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How B Cells Influence Bone Biology in Health and Disease

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Abstract

It is now well established that important regulatory interactions occur between the cells in the hematopoietic, immune and skeletal systems (osteimmunology). B lymphocytes (B cells) are responsible for the generation and production of antibodies or immunoglobulins in the body. Together with T cells these lymphocytes comprise the adaptive immune system, which allows an individual to develop specific responses to an infection and retain memory of that infection, allowing for a faster and more robust response if that same infection occurs again. In addition to this immune function, B cells have a close and multifaceted relationship with bone cells. B cells differentiate from hematopoietic stem cells (HSCs) in supportive niches found on endosteal bone surfaces. Cells in the osteoblast lineage support HSC and B cell differentiation in these niches. B cell differentiation is regulated, at least in part, by a series of transcription factors that function in a temporal manner. While these transcription factors are required for B cell differentiation, their loss causes profound changes in the bone phenotype. This is due, in part, to the close relationship between macrophage/osteoclast and B cell differentiation. Cross talk between B cells and bone cells is reciprocal with defects in the RANKL-RANK, OPG signaling axis resulting in altered bone phenotypes. While the role of B cells during normal bone remodeling appears minimal, activated B cells play an important role in many inflammatory diseases with associated bony changes. This review examines the relationship between B cells and bone cells and how that relationship affects the skeleton and hematopoiesis during health and disease.

Keywords

B cell differentiation; Macrophage/osteoclast differentiation; B cell transcription factors and bone; IL-7; Regulation of hematopoiesis by osteoblast lineage cells; Regulation of B cell development by RANKL-RANK signaling; B cell activation

Introduction

All of the blood borne elements arise from pluripotent hematopoietic stem cells (HSCs) that reside in growth and differentiation supportive structures, known as niches, which are found predominantly on endosteal bone surfaces in contact with the bone marrow. HSC are also in contact with cells that are adherent to the endosteal bone surface. These support cells secrete or express on their cell surface cytokines and growth factors that facilitate HSC growth and differentiation [1–3]. Often these cells are referred to as osteoblasts (OBs) or stromal cells, and

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although they are in the osteoblast lineage their exact stage of differentiation is unknown. However, it is unlikely that they are mature osteoid secreting osteoblasts.

Long-term reconstituting HSCs (LT-HSC) are multipotential in that they have the capacity for self-renewal as well as producing daughter cells that at each stage of differentiation become more restricted in cell lineage. LT-HSCs give rise to short-term reconstituting HSCs that differentiate to multipotent progenitors (MPPs). It is at this stage that an important separation of the lineages occurs (Fig 1). MPP differentiate into the common myeloid progenitor (CMP), which gives rise to the granulocyte-macrophage progenitor (GMP) and then, in turn, to osteoclasts (OC), macrophages, granulocytes and dendritic cells [4]. The CMP also produces the megakaryocyte-erythrocyte progenitor in a separate lineage. Alternatively, MPPs can also differentiate into the common lymphoid progenitor (CLP) which gives rise to B, T and natural killer cells. Thus, it is at the MPP stage that OC (myeloid) and B cell (lymphoid) differentiation share a common progenitor and critical cell fate decisions are made that dictate whether subsequent differentiation will result in OCs or B cells. These cell fate decisions are made by the expression or repression of proteins under the control of a hierarchy of transcription factors (TFs), which include PU.1, Ikaros, E2A, Ebf1 and Pax5. These TFs function in a network that regulates B cell differentiation [5]. Whether these TFs function in a similar network to regulate macrophage-OC differentiation remains to be elucidated.

B Cell Differentiation

B lymphopoiesis is a highly ordered process proceeding from progenitor cells in the fetal liver, to the bone marrow (BM), to mature B cells in the secondary lymphoid organs. The mature B cell, following activation and T cell derived help, terminally differentiates into immunoglobulin (Ig)-secreting plasma cells [6]. B cell development is organized around the assembly of a functional B cell receptor through a process of gene rearrangement called V(D)J recombination [7]. The BM B cell developmental pathway can be divided into several distinct stages based on the recombination status of the Ig genes and expression of surface antigens [8]. The earliest characterized committed B cell progenitor (pre-pro-B) expresses the cell surface markers CD45R/B220 and AA4.1 and has its Ig heavy (IgH) chain locus in the germ-line configuration (not rearranged) [9]. Subsequent differentiation generates pro-B cells that harbor rearranged IgH D and J genes [10]. As the cells mature, rearrangements occur in V gene segments of the Ig heavy chain gene and then finally in the Ig light chain genes culminating in a functional surface antigen receptor [7,11].

The molecular dissection of the B cell differentiation pathway has been greatly facilitated by the identification of TFs required for developmental transitions. Loss of these specific factors precludes the cells from continued maturation, and results in a developmental block of cells at the latest stage of differentiation prior to the arrest. The selective loss of two different basic helix-loop-helix proteins encoded by the E2A gene or the Ebf1 gene has been shown to play a critical role in the initiation of B cell differentiation. Absence of either of these proteins halts B cell development at the earliest stage, before D-J rearrangement of the IgH gene [8,12,13]. Commitment and maintenance of progenitors to the B cell lineage is accomplished by the expression of Pax5, [14,15]. Ebf1 and E2A are key factors for the specification and progression of lymphoid progenitors into the B-lymphoid pathway but these function before commitment of the cell, which requires Pax5 expression.

Osteoblast lineage cells support B lymphopoiesis

Osteoblast lineage cells carry out at least three major functions: 1) they make bone; 2) they regulate osteoclast differentiation; and 3) they support hematopoietic cell differentiation and growth. In addition to supporting HSC growth and survival, osteoblast lineage cells also provide support for more differentiated cells in specific lineage niches including B cells [16].

Osteoblasts support commitment and differentiation of all stages of B cell development. Production of B cell precursors from progenitors *in vitro* required contact with osteoblasts and expression of CXCL12 (SDF-1) and IL-7, which was induced by parathyroid hormone (PTH) [1,3]. Interestingly, addition of stem cell factor, IL-6 and IL-3 redirected differentiation away from B lymphopoiesis and toward myelopoiesis. Selective elimination of OBs by treatment of Col2.3d-TK transgenic mice with gancyclovir also severely depleted pre-pro B cells from the BM confirming the supportive role of OBs in B cell development [2].

It is now known that signaling through the PTH/PTH-related peptide receptor (PPR) in osteoblastic cells increases trabecular bone and importantly increase HSCs [1]. PTH is known to increase production of CXCL12 and IL-7 by osteoblastic cells *in vitro* suggesting that downstream signaling through the PPR could regulate B cell development [1,3]. Mice made deficient in PTH signaling specifically in osteoblasts by ablation of the G protein α subunit $G_{s\alpha}$ had a striking decrease in trabecular bone and an almost 50% reduction in BM B cells while other hematopoietic lineages were unaffected [16]. In addition, IL-7 expression was reduced in $G_{s\alpha}$ deficient osteoblasts, confirming the importance of osteoblast lineage cells in B cell growth and differentiation.

IL-7

IL-7 is a cytokine that has diverse effects on the hematopoietic and immunologic systems and is best known for its non-redundant role in supporting B- and T-lymphopoiesis [17]. IL-7 is the major growth factor for B cells and is apparently expressed by BM stromal cells and osteoblasts [3,16,18]. The IL-7 receptor (IL-7R) is expressed on progenitor B cells and is composed of the common γ chain and the IL-7R α chain [8,18]. Signals from the IL-7R are required during the pro-B-cell stage for further differentiation, and deficiencies in either IL-7 or the IL-7R result in severe defects in B-cell development. However, both IL-7 and IL-7R α -deficient mice possess readily detectable numbers of peripheral B cells, indicating that the block in B lymphopoiesis is not absolute in these animals. As stated above, production of IL-7 by osteoblast lineage cells appears critical for normal B-lymphopoiesis [16].

Studies have demonstrated that IL-7 also plays an important role in the regulation of bone homeostasis [19,20]. However, the precise nature of how IL-7 affects osteoclasts and osteoblasts is controversial, because it has a variety of actions in different target cells. Systemic administration of IL-7 increased osteoclast formation from human peripheral blood cells by increasing osteoclastogenic cytokine production in T cells [21]. Furthermore, mice with global over expression of IL-7 had a phenotype of decreased bone mass with increased osteoclasts and no change in osteoblasts [22]. However, the interpretation of results from *in vivo* IL-7 treatment studies is complicated by secondary effects of IL-7, which result from the production of bone-resorbing cytokines by T cells in response to activation by this cytokine [21,23,24]. Consistent with this conclusion, IL-7 administration did not induce bone resorption or bone loss in T-cell-deficient nude mice [23].

In contrast with previously reported studies, we found differential effects of IL-7 on osteoclastogenesis [19,21,23,25]. IL-7 inhibited osteoclast formation in murine bone marrow cells that were cultured for 5 days with M-CSF and RANKL [25]. We also found that IL-7-deficient mice had markedly increased osteoclast number and decreased trabecular bone mass compared to wild-type controls [26].

The role of IL-7 in the effects of estrogen on bone is also controversial. Treatment of mice with a neutralizing anti-IL-7 antibody inhibited ovariectomy-induced bone loss and the proliferation of early T cell precursors in the thymus [27]. However, we found that trabecular bone loss after ovariectomy was similar in wild type and IL-7-deficient mice [26]. Curiously, IL-7 mRNA

levels in bone increase with ovariectomy and this effect may be linked to alterations in osteoblast function, which occur with estrogen withdrawal [20,28].

Treatment of newborn murine calvaria cultures with IL-7 inhibited bone formation, as did injection of IL-7 above the calvaria of mice *in vivo* [20]. However, when IL-7 was transgenically over expressed in osteoblasts, trabecular bone mass was increased compared with wild-type mice [29]. Furthermore, targeted expression of IL-7 in the osteoblasts of IL-7-deficient mice rescued the osteopenic bone phenotype of the IL-7-deficient mice [30].

PU.1, Ikaros, E2A, Ebf1 and Pax5 a Network of Transcription Factors

(The TFs are discussed in hierarchical order from the earliest to latest acting in B cell differentiation)

PU.1 is a member of the Ets family of transcription factors and is unique among hematopoietic TFs because it is required for both myeloid and lymphoid lineage development [31]. PU.1^{-/-} mice die during fetal development by day E18.5. They lack B and T cells and fail to develop both macrophages and OCs [32,33] (Fig 2). Due to a lack of OCs their bones are severely osteopetrotic. These data were central in showing that OC differentiation required PU.1 expression early in lineage development and OCs and macrophages were members of the myeloid lineage. In one study PU.1^{-/-} MPP cells isolated from fetal liver (E14.5) were lineage depleted using flow cytometry, then transduced with PU.1 or control virus using retroviral vectors and grown on M-CSF-deficient OP9 stromal cells. PU.1^{-/-} MPPs before transduction had reduced expression of c-fms, GM-CSFR α and G-CSFR, suggesting a reduced ability to differentiate into all myeloid lineages [34]. PU.1^{-/-} MPP transfected with PU.1 produced 56% macrophages and only 19% pro-B cells [35] while PU.1^{+/-} MPPs transfected with control virus generated 7% macrophages and 86% pro-B cells. PU.1^{-/-} MPPs transfected with control virus failed to grow because they could not respond to added IL-7, due to the absence of IL-7Rs, which are regulated by PU.1 [36]. Failure to respond to IL-7 precludes further B cell differentiation. These data suggest that the increased number of macrophages in the PU.1 rescued cells was not simply due to increased M-CSF. These authors went on to show that higher levels of PU.1 expression promotes macrophage differentiation and blocks B cell development [35]. Conversely, low concentrations of PU.1 protein promote B cell differentiation. These data are consistent with reports showing that wild-type (WT) macrophages express higher levels of PU.1 than wild-type pro-B cells and that PU.1 mRNA is expressed at higher levels in myeloid than in B lymphoid cell lines [35,37,38]. In agreement with these data, the amount of PU.1 expressed by OCs is 3 fold more than in BM macrophages [32]. PU.1^{-/-} MPPs have impaired expression of Ebf1 and Pax5 [35]. Transduction of MPPs with an Ebf1 but not Pax5-containing retroviral vector rescued IL-7R expression and pro-B cell development in the PU.1^{-/-} MPPs. Whether these rescued cells can develop into OCs is unknown. Thus, PU.1 is required for the generation of lymphoid progenitors but not for their further differentiation. That is the responsibility of E2A, Ebf1 and Pax5. In the myeloid lineage, PU.1 is required for the generation of myeloid progenitors and the differentiation of macrophages and OCs [32,34].

Ikaros is a member of a Kruppel-like zinc finger family of transcription factors that also includes Helios and Aiolos [39,40]. The Ikaros gene (*Ikzf1*) is widely expressed in hematopoietic lineage cells including self-renewing HSCs and MPPs [41]. Mice deficient in *Ikzf1* have substantially less HSC activity and their MPPs are inhibited from differentiating into the CLP. This results in a complete failure of the B cell lineage to develop [42,43]. *Ikzf1*^{-/-} mice have altered T cell development in the thymus and functional changes in peripheral T cells [44]. Ikaros also regulates cytokine gene expression [44]. Ikaros deficient mice have increased myelopoiesis with increased numbers of Mac-1⁺ cells in the BM and spleen [43]. To examine hematopoietic progenitor differentiation, BM derived MPPs

(lin⁻Sca-1^{hi}c-Kit^{hi}) from *Ikzf1*^{-/-} or WT bone marrow were transduced with a retrovirus encoding EBF-GFP or control virus. Cells were grown on a stromal cell monolayer in the presence of B cell promoting cytokines Flt3 ligand and IL-7. The GFP⁺ cells were sorted and grown on a fresh stromal cell monolayer in the presence of the cytokines and monitored for differentiation. WT MPPs transduced with control virus developed into CD19⁺ B cell precursors as expected [45]. Expression of EBF in these cells increased the development of mature B cell progeny. Importantly, *Ikzf1*^{-/-} MPPs transduced with control virus differentiated not into B cells, but rather gave rise to CD11b⁺/Mac-1 myeloid progenitors [45]. Transduction of these cells with EBF blocked myelopoiesis and after 2–3 weeks of culture CD19⁺ B cell precursors developed. These results suggest that *Ikzf1* deficiency leads to increased myelopoiesis in vitro and is consistent with the in vivo data. Whether the increase in myelopoiesis reflects an increase in osteoclastogenesis is unknown. At present no data is available on the status of the skeleton of *Ikzf1*^{-/-} mice.

E2A is a member of the E-protein family of basic helix-loop-helix proteins, which bind E-box elements to regulate transcription. The E2A gene encodes two E proteins, E12 and E47, which are produced by differential splicing [46]. E2A proteins are expressed in HSCs and in different subsets of hematopoietic progenitors. As an example, BM from E2A^{-/-} mice had reduced numbers of long-term HSCs, granulocyte-macrophage progenitors and severe depletion of erythroid progenitors [47]. Although these data suggest E2A^{-/-} mice may have a bone phenotype they remain unexamined. Loss of E2A results in arrest of B cell development at the earliest appearance of B cell lineage genes and before rearrangement of the IgH locus [48]. It appears that E2A and Ebf1 work together to specify progenitors to enter B cell differentiation. To investigate the role E2A plays in myeloid and B cell differentiation MPPs (Lin⁻c-Kit^{hi}Sca-1^{hi}CD27⁺) were isolated from BM and transduced with GFP-expressing retroviral constructs encoding E12, E47 or control and cultured in B cell supporting conditions (grown on stromal cells in the presence of SCF, Flt3L, IL-7) [49]. MPPs transduced with control virus produced mainly CD11b/Mac-1⁺ myeloid cells. MPPs transduced with either E12 or E47 were unable to induce B cell differentiation suggesting that E2A alone is not sufficient to specify B cell differentiation. In contrast, transduction of MPPs with Ebf1 induced B cell differentiation (see below). These data suggest that E2A alone is not able to shift cell fate allocation from myeloid to B lymphoid.

Ebf1 is the founding member of small multigene family of helix-loop-helix proteins that are evolutionarily conserved with defined roles in cellular differentiation and function. Ebf1 is expressed early in the B cell lineage (fetal liver), in adipocytes, and we have shown it is expressed in osteoblast lineage cells including mesenchymal stem cells [13,50,51]. We have reported that all bone formation parameters (osteoblast number, osteoid thickness, bone volume and bone formation rate) are significantly increased in Ebf1^{-/-} mice as compared to their age-matched littermate controls [50]. OC numbers increase *in vivo* as the mice age. Thus, Ebf1^{-/-} mice have a bone phenotype strikingly different than PU.1^{-/-} mice. B cell development is blocked at its earliest stage in Ebf1^{-/-} mice [50]. To investigate the role Ebf1 plays in myeloid and B cell differentiation MPPs from Ebf1^{-/-} fetal livers were isolated as described for PU.1^{-/-} MPPs. Cells grown in B cell supporting conditions (SCF, Flt3L, IL-7) express a lymphoid phenotype (IL-7R⁺CD45R/B220⁺CD19⁻) [49]. Cells grown in myeloid supporting conditions (SCF, Flt3L, GM-CSF and M-CSF) developed macrophage-like cell morphology and were CD11b/Mac-1⁺ CD115⁺ c-fms⁺. No data is available on whether Ebf1^{-/-} MPPs are altered in their ability to differentiate into OC precursors or functional OCs. To test whether Ebf1 could regulate myeloid lineage development, Ebf1^{-/-} MPPs were transduced with a control GFP retroviral vector or an Ebf1-GFP retroviral vector and the cells cultured in myeloid supporting conditions. Cells transduced with GFP developed CD11b/Mac-1⁺ cells. In contrast, cells transduced with Ebf1-GFP caused an 85% reduction in CD11b/Mac-1⁺ cells. These data indicate that Ebf1 negatively regulates myeloid development and suggests that Ebf1 deficient

mice should have markedly increased OCs. However, the long bones of young (4 week-old) $Ebf1^{-/-}$ mice actually had a reduced number of OCs although this reduction was lost as the mice age. In addition, there was no difference in *in vitro* osteoclast differentiation from BM macrophages from $Ebf1^{-/-}$ mice compared to controls. This discrepancy suggests additional regulation of OC differentiation in the $Ebf1^{-/-}$ mice *in vivo*.

To determine the ability of $Ebf1$ to reconstitute multiple lineages competitive adoptive transfer experiments were performed. $Ebf1^{-/-}$ cells ($CD45.2^{+}$) that were maintained in B cell promoting conditions were injected, along with WT BM cells ($CD45.1^{+}$), into lethally irradiated $CD45.1^{+}$ mice. At 5 weeks the BM and thymus of the recipient mice were examined for lineage reconstitution. Cells derived from $Ebf1^{-/-}$ mice were recognized on the basis of $CD45.2$ expression by flow cytometry. $Ebf1^{-/-}$ progenitor cells gave rise to $Mac-1^{+}$ macrophages and $CD11c^{+}$ dendritic cells in the BM and $CD4^{+}CD8^{+}$ T cells were detected in the thymus. These data indicate that $Ebf1^{-/-}$ progenitors, even when grown in B cell promoting conditions, are capable of developing myeloid and lymphoid lineage cells [49]. The ability of these cells to differentiate into OCs *in vivo* remains to be determined.

Pax5 is a member of a multigene family that encodes the paired box (Pax) transcription factors. The Pax5 gene codes for the transcription factor B cell lineage specific activation factor (BSAP) [14]. Pax5 is expressed in the fetal liver where it correlates with the onset of B lymphopoiesis. Within the hematopoietic system, BSAP is expressed exclusively in the B lymphocyte lineage extending from pro-B cells ($CD45R/B220^{+}$) to mature B cells, but not in terminally differentiated plasma cells [14,52].

Loss of Pax5 results in an unanticipated massive decrease in trabecular bone in both the tibia and femur of 15-day-old mice [53]. Bone volume (tibia) was reduced by 67% and osteoid volume was reduced by 55%. This was found to be the result of increases in bone resorption and can be accounted for by the >100% increase in the number of OCs in $Pax5^{-/-}$ bone. These data not only indicate a marked increase in the number of OCs but also suggest that they are functional. The number of OBs in the mutant mice was reduced, although not significantly, to that of controls. This implies that the osteopenia was due, in large part, to the increase in OCs. Thus, $Pax5^{-/-}$ mice have a bone phenotype distinct from PU.1 and $Ebf1$ deficient mice.

Culture of spleen cells from $Pax5^{-/-}$ mice for 10 days results in formation of a lawn of adherent, elongated cells that morphologically appear similar to spleen macrophages [50]. These cells can be recovered from culture, passaged *in vitro* and developed into cell lines (SCL). The SCLs are 97% $CD11b^{+}/Mac-1$, 96% $CD16/32^{+}/Fc\gamma R$, and >50% $CD115^{+}$ (c-fms) and do not express $CD19$, $CD45R/B220$, $CD117/c-Kit$, $Ly6A/E/Sca-1$, $NK1.1$ and $TCR\alpha/\beta$ as determined by flow cytometry. These results indicate that the cells are in the myeloid lineage and are similar to macrophages. Importantly, these cell lines can be passaged *in vitro* for more than 10 generations in the absence of any added growth factors. WT spleen cells cultured in a similar manner die by the first passage. Culture of SCL with WT BM or spleen cells separated by a cell impermeable membrane (transwells) resulted in both BM and spleen cell proliferation (Horowitz unpublished). These data suggested that a soluble factor secreted by the SCL was responsible for the BM cell proliferation. To address this possibility we cultured WT BM cells with conditioned medium (CM) from the $Pax5^{-/-}$ SCL. SCL CM induced proliferation of the BM cells as measured by 3H -thymidine incorporation. Subsequent treatment of the SCL CM treated cells with RANKL and M-CSF, M-CSF alone or GM-CSF plus IL-4 resulted in the formation of a large number of functional OCs, macrophages and dendritic cells respectively. These data suggest that the $Pax5^{-/-}$ SCL CM induces the expansion of a subset of myeloid progenitors in WT cells. Although more restricted in its developmental plasticity, a parallel can be drawn between the SCL and the pro-B cells identified in $Pax5^{-/-}$ BM, which can differentiate into most hematopoietic cell lineages (with the exception of megakaryocytes and

red blood cells) [54]. However, the pro-B cells from Pax5^{-/-} BM require growth factor supplementation to proliferate *in vitro* making the SCL unique in its ability to grow in vitro without added growth factors.

Although OC and B cells arise from different progenitors there are data to support the hypothesis that B cells and macrophages can differentiate from a common progenitor downstream from the MPP. A rare population (0.5%) of B cell progenitors was isolated from adult mouse BM cells and was CD45R/B220⁻CD19⁺. When cultured in myeloid stimulating conditions these cells gave rise to macrophages indicating the bi-potential nature of these cells [55]. The loss of Pax5 causes the expression of unwanted genes, including c-fms, the M-CSF receptor that supports macrophage and OC differentiation. Therefore, loss of Pax5 could support the bi-potential progenitor population.

Is the loss of B cells the cause of the bone phenotype?

It is possible that the loss of B cells could be responsible for the bone phenotypes that are seen in Ebf1 and Pax5 deficient mice. However, this seems unlikely because mice lacking B and T cells due to the loss of Rag-1 (C57BL/6-Rag1^{-/-}) one of the two VDJ recombination enzymes have no detectable bone phenotype by histology [53]. Rag genes control B and T cell receptor expression, therefore their loss results in B and T cell deficient mice. Loss of the Rag-1 genes arrests B cell development at approximately the same stage of development as loss of Ebf1 and Pax5. These reports are supported by the data showing that non-obese diabetic-rag^{-/-} (NOD-rag^{-/-}) and NOD-scid IL-2γ^{-/-} mice, which lack B, T and NK cells also do not have a significant bone phenotype compared to age and sex matched NOD control mice as measured by micro-CT (M.Horowitz unpublished). The NOD mouse is a model for type 1 diabetes; however, the bones from the NOD mice were collected well before the onset of diabetes. These data are consistent with the report showing that following ovariectomy T cell deficient mice (nude, Rag2^{-/-}, T cell receptor α^{-/-}) lose trabecular bone mass equivalently to WT controls [56].

It has been reported that μMT heavy chain-deficient mice, which lack mature B cells, are osteopenic due to increased bone resorption, which is caused by a decrease of B cell secreted osteoprotegerin (OPG) [57]. Although no histology is presented, the bone phenotype of these mice appears mild. We have reported that the μMT deficient mice do not have an obvious bone phenotype [53]. In contrast, mice made deficient in OPG have a striking bone phenotype with increased cortical thickness and a “worm-eaten” appearance due to massive osteoclast activity in the cortical bone [58]. The μMT^{-/-} mice reported above have decreased cortical bone. The bone phenotype in the Ebf1 and Pax5 deficient mice is much more severe than that of μMT deficient mice. Loss of Ebf1 results in the loss of B cells as early as B cells can be identified, which could account for as much as 30% of BM cells and these mice have no apparent osteoclast phenotype. Although Pax5 deficient mice do have a demonstrable osteoclast phenotype, it is not caused by the B cell deficiency (Horowitz unpublished). Rag-1 deficiency, which directly affects T and B cell differentiation, results in a failure to develop mature B and T cells and these mice do not have an obvious bone phenotype.

The RANK-RANKL Pathway and B cell differentiation

It is well accepted that the interaction between RANKL and its cognate receptor RANK is required for the differentiation of osteoclasts from precursors. However, what is often overlooked is the role the genes that encode these proteins and their downstream effector genes play in regulating B cell development. RANKL deficient mice have severe osteopetrosis due to the inability of OBs to support OC differentiation. These mice are also one of the few mutants that lack all lymph nodes. The number of splenic IgM⁺IgD⁺ or CD45R/B220⁺IgM⁺ B cells was significantly reduced in RANKL^{-/-} mice as compared to controls [59]. To examine B cell

differentiation chimeric mice were made by injecting fetal liver cells from RANKL^{-/-} or WT mice into sub-lethally irradiated Rag^{-/-} mice [59]. The BM from Rag^{-/-} mice reconstituted with RANKL^{-/-} cells had normal numbers of CD45R/B220⁺CD43⁺ and CD45R/B220⁺CD25⁻ pro-B cells but significantly reduced numbers of CD45R/B220⁺CD43⁻, CD45R/B220⁺CD25⁺ and CD45R/B220⁺sIgM⁺ B cells, indicating a conspicuous block in the progression from pro-B cells to pre-B cells in the chimeric mice. Therefore, even in the RANKL sufficient environment of the Rag^{-/-} mice, the RANKL^{-/-} cell had a defect in B lymphopoiesis [59].

The question then arises; do other members of the RANK-RANKL pathway affect B cell differentiation or activation? RANK-deficient mice are severely osteopetrotic due to a failure of osteoclast precursors to differentiate to mature osteoclasts. The number of cells in the BM of RANK^{-/-} mice is markedly reduced with few hematopoietic colonies due to the osteopetrosis [60]. As hematopoiesis relocates, the spleens of RANK^{-/-} mice are approximately twice the size of controls but have a greater than 50% reduction in CD45R/B220⁺ B cells [60]. Mature, CD45R/B220⁺IgM⁺ splenic B cells were also reduced by 50% as compared to controls. Like the RANKL-deficient mice RANK-deficient mice lack all lymph nodes [60].

Following RANKL-RANK interaction intracellular signal transduction is mediated, in part, by TNF receptor-associated factor 6 (TRAF6). TRAF6^{-/-} mice are osteopetrotic [61]. Although TRAF6^{-/-} mice have similar numbers of OCs compared to controls, they fail to resorb bone because they do not develop an attachment zone or ruffled border. Interestingly, splenic B cells from TRAF6^{-/-} mice fail to proliferate in response to anti-CD40 or lipopolysaccharide stimulation suggesting a role for TRAF6 in B cell proliferation to specific signals.

OPG is a naturally circulating decoy protein receptor that binds RANKL, reducing RANKL's availability to bind RANK, thus regulating OC differentiation. OPG-deficient mice develop severe osteoporosis with associated fractures due to increased numbers of OCs. OPG is a CD40-regulated gene in B cells and dendritic cells. To analyze the effects of OPG deficiency on B cell development, pro-B cells (CD45R/B220⁺CD43⁺) were sorted by FACS from BM and cultured with IL-7. Pro-B cells from OPG^{-/-} mice proliferated 2 fold more after IL-7 stimulation than did wild-type cells [62]. FACS analysis revealed that CD45R/B220⁺CD43⁺IgM⁻ or CD45R/B220⁺CD25⁺IgM⁻ pro-B cells were increased in the BM of OPG^{-/-} mice as compared to WT controls. In addition, the number of CD45R/B220⁺CD19⁺ B cells in the spleen and lymph nodes was greater in OPG^{-/-} mice than in controls.

These data show that the loss of OPG increases pro-and mature B cells, while the loss of RANKL or RANK decreases these populations of cells. In the RANKL^{-/-} mice B cell differentiation is arrested between the pro- and pre-B cell stage of differentiation. The stage of differentiation arrest in the RANK^{-/-} mice is unknown.

Estrogen and B cells

Partially purified populations of B-lymphocytes from murine bone marrow are reported to form osteoclasts *in vitro* when they were treated with M-CSF and RANKL [63–66]. In addition, production of osteoclastogenic activity in these populations was increased after ovariectomy. However, when isolated to very high purity, purified B cells failed to differentiate into osteoclasts *in vitro* [67]. These results demonstrate that the osteoclastogenic potential of B cell populations in murine bone marrow is dependent on contaminating cells. Most recently, we and others have found that trabecular bone loss after ovariectomy was similar in wild type mice and mice, which were deficient in the majority of there mature B cells [26,56,68]. Estrogens are potent regulators of B-lymphopoiesis at a very early stage [69]. In mice the absolute number

of B cells (defined by expression of the antigen CD45R/B200) in bone marrow roughly doubles after ovariectomy [70]. Because B cells at multiple stages of differentiation can express RANKL, it is possible that changes in early B cells with estrogen withdrawal may affect osteoclastogenesis and subsequent bone loss [71]. However, this response does not appear involved in the bone loss that occurs after estrogen withdrawal in humans because increases in B cells were not found in postmenopausal women who had their estrogen replacement stopped [72].

Activated B cells

Activation of B cells is involved in the development of inflammatory arthritis as well as periodontal disease [73,74]. Activated B cells can produce RANKL as well as other cytokines that are involved in bone resorption and bone formation [73,75] (Fig 3). In addition, a subpopulation of memory B cells also expresses RANKL [76]. A variety of B cell-related malignancies produce factors that regulate bone cells. Multiple myeloma is a malignancy of plasma cells, which derive from B cells. It often causes enhanced bone resorption and decreased bone formation [77]. Derangements of the RANK/RANKL/OPG system are frequent in this condition and may be due to production of factors by the tumor that stimulates RANKL production in the bone microenvironment or because of direct production of RANKL by the myeloma cells [78,79]. In addition, a variety of additional factors including MIP-1 α and DKK1 are produced by myeloma cells and directly affect bone cell function [77]. B cell lymphomas can also produce RANKL and through this mechanism cause bone loss and hypercalcemia [80].

Conclusions

1. Cells in the osteoblast lineage support HSC and B cell differentiation and growth in endosteal bone niches. Support of B cell differentiation requires IL-7, secreted at least in part by osteoblast lineage cells.
2. The role of IL-7 in regulating bone homeostasis remains controversial.
3. A network of TFs is required for early B cell differentiation and function in a hierarchical order. Deletion of these transcription factors results in mice with striking bone phenotypes. Importantly, the bone phenotypes are markedly different with PU. 1 and Pax5 regulating OC development while Ebf1 regulates OB and adipocyte differentiation. One of the major functions of Ebf1 and Pax5 is the repression of unwanted genes, which helps maintain B lineage fidelity and may be the mechanism underlying the bone phenotypes. This suggests that analysis of Ikaros and E2A deficient animals will also provide important insights into the regulation of bone by the TFs.
4. In BM a series of interactions occur, some of which are mediated by B cell TFs that regulate the balance between B cell and macrophage differentiation from progenitors. Loss of these key regulatory TFs (e.g. Ikaros, Ebf1 and Pax5) results in lineage allocation being shifted away from B cell differentiation and towards myeloid lineage differentiation.
5. Loss of members of the RANK, RANKL, OPG pathway not only affects OC differentiation but also B cell development.
6. B and T cells do not have a major regulatory role in early bone development and normal bone remodeling. However, activated B and T cells can be potent regulators of bone resorption.

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Fig. 1.

Hematopoietic cell differentiation. All hematopoietic cells arise from hematopoietic stem cells (HSC) that give rise to multipotential progenitor cells (MPP, blue). B cells differentiate from the common lymphoid progenitor (CLP, green) and osteoclasts arise from the common myeloid progenitor (CMP, pink). There appears to exist a distinct lineage derived from the MPP that expresses both macrophage and B cell characteristics (B/Mφ bipotent), which can differentiate to B cells, macrophages and possibly osteoclasts (purple). (Adapted from Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL *Ann. Rev. Immunol.* 2003; 21:759–806).

Fig. 2.

Transcriptional regulation of B cell differentiation. B cell differentiation is regulated, in part, by the expression of a series of transcription factors that function in a temporal manner. These transcription factors include; PU.1, Ikaros, E2A, Ebf1 and Pax5. Loss of these specific factors precludes the cells from continued maturation, and results in a developmental block of cells at the latest stage of differentiation prior to the arrest. In addition to the absence of B cells, mice deficient in PU.1, Ebf1 and Pax5 have profound changes to their skeletons. No data is available on the bone phenotype in mice deficient in Ikaros or E2A.

Fig. 3.

Activated B cells induce osteoclast differentiation. B cells activated by the adaptive immune system (antigen specific) or through the innate immune system (LPS-Toll receptor) results in B cells that secrete or express on their cell surface molecules like RANKL that induce osteoclastogenesis. (Adapted from Boyle WJ, Simonet WS, Lacey DL. *Nature* 2003; 423:337–342).