osteoclasts. Then, the plates were treated with 0.25% trypsin to dislodge the nonosteoclast adherent cells. Osteoclasts adhere to the culture plates so firmly that they are not dislodged by trypsin treatment. After three times trypsin treatment, every cell remaining on the culture plates was examined under the microscopy to confirm that all were typical multinucleated osteoclasts. The osteoclastic cells on the plates were then treated with proteinase K (0.15 mg/ml) at 50°C for 3 h. The genomic DNA was precipitated by adding isopropanol to the cell lysate.

Magnetic cell sorting of B-lymphoid lineage cells

B-lymphoid lineage cells were isolated with a magnetic cell-sorting system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). After washing steel-wool columns with PBS, the matrix of the column was incubated with 1% BSA in PBS for 15 min to saturate nonspecific binding sites. After the column was flushed with ice-cold PBS/1% BSA, cells labeled with anti-B220, CD19, or CD43 Ab-conjugated magnetic beads were applied to the column. Unbound cells were then washed out with 2–4 column volumes of PBS/1% BSA or until no cells were collected at flow rates of 10 ml/h. The column was further washed with >3 volumes of PBS/1% BSA at an increased flow rate. After the steel-wool column was removed from the external magnetic field, bound cells were eluted and analyzed by flow cytometry. We repeated the sorting steps until >99% of eluted cells were positive for each marker.

Analysis of ODF/RANKL expression

Bone marrow cells were flushed out from femora and tibiae of 6-wk-old $KL^{+/+}$ mice with PBS^- and fractionated into $B220^+$ and $B220^-$ cells as described above. Poly$(A)^+$ RNA and total protein were extracted from $B220^+$ cells, $B220^-$ cells, whole bone marrow cells, and residual bones (bones without the bone marrow cells). Poly$(A)^+$ RNA was extracted using oligo(dT) columns (Pharmacia, Peapack, NJ). The poly$(A)^+$ RNA (500 ng) was reverse-transcribed into single-strand cDNA with random hexamer and amplified with LA-*Taq* DNA polymerase with a commercially available kit (LA RT-PCR Kit; Takara, Kyoto, Japan), using primers specific for mouse ODF/RANKL (5′-CTCCGAGCTGGTGAAGAAA-3′ and 5′-CAGGGGAATTACAAAGTGC-3′) or G3PDH (5′-CATGTAGGCCAT GAGGTCCACCAC-3′ and 5′-TGAAGGTCGGTGTGAACGGATTTG GC-3′). Protein was extracted using TNE buffer (10 mM Tris-HCl, 150

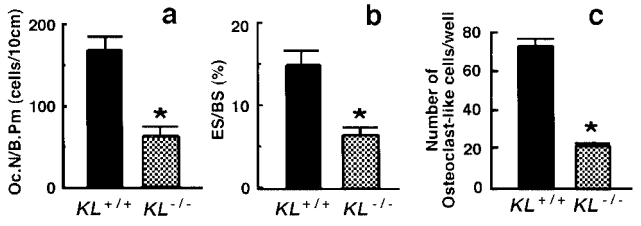

FIGURE 1. *a* and *b*, Histomorphometric analysis of the bone in $KL^{+/+}$ and $KL^{-/-}$ mice. Oc.N/B.Pm, Number of mature osteoclasts/10 cm of bone perimeter; ES/BS, percentage of eroded surface. Data are shown as means (bars) ± SEMs (error bars) for 7 mice/group. *, $p < 0.01$ vs $KL^{+/+}$ mice. *c*, Osteoclast-like cell formation from whole bone marrow cells. Bone marrow cells (1.0×10^6/well) from $KL^{+/+}$ or $KL^{-/-}$ mice were cultured in the medium containing 1,25(OH)$_2$ vitamin D$_3$ and PGE$_2$ for 8 days. The numbers of osteoclast-like cells are indicated as means (bars) ± SEMs (error bars) for eight wells. *, $p < 0.01$ vs $KL^{+/+}$ mice.

mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM aminoethyl-benzenesulfonyl fluoride, and 10 μg/ml aprotinin) and quantified with the BCA Protein Assay kit (Pierce, Rockford, IL). The same amounts of protein were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. ODF/RANKL was detected using the anti-ODF/RANKL Ab, an HRP-conjugated goat anti-mouse IgG (1 μg/ml; Amersham, Arlington Heights, IL), and ECL Western blotting detection reagents (Amersham). The membrane was stained with Amido Black (Sigma) and reprobed with anti-p38 MAPK Ab (New England Biolabs, Beverly, MA) to confirm that equivalent amounts of protein were loaded in each lane.

Genotyping at the mouse klotho *locus*

Genotypes at the mouse *klotho* locus in the osteoclasts were determined by PCR using three primers (5′-CAAGGACCAGTCATCATCG-3′, 5′-TTA AGGACTCCTGCATCTGC-3′, and 5′-TGGAGATTGAAGTGGACG-3′) specific to the mutated allele, wild-type allele, and both alleles, respectively. The mutated *klotho* allele (*kl*) and wild-type *klotho* allele gave PCR products of 920 and 458 bp, respectively.

Statistical analysis

Means of groups were compared by ANOVA, and significance of differences was determined by post hoc testing using the Bonferroni method.

Results

Impairment of osteoclastogenesis and B lymphopoiesis in $KL^{-/-}$ *mice*

Histomorphometric analysis of the bone of $KL^{-/-}$ mice revealed a significant reduction in the number of osteoclasts and bone resorption in vivo (Fig. 1, *a* and *b*) (17). In addition, the number of osteoclastic cells generated in vitro from whole bone marrow cells of $KL^{-/-}$ mice was significantly lower than that of wild-type ($KL^{+/+}$) mice when osteoclastogenesis was induced with 1,25(OH)$_2$ vitamin D$_3$ and PGE$_2$ (Fig. 1*c*). Three possible mechanisms were considered to explain the impairment in osteoclast formation in $KL^{-/-}$ mice. First, the number of osteoclast progenitors may be reduced in the bone marrow. Second, the cells and/or molecules that support the differentiation of osteoclast progenitors may be defective. Third, the osteoclast progenitors may have an intrinsic defect in differentiation. Although distinct, these possibilities are not mutually exclusive.

Because it has been established that osteoclasts differentiate from macrophage/monocyte lineage cells in the bone marrow, we examined whether the number of bone marrow cells of this particular lineage was decreased in $KL^{-/-}$ mice. Flow cytometric analysis of bone marrow cells indicated that there was no significant difference in the frequency of F4/80-positive cells between $KL^{+/+}$ and $KL^{-/-}$ mice, which contain the macrophage/monocyte lineage. This result is consistent with previous findings that no difference in the number of Mac-1-positive cells was observed (18). In contrast, the frequency of B220-positive ($B220^+$) cells was dramatically decreased in $KL^{-/-}$ mice (Table I). Considering that the number of total nucleated cells in $KL^{-/-}$ mice was lower than that in $KL^{+/+}$ mice (Table I), the decrease in the number of $B220^+$ cells in $KL^{-/-}$ mice was even larger. B220 is a cell surface marker expressed on B-lymphoid lineage cells at all stages of differentiation. Among the $B220^+$ cells, those expressing low levels of B220 ($B220^{low}$) were most significantly decreased in the bone marrow of $KL^{-/-}$ mice (Fig. 2). The majority of $B220^{low}$ cells were negative for IgM-μ-chain, which is a marker of immature and mature B lymphocytes (Fig. 2). The number of $B220^+$ cells in the spleen and peripheral blood of $KL^{-/-}$ mice was also lower than in $KL^{+/+}$ mice (18). In addition, we previously reported that the number of pro-B, pre-B, and mature B cells defined as $B220^+CD43^+IgM^-$, $B220^+CD43^-IgM^-$, and $B220^+IgM^+$ cells, respectively, was significantly lower in $KL^{-/-}$ mice (18). These data suggest that $KL^{-/-}$ mice have a defect in early stages of B cell development.

There was no difference in the frequency of granulocytes ($Gr\text{-}1^+$ cells) or erythrocytes ($TER119^+$ cells) between $KL^{+/+}$ and $KL^{-/-}$ mice (Table I). The frequency of T lymphoid lineage cells ($CD3^+$ cells) was slightly higher in $KL^{-/-}$ mice; however, the slight increase did not translate into an increase in the number of $CD3^+$ cells because of the decrease in the number of total nucleated cells in $KL^{-/-}$ mice.

Expression of ODF/RANKL on B-lymphoid lineage cells

To test whether $KL^{-/-}$ mice have a defect in cells and/or molecules that support osteoclast differentiation, we examined expression of ODF/RANKL in the bone marrow. Dual-color flow cytometric analysis using anti-ODF/RANKL Ab and Abs against several lineage-specific markers revealed that ODF/RANKL is expressed primarily on the surface of B-lineage cells expressing B220 (Fig. 3*a*). Because the number of $B220^+$ cells in the bone marrow is significantly decreased in $KL^{-/-}$ mice, the percentage of cells expressing ODF/RANKL is also decreased from 13.3% in $KL^{+/+}$ to 6.2% in $KL^{-/-}$ mice.

Most of the ODF/RANKL-positive cells are also positive for B220 in $KL^{+/+}$ mice (Fig. 3*a*). RT-PCR and immunoblot analysis confirmed that $B220^+$ cells are a major source of endogenous ODF/RANKL in the bone (Fig. 3*b*). Expression of ODF/RANKL in various tissues and cell lines has been described; however, there had been no report on its expression in the bone marrow until quite recently. These data indicate that ODF/RANKL is principally expressed on B-lymphoid lineage cells in the bone. This finding is in agreement with a recent study reporting that B lymphocytes and the pre-B cell line 70Z/3 express ODF/RANKL more abundantly than bone marrow adherent cells, which contain stromal cells (19). Taken together, these observations have raised the possibility that ODF/RANKL expressed on B-lymphoid lineage cells may be involved in osteoclastogenesis.

B-lymphoid lineage cells support osteoclast differentiation

We tested whether ODF/RANKL expressed on $B220^+$ cells could support osteoclast formation in vitro. Bone marrow cells from $KL^{+/+}$ mice were separated into $B220^+$ and $B220^-$ cell fractions using magnetic cell sorting. The sorted cells were cultured in medium containing 10^{-8} M 1,25(OH)$_2$ vitamin D$_3$ and 10^{-6} M PGE$_2$ to induce osteoclast differentiation. According to the current consensus on osteoclast differentiation, osteoclast progenitors belong not to the lymphoid but to the monocyte/macrophage lineage and should be enriched in the non-B ($B220^-$) cell fraction. Therefore, we expected that robust osteoclast formation from $B220^-$ cells

Table I. *Expression of lineage markers in bone marrow cells of* $KL^{+/+}$ *or* $KL^{-/-}$ *mice*[a]

	Total Nucleated Cells ($\times 10^7$)	B220 (%)	Gr-1 (%)	CD3 (%)	TER119 (%)	F4/80 (%)
$KL^{+/+}$	2.57 ± 0.27	29.6 ± 1.8	46.8 ± 2.1	4.2 ± 0.6	17.2 ± 1.5	15.0 ± 1.8
$KL^{-/-}$	1.44 ± 0.17*	8.9 ± 1.3**	52.8 ± 3.0	8.3 ± 1.0*	24.5 ± 1.9	12.3 ± 1.6

[a] The percentage of positive cells for each marker was indicated as means ± SEM of 15 animals. The total number of nucleated cells from bilateral tibiae and femora was counted in nine $KL^{+/+}$ mice and eight $KL^{-/-}$ mice.
*$p < 0.05$ vs $KL^{+/+}$; **$p < 0.001$ vs $KL^{+/+}$.

would be observed. Contrary to our expectation, very few osteoclasts were generated by $B220^-$ cells alone (Fig. 4*a*). However, when $B220^+$ cells were added back to the fixed number of $B220^-$ cells, osteoclast formation was significantly augmented in proportion to the number of $B220^+$ cells added (Fig. 4*c*). $B220^+$ cells alone gave rise to very few osteoclasts under these conditions (Fig. 4*a*). These results clearly demonstrated that interactions between $B220^+$ and $B220^-$ cells potentiated differentiation of osteoclasts. Osteoclast formation was completely abolished by the addition of anti-ODF/RANKL Ab or recombinant osteoprotegerin (100 ng/ml), a soluble decoy receptor of ODF/RANKL, indicating that endogenous ODF/RANKL is essential for osteoclastogenesis under these culture conditions.

To determine whether osteoclast progenitors in the bone marrow of $KL^{-/-}$ mice have an intrinsic defect in differentiation into mature osteoclasts, we prepared $B220^+$ and $B220^-$ cells from $KL^{-/-}$ and $KL^{+/+}$ mice and did coculture experiments. According to the origin of the cells, four different cell combinations were tested (Fig. 4, *c–f*). $B220^+$ cells from $KL^{-/-}$ mice supported osteoclast formation to the same extent as those from $KL^{+/+}$ mice. In addition, the $B220^-$ cells from $KL^{-/-}$ mice generated almost the same number of osteoclasts as those from $KL^{+/+}$ mice. Thus, the bone marrow cells of $KL^{-/-}$ mice showed no difference in osteoclastogenesis in vitro from those of $KL^{+/+}$ mice. Nevertheless, the number of osteoclasts generated from whole bone marrow cells of $KL^{-/-}$ mice was reduced to approximately one-third that of $KL^{+/+}$ mice (Fig. 1*c*). It should be noted that, in $KL^{-/-}$ mice, the frequency of $B220^+$ cells is also reduced to almost one-third (Table I). When the $B220^+/B220^-$ ratio was reduced to that found in $KL^{-/-}$ mice (1×10^5 $B220^+$ cells with 1×10^6 $B220^-$ cells), the number of osteoclasts generated was almost the same as that generated from the whole bone marrow mixture of $KL^{-/-}$ mice. The results were indifferent to the origin of the cells (Fig. 4, *c–f*). Therefore, it is unlikely that the reduction of osteoclast formation from whole bone marrow cells of $KL^{-/-}$ mice in vitro is caused by an inability of bone marrow cells to support osteoclast differentiation or by an intrinsic defect in osteoclast progenitors to differentiate. Rather, the reduction in osteoclastogenesis could simply be caused by the reduction in the number of $B220^+$ cells.

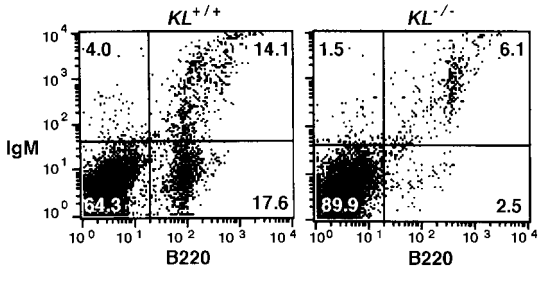

FIGURE 2. Two-color immunofluorescence analysis of B220 and IgM-μ-chain in bone marrow cells from $KL^{+/+}$ and $KL^{-/-}$ mice. Respective typical patterns from five $KL^{+/+}$ and $KL^{-/-}$ mice are shown.

The most likely explanation for the results of the coculture experiment is that ODF/RANKL expressed on the surface of $B220^+$ cells supported differentiation of osteoclast progenitors in $B220^-$ cell fraction. In fact, the effect of $B220^+$ cells could be replaced by soluble ODF/RANKL (Fig. 4*b*) and abolished with anti-ODF/RANKL Ab or osteoprotegerin. If $B220^+$ cells serve exclusively as a source of ODF/RANKL, osteoclasts generated in the coculture experiment should have originated only from the $B220^-$ cell fraction that contains osteoclast progenitors. To test this possibility, we examined the origin of the osteoclasts. We generated osteoclasts by coculture of $B220^+$ cells from $KL^{-/-}$ mice and $B220^-$ cells from $KL^{+/+}$ mice (and vice versa) and determined *klotho* locus genotypes of the osteoclasts. Semiquantitative PCR demonstrated that ~75% of osteoclasts originated from $B220^-$ cells and that 25% came from $B220^+$ cells (Fig. 5). The majority of osteoclasts originated from the $B220^-$ cell fraction as expected, verifying the idea that $B220^+$ cells support differentiation of osteoclast progenitors in the $B220^-$ cell fraction. At the same time, however, these observations imply that a significant portion of osteoclasts (~25%) came from $B220^+$ cells. This high percentage cannot be explained by contamination of $B220^-$ cells in the $B220^+$ cell fraction, because the contamination was determined to

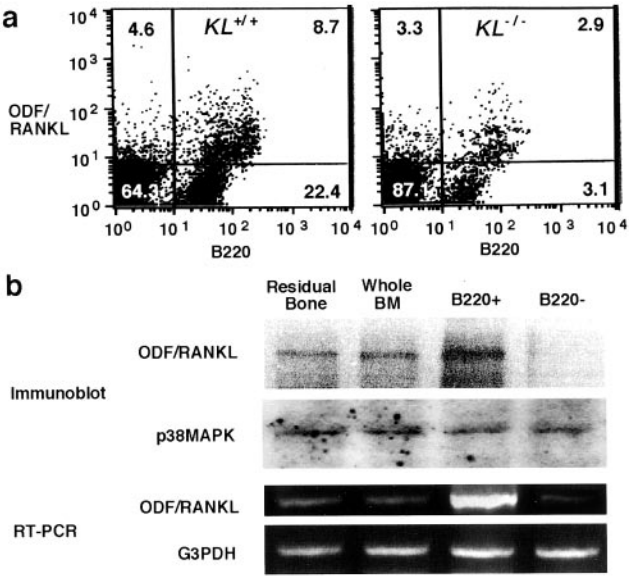

FIGURE 3. Expression of ODF/RANKL on the surface of B-lymphoid lineage cells. *a*, Two-color immunofluorescence analysis of B220 and ODF/RANKL expression on bone marrow cells from $KL^{+/+}$ and $KL^{-/-}$ mice. Respective typical patterns from five $KL^{+/+}$ and $KL^{-/-}$ mice are shown. *b*, Expression of ODF/RANKL at the protein and messenger RNA levels in bones without the bone marrow cells (Residual Bone), whole bone marrow cells (Whole BM), $B220^+$ cells (B220+), and $B220^-$ cells (B220−) from $KL^{+/+}$ mice were detected by immunoblotting and RT-PCR. Expression of p38 MAPK protein and G3PDH mRNA are shown as internal controls.

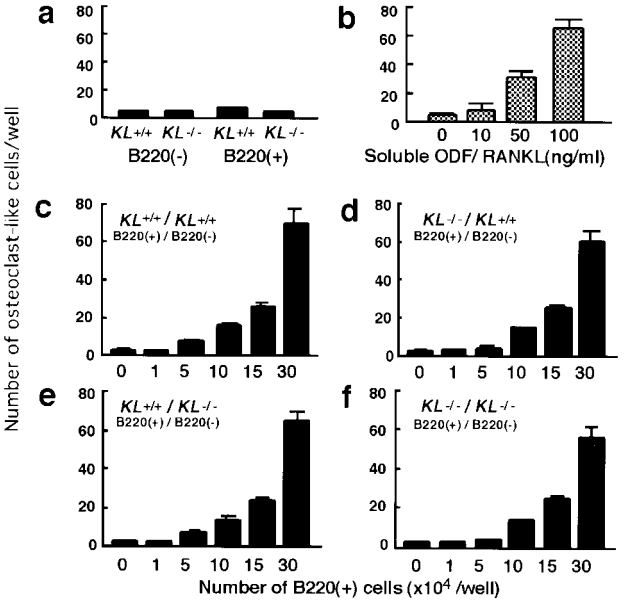

FIGURE 4. Osteoclast formation in coculture of $B220^+$ and $B220^-$ cells from the bone marrow. *a*, Number of osteoclast-like cells generated from $B220^-$ cells (*left*) or $B220^+$ cells (*right*) alone. *b*, Induction of osteoclast differentiation with soluble ODF/RANKL protein. Recombinant soluble ODF/RANKL protein was added to the $B220^-$ bone marrow cells from wild-type mice at the indicated concentration. *c–f*, Number of osteoclast-like cells generated by adding $B220^+$ cells (from 0 to 30×10^4/well) to a fixed number of $B220^-$ cells (1×10^6/well). The origins of $B220^+$ and $B220^-$ cells are indicated. Data are shown as means (bars) ± SEMs (error bars) for eight wells per group.

be <1% by FACS analysis. Rather, it suggests that $B220^+$ cells actually contributed to osteoclast formation. These observations raised the possibility that the B-lymphoid lineage cells functioned not only as a source of ODF/RANKL but also as osteoclast progenitors.

Osteoclast formation from $B220^+$ cells

To confirm that $B220^+$ cells are able to give rise to osteoclasts, we isolated cells from the bone marrow of $KL^{+/+}$ mice by cell sorting using several different B-lineage markers (CD43, B220, CD19) and induced osteoclast differentiation with soluble ODF/RANKL and M-CSF (Fig. 6). Under these conditions, $CD43^+B220^+$ cells gave rise to the highest number of osteoclasts. $B220^+$ cells and $CD19^+$ cells gave rise to equivalent numbers of osteoclasts as did $B220^-$ and $CD19^-$ cells, respectively. However, $B220^+$ and $CD19^+$ cells from the spleen, most of which are positive for IgM-

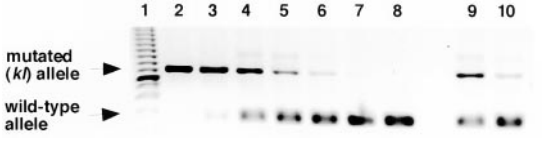

FIGURE 5. Contribution of $B220^+$ cells to osteoclasts. Genotypes of the osteoclasts generated by coculture of $B220^+$ cells from $KL^{+/+}$ mice and $B220^-$ cells from $KL^{-/-}$ mice (*lane 9*) or $B220^+$ cells from $KL^{-/-}$ mice and $B220^-$ cells from $KL^{+/+}$ mice (*lane 10*) were determined at the *klotho* locus by the 3-primer-PCR (see *Materials and Methods*). Genomic DNA of tails from $KL^{-/-}$ and $KL^{+/+}$ mice was mixed in various ratios to obtain a standard for semiquantification (*lanes 2–8*). Ratios of the genomic DNA of $KL^{-/-}$ mice to that of $KL^{+/+}$ mice were 100% (*lane 2*), 95% (*lane 3*), 75% (*lane 4*), 50% (*lane 5*), 25% (*lane 6*), 5% (*lane 7*), and 0% (*lane 8*), respectively. *Lane 1*: 100 bp ladder.

μ-chain, gave rise to very few osteoclasts. These splenic B-lineage cells are regarded as having already committed to the formation of mature B lymphocytes, and lost the capability of differentiating into other cell lineages.

Within the $B220^+$ population of bone marrow cells, CD43 (leukosialin) is a marker for B-lymphoid cells in early differentiation stages. It is expressed on the surface of pro-B, pre-BI, and early pre-BII cells and down-regulated thereafter (20). CD19 is another pan-B-lineage marker like B220, expression of which is dependent on expression of Pax5 (21), a transcription factor essential to the commitment of pre-BI cells to the B-lymphoid lineage (15). Considering that $CD43^+$ cell fraction within the $B220^+$ population gave rise to more osteoclasts than did any other fractions, osteoclast progenitors are enriched in the differentiation stages earlier than the pre-BII stage. In fact, the number of osteoclasts generated from $CD43^-B220^+$ cells (pre-BII or later) was less than one-fourth that from the total $B220^+$ cell pool (Fig. 6). Therefore, we conclude that B-lymphoid lineage cells in earlier developmental stages, before the pre-BII stage, contain significant osteoclast progenitor activity.

Discussion

Numerous reports have verified that osteoclasts can arise from the monocyte/macrophage lineage cells under the support of osteoblastic and/or stromal cells that express ODF/RANKL and M-CSF. Our present study demonstrates the existence of another pathway of osteoclast differentiation. A subset of normal B-lymphoid lineage cells may hold a potential to give rise to osteoclasts. Moreover, B-lymphoid cells are the major source of ODF/RANKL in the bone marrow and support osteoclast differentiation. Among B-lymphoid lineage cells, those in late developmental stages (immature/mature B cells) express ODF/RANKL abundantly, but do not differentiate into osteoclasts. In contrast, B-lymphoid lineage cells in early developmental stages (before the pre-BII stage) express lower levels of ODF/RANKL than those in late stages, but have the potential to differentiate into osteoclasts. In addition to the current paradigm of osteoclast differentiation pathway, we propose a novel pathway that involves B-lymphoid cells at various differentiation stages.

Recent studies challenge the current concept of lineage commitment for B-lymphoid cells. Nutt et al. (15) showed that Pax5-deficient pro-B cells have multilineage potential. Bone marrow

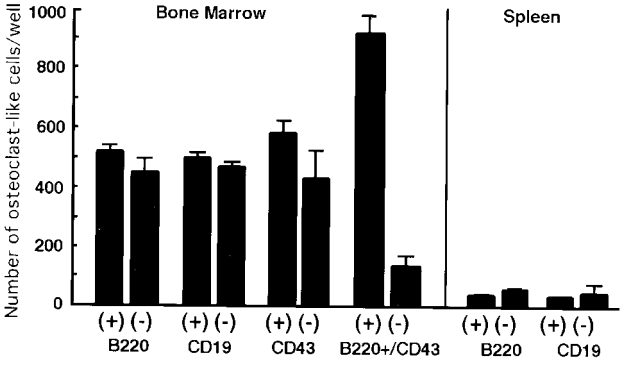

FIGURE 6. Generation of osteoclast-like cells from B-lymphoid lineage cells. Bone marrow cells and spleen cells from wild-type mice were sorted according to the presence (+) or absence (−) of the indicated B cell markers. $B220^+$ cells were further sorted with CD43 (the *right two columns* under Bone Marrow). The sorted cells (2×10^6/well) were cultured with the medium containing soluble ODF/RANKL and M-CSF for 8 days. Data are shown as means (bars) ± SEMs (error bars) for eight wells per group.

progenitors from Pax5$^{-/-}$ mice are unable to differentiate beyond the pre-BI stage of the B-lymphoid lineage. However, when induced with appropriate cytokines, these early pre-B cells can give rise to several different lineage cells including macrophages, osteoclasts, dendritic cells, granulocytes, and NK cells. The lineage switch of Pax5$^{-/-}$ pro-B cells to osteoclasts may not be an abnormal pathway due to the blockade in the normal B cell differentiation, because our present data indicate that normal B-lymphoid lineage cells may also have the potential to differentiate into osteoclasts when induced by soluble ODF/RANKL and M-CSF. We conclude that a subset of normal pro-B cells is uncommitted and may still hold a potential to give rise to osteoclasts.

The fact that the pro-B cell fraction contains osteoclast progenitors may have implications in pathophysiology of osteoporosis because it is known that the number of pro-B cells is positively correlated to the number of osteoclasts in vivo in various pathological conditions. Estrogen withdrawal causes a marked increase in the number of both B220^{low} cells (containing pro-B cells) and osteoclasts, which leads to bone loss in humans (postmenopausal osteoporosis), and mice (osteopenia caused by ovariectomy) (1, 22, 23). In addition, IL-7, a cytokine that primarily stimulates B lymphopoiesis, also causes an increase in osteoclasts and bone loss when injected into mice (24). In contrast, a decrease in B220^{low} cells is accompanied by a decrease in osteoclast numbers as observed in IL-7 receptor knock-out mice (24) and in *KL*$^{-/-}$ mice. Interestingly, the number of both osteoclasts and B220^{low} cells decreases with age in mice (25) and humans (26). The positive correlation between the number of B220^{low} cells and osteoclasts in various pathological conditions and the natural aging process may be partly explained by the possibility that pro-B cells actually serve not only as B cell progenitors but also as osteoclast progenitors in vivo.

ODF/RANKL expressed on B220^{+} cells may be another factor that is involved in pathophysiology of osteoporosis. Although our present study showed that B220^{low} cells express less ODF/RANKL than B220^{high} cells (Fig. 3*a*), a significant increase in B220^{low} cells could result in a substantial increase in total amount of ODF/RANKL in the bone marrow, which may contribute to acceleration of osteoclastogenesis. A recent report indicated that the number of B220^{low} cells that express ODF/RANKL was higher in bone marrow from ovariectomized mice than in bone marrow from sham-operated mice (27). Because the expression level of ODF/RANKL in each B220^{low} cell was not significantly different, the increase in the number of B220^{low} cells means a net increase in the amount of ODF/RANKL expression. Conversely, the decrease in B220^{low} cells in *KL*$^{-/-}$ mice results in a net decrease in ODF/RANKL expression (Fig. 3*a*). Based on these observations, the number of B220^{+} cells could affect osteoclastogenesis and bone resorption in two ways: a change in the amount of ODF/RANKL in the bone marrow and a change in the number of osteoclast progenitors.

The mechanism by which B lymphopoiesis is impaired in *KL*$^{-/-}$ mice remains to be elucidated. A recent study indicated that the number of IL-7-responsive clonogenic progenitors (B-lymphoid progenitors) in bone marrow cells was markedly decreased in *KL*$^{-/-}$ mice (18). However, the number of hemopoietic stem cells and their capacity for B lymphopoiesis were normal (18), suggesting that a defect in hemopoietic environment might be responsible for poor B lymphopoiesis or survival in *KL*$^{-/-}$ mice. It remains to be determined whether the decrease in B220^{+} cells is the only explanation for the poor osteoclastogenesis in *KL*$^{-/-}$ mice. In addition to the decrease in B-lineage cells, it is possible that local and/or systemic factors affect osteoclast differentiation in *KL*$^{-/-}$ mice. In fact, we previously demonstrated that *KL*$^{-/-}$ mice had significantly higher blood osteoprotegerin levels than *KL*$^{+/+}$ mice (17), which could also inhibit osteoclast formation in *KL*$^{-/-}$ mice in vivo. However, there was no significant difference in M-CSF mRNA levels between *KL*$^{+/+}$ and *KL*$^{-/-}$ mice (18).

To our knowledge, there are no reports of osteoclast abnormality in B cell-deficient mice. There are two major differences between *KL*$^{-/-}$ mice and other B cell-deficient mice. First, although many B cell-deficient mouse strains, such as RAG2$^{-/-}$ and λ5$^{-/-}$ mice (28, 29), lack IgM^{+} cells, they have normal numbers of pro-B cells, which contain the osteoclast progenitors. In contrast, *KL*$^{-/-}$ mice show losses in both the IgM^{+} and pro-B cells, which results in a simultaneous reduction in both ODF/RANKL expression levels and the number of osteoclast progenitors. Therefore, *KL*$^{-/-}$ mice would be expected to have a more severe defect in osteoclastogenesis than the other B cell-deficient strains. Second, B-lymphoid lineage cells in the other B cell-deficient strains have intrinsic defects in the ability to differentiate into mature B lymphocytes. In contrast, the impairment of B lymphopoiesis in *KL*$^{-/-}$ mice was attributed to a defect in the "microenvironment" in the bone marrow (18), which could also affect osteoclast differentiation.

Our present study has indicated that B220^{+} cell fraction contains both ODF/RANKL-expressing cells and osteoclast progenitors. However, B220^{+} cells alone, when stimulated with 1,25(OH)$_2$ vitamin D$_3$ and PGE$_2$, gave rise to many fewer osteoclasts than those cocultured with B220^{-} cells (Fig. 4*a*). The poor osteoclast formation was not completely restored by adding M-CSF (data not shown), suggesting that endogenous ODF/RANKL was insufficient for the effective osteoclast formation from B220^{+} cells and that B220^{-} cells played an important role in the osteoclast differentiation. Cell-cell interaction between osteoclast progenitors and stromal cells is believed to be indispensable for efficient osteoclast differentiation. Stromal cells express several adhesion molecules including VCAM-1 that binds to very late Ag-4 expressed on B-lymphoid lineage cells. Previous studies showed that very late Ag-4/VCAM-1 interactions are important in the adhesion of B cells to the bone marrow microenvironment and B cell differentiation (30). It is possible that stromal cells, which reside in the B220^{-} cell fraction, act as scaffolding to tether B220^{+} cells that express ODF/RANKL to produce a microenvironment where ODF/RANKL is focally concentrated. Such scaffolding may no longer be necessary when excessive ODF/RANKL is present, as we observed robust osteoclast formation from B220^{+} cells stimulated with M-CSF and soluble ODF/RANKL (Fig. 6).

In conclusion, we propose two possible mechanisms by which B-lymphoid lineage cells regulate osteoclastogenesis: they act as the major source of ODF/RANKL and support differentiation of osteoclast progenitors into mature osteoclasts. In addition, normal pro-B cells may be able to serve as osteoclast progenitor cells. The close association between B-lymphoid cell and osteoclast differentiation pathways may also imply involvement of B-lymphoid cells in the pathophysiology of bone disorders such as osteoporosis.

Acknowledgments

We thank Errol C. Friedberg for critical reading of the manuscript.

References

1. Manolagas, S. C., and R. L. Jilka. 1995. Bone marrow, cytokines, and bone remodeling: emerging insights into the pathophysiology of osteoporosis. *N. Engl. J. Med. 332:305.*
2. Kurihara, N., C. Chenu, M. Miller, C. Civin, and G. D. Roodman. 1990. Identification of committed mononuclear precursors for osteoclast-like cells formed in long term human marrow cultures. *Endocrinology 126:2733.*
3. Hattersley, G., J. A. Kerby, and T. J. Chambers. 1991. Identification of osteoclast precursors in multilineage hemopoietic colonies. *Endocrinology 128:259.*
4. Suda, T., I. Nakamura, E. Jimi, and N. Takahashi. 1997. Regulation of osteoclast function. *J. Bone Miner. Res. 12:869.*

5. Lacey, D. L., E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, et al. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell 93:165.*
6. Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, et al. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA 95:3597.*
7. Kong, Y. Y., H. Yoshida, I. Sarosi, H. L. Tan, E. Timms, C. Capparelli, S. Morony, A. J. Oliveira-dos-Santos, G. Van, A. Itie, et al. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature 397:315.*
8. Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett III, W. N. Frankel, et al. 1997. 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. 272:25190.*
9. Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor (TNF)-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med. 186:2075.*
10. Yoshida, H., S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo, and L. D. Shultz. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature 345:442.*
11. Wiktor-Jedrzejczak, W., A. Bartocci, A. W. J. Ferrante, A. Ahmed-Ansari, K. W. Sell, J. W. Pollard, and E. R. Stanley. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (*op/op*) mouse. *Proc. Natl. Acad. Sci. USA 87:4828.*
12. Lagasse, E., and I. L. Weissman. 1997. Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in *op/op* mice. *Cell 89:1021.*
13. Borrello, M. A., and R. P. Phipps. 1996. The B/macrophage cell: an elusive link between $CD5^+$ B lymphocytes and macrophages. *Immunol. Today 17:471.*
14. Katoh, S., A. Tominaga, M. Migita, A. Kudo, and K. Takatsu. 1990. Conversion of normal Ly-1-positive B-lineage cells into Ly-1-positive macrophages in long-term bone marrow cultures. *Dev. Immunol. 1:113.*
15. Nutt, S. L., B. Heavey, A. G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature 401: 556.*
16. Kuro-o, M., Y. Matsumura, H. Aizawa, H. Kawaguchi, T. Suga, T. Utsugi, Y. Ohyama, M. Kurabayashi, T. Kaname, E. Kume, et al. 1997. Mutation of the mouse *klotho* gene leads to a syndrome resembling ageing. *Nature 390:45.*
17. Kawaguchi, H., N. Manabe, C. Miyaura, H. Chikuda, K. Nakamura, and M. Kuro-o. 1999. Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. *J. Clin. Invest. 104: 229.*
18. Okada, S., T. Yoshida, Z. Hong, G. Ishii, M. Hatano, M. Kuro-o, Y. Nabeshima, Y. Nabeshima, and T. Tokuhisa. 2000. Impairment of B lymphopoiesis in precocious aging (klotho) mice. *Int. Immunol. 12:861.*
19. Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature 390:175.*
20. Hardy, R. R., C. E. Carmack, S. A. Shinton, J. D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med. 173:1213.*
21. Nutt, S. L., A. M. Morrison, P. Dorfler, A. Rolink, and M. Busslinger. 1998. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J. 17:2319.*
22. Pacifici, R. 1996. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. *J. Bone Miner. Res. 11:1043.*
23. Masuzawa, T., C. Miyaura, Y. Onoe, K. Kusano, H. Ohta, S. Nozawa, and T. Suda. 1994. Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. *J. Clin. Invest. 94:1090.*
24. Miyaura, C., Y. Onoe, M. Inada, K. Maki, K. Ikuta, M. Ito, and T. Suda. 1997. Increased B-lymphopoiesis by interleukin 7 induces bone loss in mice with intact ovarian function: similarity to estrogen deficiency. *Proc. Natl. Acad. Sci. USA 94:9360.*
25. Zharhary, D. 1988. Age-related changes in the capability of the bone marrow to generate B cells. *J. Immunol. 141:1863.*
26. Nunez, C., N. Nishimoto, G. L. Gartland, L. G. Billips, P. D. Burrows, H. Kubagawa, and M. D. Cooper. 1996. B cells are generated throughout life in humans. *J. Immunol. 156:866.*
27. Kanematsu, M., T. Sato, H. Takai, K. Watanabe, K. Ikeda, and Y. Yamada. 2000. Prostaglandin E2 induces expression of receptor activator of nuclear factor-κB ligand/osteoprotegrin ligand on pre-B cells: implications for accelerated osteoclastogenesis in estrogen deficiency. *J. Bone Miner. Res. 15:1321.*
28. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, and A. M. Stall. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell 68:855.*
29. Kitamura, D., A. Kudo, S. Schaal, W. Muller, F. Melchers, and K. Rajewsky. 1992. A critical role of λ5 protein in B cell development. *Cell 69:823.*
30. Miyake, K., K. Medina, K. Ishihara, M. Kimoto, R. Auerbach, and P. W. Kincade. 1991. A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J. Cell Biol. 114:557.*