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Perspective Article

Hylonome

Characterization of osteoclast precursor cells in murine bone marrow

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Relationship of osteoclast precursor cells to other hematopoietic cell lineages

The osteoclast is a terminally differentiated multinuclear cell with the unique ability to resorb bone[1]. It is now well established that the osteoclast originates from a hematopoietic precursor cell[2,3] but the exact relationship of the osteoclast precursor cell to other hematopoietic lineages is uncertain. Osteoclasts morphologically resemble macrophages[4]. However, unlike macrophages, they express membrane receptors for calcitonin, form a ruffled border, and resorb bone[5].

The mononuclear precursor cell that differentiates into the osteoclast circulates in the monocyte population[6]. A number of cytokines enhance osteoclast precursor cell development or differentiation. GM-CSF appears to induce the differentiation of multipotential progenitor cells towards the osteoclast phenotype[7]. Mice lacking M-CSF fail to form osteoclasts early in their development[8], 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[9] and respond to M-CSF by decreasing their apoptosis rate[10].

Recently, a new cytokine system was shown to be essential for osteoclast formation[11-14]. Receptor activator of nuclear factor kB ligand (RANKL), first identified as a product of activated T-lymphocytes, regulates osteoclast formation. It is produced by stromal and osteoblastic cells in response to stimulators of resorption and interacts with its receptor, RANK, on the cell membrane of osteoclast precursors to activate their differentiation into mature osteoclasts. RANKL can also bind to osteoprotegerin (OPG), a soluble "decoy" receptor[15], which inhibits RANKL/RANK interactions. OPG is produced widely and a number of stimulators of resorption regulate its production[16,17].

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[22]. In addition, pregnancy in mice is associated with a marked inhibition of B-lymphopoiesis through an estrogen-mediated mechanism[20,21].

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)[23] contain a population of precursors that can differentiate into osteoclast-like (OCL) cells *in vitro*[24,25]. In addition, ovariectomy was found to increase the number of OCLs that formed when CD45R-expressing cells were cultured with ST2 cells and treated with 1,25 $(OH)_2$ vitamin D_3[25]. This result implied that ovariectomy increased the number of CD45R+ osteoclast precursor cells in bone marrow.

We recently examined the ability of RANKL and M-CSF to stimulate osteoclast-like cell (OCL) formation in cultures of murine bone marrow cells that expressed or did not express CD45R. Cells were derived from either wild type 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[26]. We postulated that the lack of a response to ovariectomy in this model involved changes in osteoclast precursor cells.

We found that a highly enriched population of CD45R+ cells (greater than 98% pure) was capable of differentiating into OCLs with many characteristics of authentic osteoclasts. These included multinucleation, calcitonin receptor expression, TRAP production, and the ability to resorb bone. In addition, we found that the number of OCLs that form in the

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cultures of CD45R+ bone marrow cells increased in bone marrow cell cultures from Ovx animals.

We also found that highly enriched CD45R– murine bone marrow cell cultures did not demonstrate an increase in OCL number after ovariectomy. Furthermore, we found that OCL formation *in vitro* was not increased by estrogen withdrawal in either unfractionated, CD45R+ or CD45R– bone marrow cells from IL-1R1 KO mice, which do not lose bone mass after estrogen withdrawal[26]. Hence, our data demonstrate that the increase in OCL formation, which occurs in unfractionated murine bone marrow cell cultures after ovariectomy, is only reproduced in the CD45R+ subpopulation when bone marrow is fractionated into CD45R+ and CD45R– cells. In addition, our finding that ovariectomy 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 predicts 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[27,28]. 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[28]. 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 or are CD24–[29]. 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 3 transcription factors: the basic helix-loop-helix (bHLH) proteins E2A and early B-cell factor (EBF), and the transcription factor Pax5[30]. In mice, deletion of any of these genes causes the complete arrest of B-lymphopoiesis[31-33]. 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. Interestingly, expression of Pax5 by retroviral transduction in Pax5 -/- pre-B cells downregulated expression of myeloid genes like c-Fms[33]. 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[33]. 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 OCLs that formed in cultures from CD45R+ cells increased proportionally to that of pre-B-lymphocytes in marrow implies that these events are related. 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.

Role of IL-7 in osteoclastogenesis

Multiple cytokines; i.e., interleukins (IL-), 1, 6 and 7, are involved in the growth and differentiation of hematopoietic cells. Most of these also regulate bone remodeling and are products of bone marrow stromal cells, which play a key role in hematopoiesis[34]. B-lymphocyte development is supported by stromal cells in bone marrow and these cells produce IL-7[35]. This cytokine was originally identified as a stimulator of pre-B cells proliferation in a long-term mouse bone marrow lymphoid culture system. Pre-B cells do not grow or differentiate in co-culture with stromal cells that fail to produce IL-7[36]. Therefore, this cytokine is critical for *in vitro* B-lymphopoiesis. In both IL-7 and IL-7 receptor deficient mice, which are generated by gene inactivation, the number of B lymphocytes is markedly reduced[37,38]. These animals also show markedly decreased B-lymphopoiesis and a block in B cell development at the pro-B cell stage in bone marrow. However, the inhibition of B-lymphopoiesis is not complete and some B-cells do undergo maturation in the absence of functional IL-7 receptor or IL-7. Analysis of the bone marrow from IL-7 KO animals indicated that the percentage of pre-pro-B cells was normal. However, the proportions of pro-B cells were reduced by 30% and cells in transition between pro-B cells and early pre-B cells were almost completely absent. Administration of IL-7 *in vivo* into normal mice induced B-lymphopoiesis and resulted in marked bone loss[39]. IL-7 induces proliferation of IL-7 receptor (IL-7R)-bearing precursor B cells both *in vitro* and *in vivo* when administered systemically[40,41]. In mice that over-express IL-7 globally under control of a major histocompatibility complex (MHC) class II Ea promoter, B cell development is dramatically affected. In these animals the number of pro-, pre- and immature B cells in bone marrow is greatly increased and myeloid cells are virtually absent. In addition, the diameter of the femur is increased, the marrow cavity is enlarged, the periosteum adheres to the underlying bone and there is evidence of focal osteolysis[42]. Recently, it was shown that systemic administration of IL-7 to mice stimulated osteoclast formation by increasing osteoclastogenic cytokine production in T cells[43] and that RANKL and tumor necrosis factor α were the principal cytokines responsible for this effect[44]. These results demonstrate that over-expression or administration of IL-7 stimulates additional cytokines, which activate osteoclast-mediated bone resorption.

To investigate the relationship between osteoclastogenesis and lymphopoiesis further, we examined the direct effects that IL-7 had on osteoclastogenesis in murine bone marrow cultures. We also compared OCL formation rates in bone

marrow cultures from wild type mice with those from mice that were deficient in either IL-7 (IL-7 KO) or IL-7 receptor (IL-7R KO). We found that IL-7 was a potent inhibitor of osteoclastogenesis in bone marrow cultures, which were stimulated with a variety of agents. In contrast, osteoclastogenesis was increased in bone marrow cultures from IL-7 deficient (KO) mice and this response was associated with an increase in CFU-GM, an index of osteoclast precursor number. Hence, it appears that IL-7 regulates the number of osteoclast progenitor cells in murine marrow both *in vitro* and *in vivo*.

IL-7 is a critical regulator of B-lymphopoiesis since IL-7 -/- mice demonstrate a marked decrease in mature B-lymphocytes[38,45] and we found that treatment of murine bone marrow cultures with IL-7 markedly increased the number of cells expressing CD45R (a B-lymphocyte-associated surface antigen) and decreased the number of cells expressing Mac-1, a myeloid cell marker. Given the existence of a common lymphoid-myeloid progenitor cell in marrow and the ability of a subset of cells that express CD45R to differentiate into osteoclasts, it seems plausible that IL-7 inhibited osteoclast formation directly by preventing the differentiation of a lymphoid-osteoclast precursor cell towards the osteoclast lineage.

Curiously, we also found that bone marrow cultures from IL-7 receptor deficient (IL-7R KO) mice formed decreased numbers of OCLs when stimulated with multiple cytokines. The reason for the discrepancy of *in vitro* osteoclastogenesis between IL-7 KO and IL-7R KO mice is unknown. However, the IL-7 receptor can bind additional cytokines besides IL-7 including thymic stromal lymphopoietin (TSLP)[45]. Hence, the disparity between osteoclastogenesis in cell cultures from IL-7 KO and IL-7R KO mice may result from the inability of osteoclast precursor cells in IL-7R KO mice to respond to cytokines other than IL-7. Consistent with our results from *in vitro* osteoclastogenesis assays, previous studies demonstrated that IL-7R KO mice have a markedly increased bone mass[39]. In addition, we found that CFU-GM formation in marrow cultures from IL-7R KO mice was significantly less than in wild type controls.

Our finding that IL-7 KO mice have increased OCL formation *in vitro* is unlikely to result solely from changes in the bone marrow cell populations in these mice producing fewer B-lymphocytes and more myeloid cells. We believe this because OCL formation in purified CD45R+ murine marrow cells from IL-7 KO murine marrow cultures was increased compared to similar cultures from wild type animals. In addition, IL-7R KO mice, which have a similar decrease in the percentage of B lymphocytes and a similar increase in the percentage of myeloid cells in their marrow, had decreased OCL formation *in vitro*. These results argue that IL-7 regulates the lineage commitment of a common lymphocyte/osteoclast precursor cell. We hypothesize that in the absence of IL-7, this cell, which is CD45R+, commits more to the osteoclast lineage. In contrast, when cultures are treated with IL-7, the commitment of this cell is driven towards the B-lymphocyte lineage and away from the osteoclast lineage, which decreases *in vitro* osteoclastogenesis.

Summary

Our data argue that osteoclasts can originate from two hematopoietic lineage pathways: the monocyte-macrophage pathway and the B-lymphocyte pathway. The importance of the B-lymphocyte pathway is demonstrated by the finding that estrogen withdrawal, which is known to increase osteoclast formation both *in vivo* and *in vitro* and B-lymphopoiesis *in vivo*, increased *in vitro* osteoclastogenesis only in CD45R+ cells. Our finding that IL-7 regulates osteoclastogenesis supports this hypothesis.

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