

Osteoimmunology: Interactions of the Bone and Immune System

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Bone and the immune system are both complex tissues that respectively regulate the skeleton and the body's response to invading pathogens. It has now become clear that these organ systems often interact in their function. This is particularly true for the development of immune cells in the bone marrow and for the function of bone cells in health and disease. Because these two disciplines developed independently, investigators in each don't always fully appreciate the significance

that the other system has on the function of the tissue they are studying. This review is meant to provide a broad overview of the many ways that bone and immune cells interact so that a better understanding of the role that each plays in the development and function of the other can develop. It is hoped that an appreciation of the interactions of these two organ systems will lead to better therapeutics for diseases that affect either or both. (Endocrine Reviews 29: 403–440, 2008)

- I. Introduction
- II. The Origins of Bone Cells
 - A. Osteoclasts
 - B. Osteoblasts
- III. Role of Osteoblasts in Hematopoiesis
- IV. Role of Osteoblasts in Bone Marrow Cell Transplantation
- V. B Lymphocyte Differentiation
 - A. PU.1
 - B. Early B cell factor (Ebf)
 - C. Pax5
- VI. Role of Megakaryocytes in Bone Turnover
- VII. Cytokines and Local Immune Cell Factors as Regulators of Bone Cell Functions
 - A. Receptor activator of nuclear factor- κ B ligand (RANKL), receptor activator of nuclear factor- κ B (RANK), and osteoprotegerin (OPG)
 - B. RANKL-RANK mediated signals for osteoclast differentiation
 - C. Costimulation in RANKL-induced osteoclast differentiation
 - D. Macrophage colony-stimulating factor
 - E. Additional colony stimulating factors
 - F. Interleukin-1
 - G. Tumor necrosis factor
 - H. Additional TNF superfamily members
 - I. Interleukin-6
 - J. Additional interleukin-6 family members
 - K. Interleukin-7
 - L. Interleukin-8 and other chemokines
 - M. Interleukin-10
 - N. Interleukin-12
 - O. Interleukin-15
 - P. Interleukin-17 and interleukin-23
 - Q. Interleukin-18
 - R. Interferons
 - S. Additional cytokines
- VIII. Regulation of Osteoblasts by Immune Cells and Cytokines
- IX. Role of Osteoclasts in Regulating Osteoblasts

- X. Role of the Immune System in Bone Disease: the Birth of Osteoimmunology
- XI. Role of Immune Cells in Estrogen-Withdrawal-Induced Bone Loss
- XII. Modulation of Immunity by the RANKL-RANK-OPG Axis
- XIII. Toll-Like Receptors, Inflammation, and Osteoimmunology

I. Introduction

BONE IS A COMPLEX organ with multiple functions. It provides structural integrity for the body, it is the site of hematopoiesis, and it is a storehouse for calcium and

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Abbreviations: AIRE, Autoimmune regulator; BMP, bone morphogenic protein; BMSC, bone marrow stromal cells; BSAP, B-cell specific activation protein; CD40L, CD40 ligand; Ebf, early B cell factor; Eph, erythropoietin-producing hepatocyte kinase; FasL, Fas ligand; Fc γ R, Fc receptor γ ; G-CSF, granulocyte colony-stimulating factor; GCV, ganciclovir; GM-CSF, granulocyte M-CSF; HSC, hematopoietic stem cell(s); IFN, interferon; IgH, Ig heavy; ITAM, immunoreceptor tyrosine-based activation motif; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; MK, megakaryocyte(s); MMP, matrix metalloproteinase; MyD88, myeloid differentiation factor 88; NF- κ B, nuclear factor κ B; OCL, osteoclast-like cell(s); OPG, osteoprotegerin; OSCAR, osteoclast-associated receptor; OVX, ovariectomy; Pax, paired box; PI3K, phosphatidylinositol-3-kinase; PIR-A, paired Ig-like receptor A; PLC, phospholipase-C; RANK, receptor activator of NF- κ B; RANKL, RANK ligand; ROS, reactive oxygen species; SCF, stem cell factor; SIRP β 1, signal regulatory protein β 1; TCR, T-cell receptor; T_H, T-helper; TLR, Toll-like receptor; TPO, thrombopoietin; TRAF, TNF receptor-associated factor; TRAIL, TNF-related apoptosis inducing ligand; TREM-2, triggering receptor expressed by myeloid cells-2; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing interferon- β ; XHIM, X-linked hyper IgM.

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phosphorus (1). Likewise, the immune system is complex and provides organisms with protection from invading pathogens (2). Multiple overlapping and interacting mechanisms have evolved to regulate both systems. Interactions between bone and immune cells are now well described. It has become apparent that to explain the phenotype of many *in vivo* models with abnormal bone metabolism, one can no longer view either system in isolation. Rather, to understand their function, they must be viewed as a single integrated system. Examples of recently identified interactions of bone and immune cells include the findings: 1) that cells related to osteoblasts, which form bone, are critical regulators of the hematopoietic stem cell (HSC) niche from which all blood and immune cells derive; and 2) that osteoclasts, which are the cells that resorb bone, appear to share a common origin with the myeloid precursor cells that also gives rise to macrophages and myeloid dendritic cells. It has also been shown *in vitro* that cells that are relatively far along in their differentiation toward antigen-presenting dendritic cells retain the ability to form mature bone-resorbing osteoclasts (3). Finally, over the last 30 yr, it has become well established that multiple soluble mediators of immune cell function including cytokines, chemokines, and growth factors also regulate osteoblast and osteoclast activity (4). It is likely that immune cells and cytokines are critically responsible for the changes in bone turnover and bone mass that occur in postmenopausal osteoporosis and inflammatory conditions such as rheumatoid arthritis, periodontal disease, or inflammatory bowel disease.

The regulation of bone by hematopoietic and immune cells serves a variety of functions. It is likely that developing hematopoietic cells regulate bone turnover and maintain the marrow cavity by interacting with osteoblasts and osteoclasts during normal bone development (5). Conversely, during inflammatory states either locally produced or circulating cytokines, which are the products of activated immune cells, mediate increased bone turnover and the bone pathology in diseases such as rheumatoid arthritis and inflammatory bowel disease. We are only beginning to understand the breadth of bone and immune cell interactions, and this review is by no means complete. However, by appreciating the interactions of these two systems, it is hoped that future research into this area will develop in the context of the synergies between them so that the mechanisms underlying bone and immune cell function in both health and disease can be better understood.

II. The Origins of Bone Cells

A. Osteoclasts

Osteoclasts are multinucleated giant cells that form from the fusion of mononuclear precursor cells. Mature osteoclasts are unique in their capacity to efficiently resorb bone and contain a variety of specific cell structures that facilitate this process (1). The origin of the osteoclast precursor cell has been well studied. Initial work demonstrated that osteoclasts share many characteristics with macrophages (6). Although, osteoclasts and macrophages appear to express some common antigens (7), there are also clear differences in the ex-

pression of surface antigens that separate these two cell types (8, 9). Mononuclear cells, which can differentiate into osteoclast-like cells (OCL) in a variety of *in vitro* culture systems, are present in the bone marrow and the peripheral blood (10, 11).

The availability of multiple antibodies recognizing cell surface molecules, which are expressed on hematopoietic cells (12–15), has allowed the identification of bone marrow peripheral blood and spleen cell populations that can form OCL *in vitro*. Studies from multiple laboratories have identified several candidate populations with the ability to form OCL in coculture with stromal cells, when cultured alone in liquid medium or when cultured in methylcellulose. In experiments performed before the identification of receptor activator of nuclear factor (NF)- κ B ligand (RANKL), which is the critical cytokine that regulates osteoclast formation (16), investigators relied on coculture of various fractions of cells (generally from bone marrow) with stromal or osteoblastic cells (17). In these assays, cells were stimulated to induce osteoclastogenesis by treatment with a stimulator of resorption like 1,25 OH₂ vitamin D₃ or PTH. Interestingly, these assays require cell-cell interaction between osteoblastic and osteoclastic cells because OCL did not form in these cultures if the two cell types were separated by a membrane (18). The majority of these early studies focused on myeloid lineage cells. They demonstrated that rodent cells expressing mature macrophage markers, which were isolated from the bone marrow or spleen, gave rise to OCL when they were cocultured with bone marrow stromal cells (BMSC) (19). Muguruma and Lee (20) identified an osteoclast progenitor population in murine bone marrow that was negative for mature markers of B lymphocytes (CD45R/B220), granulocytes (Gr-1), macrophages (CD11b/Mac-1), and erythroid cells (Ter-119). This population did not express Sca-1, which is a marker that is found on HSC but was positive for the progenitor marker CD117/c-kit (20). These cells could progress to tartrate-resistant acid phosphatase-expressing mononuclear cells when they were cultured in semisolid media and OCL when they were cultured with 1,25 OH₂ vitamin D₃-treated-ST2 stromal cells. However, the cells in this fraction were considered multipotential because they were also able to differentiate into granulocytes, macrophages, and erythroid cells. Interestingly, when the c-kit low population was separated, it also could generate osteoclasts, but in a more restricted fashion. Tsurukai *et al.* (9) isolated cells from coculture of murine bone marrow hematopoietic cells and osteoblastic cells by passage through a Sephadex column and found that the population that was enriched for osteoclast precursors expressed monocytic markers but not markers of B or T lymphocytes. Using a coculture assay with ST2 cells, Hayashi *et al.* (21) found that osteoclast precursors were in the c-kit-positive (+) fraction and that expression of c-fms, the macrophage colony-stimulating factor (M-CSF) receptor, inhibited the efficiency of c-kit-positive cells forming OCL in culture.

Arai *et al.* (22) used both coculture with ST2 stromal cells and direct stimulation with RANKL and M-CSF, as well as antibodies against c-fms and the monocytic marker CD11b/Mac-1, to demonstrate that murine bone marrow cell populations expressing c-kit formed OCL in culture. These au-

thors concluded that a population of murine bone marrow cells with the phenotype $c\text{-kit}^+$, $c\text{-fms}^+$ CD11b^{lo} contained a multipotential progenitor cell population that gave rise to osteoclasts with high frequency. This population did not express RANK (the receptor for RANKL) when it was isolated from bone marrow but did after it was cultured with M-CSF. Interestingly, these precursors were not completely restricted to osteoclastogenesis because in methylcellulose cultures they generated macrophages and mononuclear tartrate-resistant acid phosphatase-positive cells. Microglia, the specialized phagocytic cells in the central nervous system, also appear to arise from a precursor cell that can give rise to osteoclasts (23). We have found that the osteoclast precursor cells in murine bone marrow are negative for CD3 and CD45R, negative or low for CD11b, and positive for expression of $c\text{-fms}$ (24). Expression of $c\text{-kit}$ further separated this population of murine bone marrow into two populations: 1) cells that rapidly formed OCL *in vitro* when cultured with M-CSF and RANKL ($c\text{-kit}$ high cells); and 2) cells that formed OCL more slowly *in vitro* ($c\text{-kit}$ low to negative cells). Expression of CD11b in this population of osteoclast precursors occurred transiently during *in vitro* culture. Initially, we found the population that most efficiently formed osteoclasts to be negative to low for this antigen. However, culture with M-CSF and RANKL induced mononuclear osteoclast precursor cells to transiently express high levels of CD11b. Expression of this antigen was lost in multinucleated cells (24).

The relationship of osteoclasts to dendritic cells, which present antigen to T lymphocytes as part of the adaptive immune response (25), is now also established. Both human and murine cells, expressing early markers of the myeloid dendritic cell lineage, can differentiate into osteoclasts *in vitro* (23, 26, 27). In addition, it appears that dendritic cells, which are relatively late in their lineage development, retain the ability to form osteoclasts *in vitro*. Alnaeeli *et al.* (3) showed that murine bone marrow cells, which were treated *in vitro* with cytokines so that they could present antigen to T lymphocytes, formed OCL in culture when they were treated with M-CSF and RANKL. However, Speziani *et al.* (28) found that neither mature myeloid dendritic cells generated *in vitro* nor plasmacytoid dendritic cells generated *in vivo* formed OCL in culture.

The ability of a common progenitor cell to differentiate into macrophages, osteoclasts, and myeloid dendritic cells has been proposed for some time (26, 27). However, only recently has it been demonstrated that a myeloid murine bone marrow cell can be isolated to the purity of single cell clones and retain the capacity to differentiate into macrophages and dendritic cells (29). We now have good evidence that this macrophage/myeloid dendritic cell precursor (29) can also differentiate into an OCL *in vitro* (J. Lorenzo, unpublished data). Hence, it appears that a common precursor cell exists for macrophages, myeloid dendritic cells, and osteoclasts. Commitment of the common macrophage/myeloid dendritic cell/osteoclast precursor to the osteoclast lineage occurs relatively quickly (within 24 h) after treatment of these cells with RANKL (30).

Expression of the myeloid-specific antigen CD11b has been used by a number of investigators to identify a circulating osteoclast precursor cell (31–34). The number of these

cells in the circulation is regulated by the inflammatory state of the organism and in particular by TNF α . Most recently, Yao *et al.* (34) demonstrated that expression of CD11b and Gr-1 could be used to identify this circulating osteoclast precursor population. In humans, expression of CD14 and the lack of expression of CD16 have been used to identify osteoclast precursor cells in peripheral blood (35, 36). In addition to CD14, osteoclast precursors in human peripheral blood also express receptor activator of NF- κ B (RANK) (37). Migration and adhesion of human CD14-positive monocytes to sites of inflammation from the peripheral circulation may be mediated through activation of microvascular endothelial cells by proinflammatory cytokines (38).

One interesting aspect of osteoclastogenesis is that cells with a cell surface phenotype that is similar to that of osteoclast precursor cells in bone marrow can be identified in the spleen. However, osteoclastogenesis does not occur in the spleen under any known condition. One possible explanation for this paradox is that the population of cells found in the spleen, despite having a similar phenotype to cells found in the bone marrow, are missing crucial elements that prevent their forming osteoclasts in splenic tissues. However, this hypothesis seems improbable because multiple investigators have established the *in vitro* osteoclastogenic potential of splenocytes. Another possibility is that the microenvironment in the spleen does not allow the production of osteoclasts either because it lacks critical signaling molecules or because it produces inhibitory signals. Miyamoto *et al.* (39) proposed that to complete osteoclastogenesis an adherent condition, which is defined by the expression of specific molecules, is required. This would ensure the correct interactions between osteoclast progenitors and supporting cells that express the correspondent ligands. Osteoblast lineage cells in the bone marrow might produce these signals, whereas the spleen or other nonbone tissues would not.

The latter hypothesis is supported by the recent findings that late osteoclast differentiation and activation require a novel combination of costimulatory molecules, which act in concert with M-CSF and RANKL to complete osteoclastogenesis (40). These molecules involve proteins containing an immunoreceptor tyrosine-based activation motif (ITAM) domain. They are found in adapter molecules like DAP12 and the Fc receptor γ (FcR γ). The search for receptors associated with these ITAM adapters in myeloid cells has identified at least two candidates that associate with FcR γ [osteoclast-associated receptor (OSCAR) and paired Ig-like receptor A (PIR-A)] and two that associate to DAP12 [the triggering receptor expressed by myeloid cells-2 (TREM-2) and the signal regulatory protein β 1 (SIRP β 1)] (41). The ligands for these receptors are currently unknown.

Fusion of osteoclast precursor cells into mature resorbing osteoclasts is a regulated process (42). Recently, expression of CD200 and CD200R on osteoclasts was found to influence this fusion process because osteoclast number was decreased and bone mass was increased in CD200-deficient mice (43). CD200-deficient mice also had a normal number of osteoclast precursor cells but a decreased rate of osteoclastogenesis *in vitro*.

Although the myeloid origin of osteoclasts is well established, it has been proposed that cells of the B-lymphoid

lineage can also give rise to osteoclast progenitors. Several groups have suggested the existence of bipotential progenitors for B lymphocytes and macrophages in bone marrow, which have the ability to differentiate into osteoclasts (44–46). We have found that paired box (Pax) 5^{−/−} mice, which have a block in B lymphocyte development at the pro-B cell stage, have an increased number of osteoclasts in their bones and decreased bone mass (47). However, the osteoclast precursor cell population in Pax5^{−/−} mice is myeloid in origin. In previous work (48), we found that OCL formed in cultures of murine bone marrow cells that express the B lymphocyte marker CD45R. These studies relied on populations of CD45R-positive murine bone marrow cells that were separated by fluorescence-activated cell sorting to a purity of 98–99%. However, in more recent work (24), we found that purification of the CD45R-positive murine bone marrow population by a second round of fluorescence-activated cell sorting to a purity more than 99.9% essentially eliminated the ability of purified CD45R population to form OCL *in vitro*. Hence, it appears that the OCL, which form in cultures of CD45R-positive cells, require the presence of a contaminating non-CD45R-expressing population of cells to form OCL. We are aware of no other studies of the osteoclastic potential of CD45R-expressing murine bone marrow cells that purified their populations to the degree that we have now done. However, we suspect that additional rounds of purification of other CD45R-positive populations, which have been proposed to contain osteoclast precursors, will likely also demonstrate them to be contaminated with small amounts of non-CD45R-expressing osteoclast precursors. We believe that these contaminating cells are critical for OCL formation in these cultures and may represent osteoclast precursor cells with a high proliferative capacity.

In humans it was recently demonstrated that the nuclei of myeloma cells, which are malignant cells of B lymphocyte origin, can be identified in osteoclasts. It was further proposed that this may be a mechanism for the increased osteoclastic activity seen in this condition (49). However, it has not been demonstrated that nonmalignant B lymphocyte lineage cells integrate into osteoclasts *in vivo* in humans.

B. Osteoblasts

Osteoblasts are derived from a mesenchymal progenitor cell that is multipotential and also can differentiate into marrow stromal cells and adipocytes (50). The signals that regulate the decision of mesenchymal progenitor cells to form osteoblasts are incompletely understood. However, a number of critical paracrine signals and cell autonomous transcription factors have been identified. These include the transcription factors Runx2 and osterix, which when absent prevent osteoblast formation, and the bone morphogenic protein (BMP) family (51–53), which initiates the signals for osteoblast differentiation. Most recently, it was found that Wnt signaling pathways are involved in the decision of the mesenchymal progenitor cell to become either an adipocyte or an osteoblast (54–58).

As matrix calcifies under the influence of the osteoblast-produced enzyme, bone-specific alkaline phosphatase, a portion of the osteoblasts are entrapped in the calcified matrix

and persist in bone as unique cells called osteocytes. These cells are believed to sense mechanical force on bone and to send signals, which regulate bone turnover, to cells at the bone surface. Osteocytes interconnect with each other and the cells at the bone surface via cellular projections, which are termed dendritic processes. These reside in channels in the mineralized bone, named canaliculi (59, 60). Most recently much interest has been generated by the discovery that the relatively osteocyte-specific protein sclerostin is an important regulator of the Wnt signaling pathway in osteoblast lineage cells (61).

III. Role of Osteoblasts in Hematopoiesis

In mammals during the early stages of gestation, hematopoiesis takes place in the yolk sac and then in the fetal liver. Eventually, it migrates to the bone marrow where, unless disturbed, it remains throughout the rest of life. Several investigators have documented the close proximity and/or attachment of hematopoietic cells to bone matrix and/or bone cells. These studies showed that multipotential HSC in the bone marrow were located adjacent to the endosteal surfaces of bone. They also demonstrated that cells closest to the bone surface were more proliferative than those that were farther away from the endosteum (62–67). Electron micrographs from Deldar *et al.* (68) found that granulocytes and reticular cells were either in close juxtaposition or in contact with endosteal bone-lining cells. There is a relatively high frequency of pre-B and terminal deoxynucleotidyl transferase-positive (TdT⁺) cells near the endosteal bone surface, and this frequency declines in cells that are closer to the center of the bone marrow cavity (69). HSC differentiation was also demonstrated to occur in close proximity to endosteal osteoblasts (70). Cheng *et al.* (71) observed, when isolating BMSC (a source of osteoblast progenitors) from bone marrow, that complexes existed, which were composed of BMSC and megakaryocytes (MK). This result implied that there was a physical association between mesenchymal and hematopoietic cells. It was also found that long-term HSC were attached to spindle-shaped osteoblast-like cells on bone surfaces, which expressed N-cadherin but not CD45 (72). However, this result is controversial because other investigators do not find HSC to express N-cadherin (73). These authors also demonstrated that the majority of HSC associate with sinusoidal vessels in the bone marrow and that only a minority of HSC are in close proximity to endosteal cells (73). Other authors have found that interaction of the chemokine CXCL12 on support cells with its receptor CXCR4 on HSC is critical for maintenance of HSC in the bone marrow (74). Expression of CXCL12 on support cells is found on cells in both the vasculature and the endosteum (75). The interactions of support and hematopoietic cells lead to the concept of the hematopoietic niche, which is a specialized structure that supports HSC and facilitates their replication and differentiation (Fig. 1).

It is clear that even the use of the most vigorous methods to expel bone marrow from mouse long bones leaves many cells adherent to endosteal surfaces. In the hematopoietic literature, these cells are often referred to as osteoblasts.

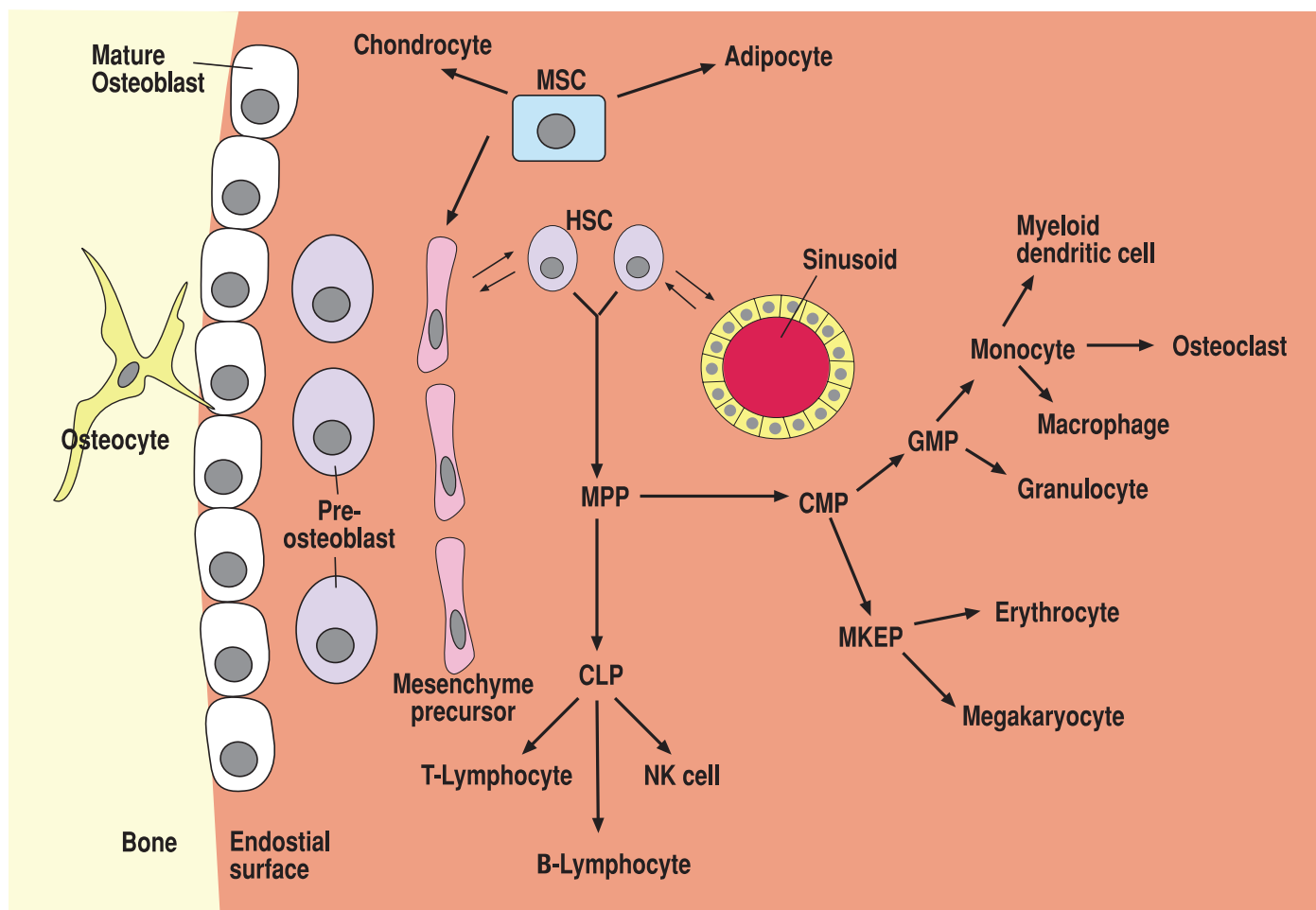


FIG. 1. Scheme for the interactions of osteoblasts with hematopoiesis. HSCs reside in the bone marrow adjacent to either osteoblast lineage cells or sinusoids. Both of these likely produce signals that control HSC replication and differentiation. HSC can remain dormant or replicate to either self-renew or differentiate into multipotential progenitor (MPP) cells. MPP can differentiate into either common lymphoid progenitors (CLP), which have the capacity to differentiate into precursors for T-lymphocytes, B-lymphocytes or natural killer (NK) cells; or MPP can become common myeloid precursor (CMP) cells, which are the precursor cells for all other hematopoietic lineages. CMP can differentiate into either granulocyte-macrophage progenitor (GMP) cells or megakaryocyte-erythroid progenitor (MEKP) cells. In turn, MEKP can differentiate into either erythrocytes or megakaryocytes. GMP can differentiate into monocytes or granulocytes. Bone marrow monocytes are precursors for myeloid dendritic cells, macrophages, and osteoclasts. Osteoblasts derive from a mesenchymal precursor cell (MSC) that is multipotential and can also differentiate into chondrocytes and adipocytes. Like the hematopoietic system, differentiation of MSC toward the osteoblast lineage involves multiple intermediates including mesenchyme precursors, preosteoblasts, and mature (matrix producing) osteoblasts. Finally, some mature osteoblasts appear to differentiate further into osteocytes, which are encased in the mineralized matrix of bone. [Derived from Ref. 578.]

Although this population predominantly contains cells of the osteoblast lineage, adherent cells of hematopoietic origin, such as osteoclasts and macrophages, are also present. It is also possible that bone-lining cells support HSC in the niche. Bone-lining cells are members of the osteoblast lineage and are thought to be mature cells that are different from osteocytes. Both bone-lining cells and osteocytes are believed to be more mature than matrix-producing osteoblasts. It has been recently demonstrated that osteoclasts are important for the release of hematopoietic progenitors from the niche (76). Mice injected with lipopolysaccharide (LPS) have increased numbers of osteoclasts on the endosteum and increased numbers of hematopoietic colony-forming cells in the peripheral blood, as a result of a marked mobilization of HSC from the bone marrow (76). Mice treated with RANKL also had an increased number of osteoclasts on their bone sur-

faces and increased levels of circulating colony-forming progenitors including Lin-Sca-1+c-kit⁺ progenitor cells (76). Conversely, mice treated with calcitonin, an inhibitor of osteoclast formation, or mice with defective osteoclasts had a reduced number of circulating progenitors (76).

Primary osteoblast lineage cells, which are established from humans and/or mice, have been shown to synthesize granulocyte colony-stimulating factor (G-CSF), granulocyte M-CSF (GM-CSF), M-CSF, IL-1, IL-6, lymphotoxin, TGF β , TNF α , leukemia inhibitory factor (LIF), and stem cell factor (SCF) or c-kit ligand (77–89). Importantly, all of these cytokines have been demonstrated to play a role in hematopoiesis (90,91) and many are also involved in osteoclast development.

One of the first definitive findings regarding the role for osteoblast lineage cells in hematopoiesis was the demonstration that when CD34⁺ hematopoietic progenitors were cul-

tured on a monolayer of osteoblast lineage cells for 2 wk, there was an 8-fold increase in hematopoietic cell number (89). It was then demonstrated that human osteoblast lineage cells constitutively produce membrane-bound G-CSF and that osteoblast lineage cell-bound G-CSF was responsible for approximately 55% of the increase in hematopoietic cell number (89). Assessment of the morphology of hematopoietic progenitors, which were cultured with osteoblast lineage cells, showed that the osteoblast lineage cells were able to support the survival of immature hematopoietic cells such as the long-term culture-initiating cells (92). In addition, osteoblast lineage cells were able to support the survival and, to a smaller extent, the proliferation of early myeloid progenitors (89). Interestingly, the coculture of CD34⁺ bone marrow cells with osteoblast lineage cells did not alter the secretion of G-CSF, GM-CSF, or TGF β -1 by the osteoblast lineage cells (93). However, the coculture of CD34⁺ bone marrow cells with osteoblast lineage cells minimally elevated LIF secretion and markedly elevated osteoblast lineage cell IL-6 production (93). Although the exact mechanism(s) responsible for the increase in osteoblast lineage cell-synthesized IL-6 remains to be determined, it appears to be regulated by unknown factor(s), which are secreted by CD34⁺ cells. Two likely candidates, IL-1 β and TNF α , were found not to be responsible (93). Transgenic mice with a constitutively active PTH/PTHrP receptor, whose expression is restricted to osteoblast lineage cells, had increased trabeculae and trabecular osteoblasts (94). Bone marrow from these transgenic mice had an increased number of Lin-Sca-1+cKit⁺ HSC compared with controls (94). The increase in HSC was due to the increased ability of the stromal cells from the transgenic mice to support HSC growth and differentiation. Injection of wild-type mice with PTH also produced an increase in these HSC (94). These data support the idea that cells of the osteoblast lineage are important regulators of the bone marrow hematopoietic niche.

Recently, significant insights have been generated concerning the molecular mechanisms regulating osteoblast lineage cell-hematopoietic cell interactions (72, 94, 95). Long term HSC were shown to be adjacent to osteoblast-lineage cells, and their number was increased by approximately 2.3-fold in mice upon deletion of the bone morphogenetic protein receptor 1A. Significantly, bone morphogenetic protein receptor 1A-deficient mice also had a similar increase in osteoblast-lineage cell number (72). Similarly, it was demonstrated that expansion of the osteoblast-lineage cell population in bone by stimulation of the PTH/PTHrP receptor increased the number of HSC in bone marrow (94). This effect appeared to be mediated by Jagged-1-Notch-1 signaling because Jagged-1 levels were increased in mice with osteoblast-targeted activation of the PTH/PTHrP receptor. In addition, the increase in the number of HSC in cultures of cells from transgenic mice with osteoblast-lineage cell-targeted activation of the PTH/PTHrP receptor was abrogated by inhibitors of Notch signaling. It has also been shown that PTH directly stimulates production of Jagged-1 by osteoblast-lineage cells (96). In a converse experiment, it was found that targeted destruction of osteoblast-lineage cells in mice led to a decrease in HSC in bone marrow (95). Interactions of HSC and osteoblast lineage cells appear to be mediated by interactions

of Tie2 on HSC and angiopoietin-1 on osteoblast lineage cells. This signaling system appears to inhibit cell division in HSC, while maintaining their capacity for self-renewal (97, 98).

Annexin II also appears to be involved in osteoblast-lineage cell-HSC interaction (99). Osteoblasts express this protein, which appears to mediate HSC adhesion, and HSC number in the marrow of annexin II-deficient mice was significantly reduced. Production of IL-10 by osteoblasts has also been shown recently to promote the self-renewal of HSC in the bone marrow (100).

Erythropoietin-producing hepatocyte kinases (Ephs) are small receptor tyrosine kinases that function to regulate a variety of cellular systems including immune and bone cells (101, 102). The Eph family has 15 members and is separated into Eph A and Eph B subgroups. The ligands of Ephs are called ephrins. Both Ephs and ephrins are cell surface molecules, and both mediate cellular responses. In bone, ephrinB and EphB receptors control skeletal patterning in the developing organism (103). Mice lacking ephrinB1 have defects in rib, joint, and digit development (104). It is now known that osteoclasts express ephrinB2, whereas osteoblasts express EphB receptors, particularly EphB4 (105). Zhao *et al.* (105) demonstrated that activation of ephrinB2 in osteoclasts by EphB4 on osteoblasts had bidirectional effects, which resulted in inhibition of osteoclastogenesis and increased osteoblast differentiation. Inhibition of osteoclastogenesis by ephrinB2 signaling was mediated by decreases in Fos and NFATc1, whereas enhanced osteoblast differentiation required RhoA inactivation.

IV. Role of Osteoblasts in Bone Marrow Cell Transplantation

Cells of the osteoblast lineage facilitate bone marrow transplantation. Specifically, it has been shown that transplantation of donor bone (containing BMSC and/or osteoblast lineage cells) or osteoblast lineage cells isolated from mouse long bones, along with HSC or bone marrow cells, promoted hematopoietic engraftment (106–108). Indeed, this combination allowed for successful transplantation where HSC or bone marrow cells alone failed (106, 107). As another example, a transgenic mouse model was studied. This mouse expressed the herpes virus thymidine kinase gene under the control of the rat collagen α 1 type I promoter. The transgene conferred lineage-specific expression of thymidine kinase in developing and mature osteoblast lineage cells and allowed for the conditional ablation of these cells after treatment of the transgenic mice with ganciclovir (GCV) (95). After GCV treatment, these mice had marked changes in bone formation leading to a progressive bone loss. Importantly, treated animals also lost lymphoid, erythroid, and myeloid progenitors in the bone marrow, followed by decreases in the number of HSC. After withdrawal of GCV, osteoblasts reappeared in the bone, and medullary hematopoiesis was reestablished. Because PTH is able to regulate HSC number *in vivo*, PTH has therapeutic potential to enhance bone marrow transplantation. Pharmacological use of PTH increased the number of HSC that were mobilized into the peripheral blood, protected stem cells from repeated exposure to cytotoxic chemother-

apy, and expanded stem cells in transplant recipients (36, 109).

V. B Lymphocyte Differentiation

B lymphopoiesis is a highly ordered process proceeding from progenitor cells in the fetal liver to development in the bone marrow and to mature B cells in the secondary lymphoid organs. The mature B cell terminally differentiates into Ig-secreting plasma cells after activation (110). 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 (111). The bone marrow B cell developmental pathway can be divided into several distinct stages, based on the recombination status of the immunoglobulin genes and the expression of surface antigens (112–114). The earliest characterized committed B cell progenitor (pre-pro-B) expresses the cell surface markers CD45R and AAR.1 and has its Ig heavy (IgH) chain locus in the germ-line configuration (not rearranged) (42, 115). Subsequent differentiation generates pro-B cells that harbor rearranged IgH D and J genes and express the surrogate light chains $\lambda 5$ and VpreB and signaling adapters Ig β and Ig α (116, 117). As the cells mature, rearrangements occur initially in V gene segments of the IgH chain gene and then in the Ig light chain genes, a process that culminates in a functional surface antigen receptor (111, 118). The molecular dissection of the B cell differentiation pathway has been greatly facilitated by the identification of transcription factors, which are critical for this process. These include PU.1, Ikarous, E2A, Ebf-1, and Pax5, which are required for developmental transitions during B lymphopoiesis. 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 before the arrest.

Three transcription factors (PU.1, Ebf-1, and Pax5), which act early in B cell differentiation, surprisingly also have profound effects on bone cell development. Because these proteins function in close temporal sequence during B cell development, it might be expected that loss of their function would result in similar bone phenotypes. However, with the exception of being runted and lacking B cells, deletion of these transcription factors in mice produces animals with strikingly different bone phenotypes.

A. PU.1

PU.1, a member of the ETS domain transcription factors, regulates the proliferation and differentiation of B cell and macrophage lineage progenitors (119, 120). The commitment of early progenitors to the B cell lineage depends on a low level/activity of PU.1 in cells. In contrast, macrophage (osteoclasts) lineage commitment depends on high level/activity of PU.1 (121). PU.1-deficient (–/–) mice have no B cells and fail to develop either osteoclasts or macrophages (120). This observation was one of the first to definitively show that osteoclasts are members of the macrophage lineage. PU.1 regulates the lineage fate of these progenitors by directly controlling expression of the IL-7 receptor and c-fms genes (122, 123).

B. Early B cell factor (Ebf)

Ebf-1 is the founding member of a small multigene family of helix-loop-helix proteins that are evolutionarily conserved and have defined roles in cellular differentiation and function. This factor was cloned both from *Saccharomyces cerevisiae* in experiments aimed at identifying the olfactory-restricted olfactory marker protein-1 promoter (124) and by biochemical purification of a factor interacting with the B lymphocyte restricted mb-1 promoter (125). It was named Olf-1, or early B cell factor (Ebf), which in turn led to its current designation as O/E-1. Mice express at least three additional members of this family, Ebf-2 (mMot1/O/E-3), Ebf-3 (O/E-2), and Ebf-4 (126–128). Isolation of the Ebf homolog Collier from *Drosophila* demonstrated the existence of a new family of evolutionarily conserved proteins Collier/Olf/EBF. Mouse Ebf-1, -2, -3, and -4 bind DNA as homo- or heterodimers (129). Ebf1 gene expression is required for B cell fate specification, whereas Pax5, which is regulated by Ebf1, is required for the differentiation of B lymphocyte lineage cells (130, 131). Mice deficient in either of these transcription factors have arrested B cell differentiation at very early stages of B lymphopoiesis (Hardy A and B) (130).

Ebf proteins are involved in both embryonic and adult development of the nervous system. Ebf-1 and -3 expression is seen in Purkinje cells and the cerebellum, and all four Ebf proteins are expressed at high levels in olfactory epithelium (132). Interestingly, OAZ is an Ebf-interacting protein involved in BMP signaling (110). OAZ interacts with Smad1 where Smads and O/E proteins compete for OAZ. Thus, it may be that Ebf proteins regulate BMP signaling. Additional support for this idea comes from the observation that Ebf-1 potentiates activation of the B cell-specific gene mb-1 (CD79a) by Pax5 (133). Importantly, Runx1 and Ebf-1 synergized to activate mb-1. This may be important because Runx1, a runt homology domain transcription factor, which is required for hematopoiesis during embryonic development, is expressed in cartilaginous anlagen in the embryo, resting zone chondrocytes, and suture lines of the calvaria (134). Runx1 continues to be expressed in these tissues in adult mice, but not in mature cartilage or mineralized bone. Our preliminary data confirm the original report that Ebf-1-deficient (–/–) mice are growth retarded (130). This is most likely due to the skeletal phenotype, which appears to result from a cell autonomous role of Ebf-1 on osteoblasts. Ebf-1, like all of the known Ebf genes, is highly expressed in adipocytes (130, 135). Analysis of the preadipocyte cell lines, 3T3 L1, indicates that these genes are expressed strongly in undifferentiated cells and their expression increases with differentiation (135). Overexpression of Ebf-1 enhances terminal adipocyte differentiation in preadipocyte cell lines and induces adipogenesis in multipotential cells. The fact that Ebf genes are expressed throughout adipocyte differentiation raises the possibility that they are key regulators of the pathway. However, the exact mechanism by which Ebf-1 stimulates adipogenesis *in vitro* or *in vivo* remains to be elucidated. Ebf1 mRNA is expressed in osteoblasts at all stages of differentiation and also in adipocytes (136). Tibiae and femora from Ebf-1–/– mice had a striking increase in all bone formation parameters examined, including the number of osteoblasts, osteoid vol-

ume, and bone formation rate (136). Serum osteocalcin, a marker of bone formation, was significantly elevated in mutant mice. The number of osteoclasts in bone were normal in younger (4 wk old) *Ebf1*^{−/−} mice but increased in older (12 wk old) *Ebf1*^{−/−} mice. This correlated well with *in vitro* osteoclast development from bone marrow cells. In addition to increased osteoblastogenesis, *Ebf1*^{−/−} mice had a dramatic increase in adipocyte number in the bone marrow. Increased adiposity was also seen histologically in the liver but not in the spleen of these mice (136). Thus *Ebf1*^{−/−} mice appear to be a new model of lipodystrophy. EBF1 is a rare example of a transcription factor that regulates both the osteoblast and adipocyte lineages similarly.

It is possible that the loss of B cells could account for the changes in bone that are seen in these mutants. However, this seems unlikely because RAG-1- or μ MT-heavy chain-deficient mice, which also lack most B cells, do not have a similar bone phenotype (47). It has been reported recently that B cell-deficient mice (μ MT heavy chain-deficient) are osteopenic due to increased bone resorption caused by a decrease of B cell-secreted osteoprotegerin (OPG) (137). In contrast, *Ebf1*^{−/−} mice, which lack all but the very earliest population of pro-B cells, have increased bone mass and increased osteoclasts.

C. *Pax5*

Pax5 is a member of the multigene family that encodes the Pax transcription factors. This highly conserved motif was originally identified in *Drosophila* (138). At present, nine paired box containing genes (*Pax1*–*Pax9*) have been isolated in mammals (131, 139). Three Pax gene-deficient conditions have been studied, and all exhibit developmental mutations. The *Pax1* gene is mutated in different forms of undulated, which have skeletal changes in the vertebra (140). It is unknown whether any of these mutant mice express an altered bone phenotype. Human disorders have also been associated with mutations of Pax genes. *Pax3* is mutated in Waardenburg's syndrome, which causes deafness, and *Pax6* is altered in aniridia and in Peter's anomaly (141, 142). All of these mutations suggest the importance of Pax proteins in the specialization, proliferation, and migration of progenitor cells.

The *Pax5* gene codes for the transcription factor B-cell specific activation protein (BSAP) (143). During embryogenesis, *Pax5* is transiently expressed in the mesencephalon and spinal cord in a pattern that is different from other Pax genes (143). Later in development, expression moves to 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 cells, extending from pro-B cells to mature B cells, but it is not found in terminally differentiated plasma cells (143, 144). Testis is the only other tissue in the adult mouse that expresses BSAP.

Loss of *Pax5* results in an unanticipated massive decrease in trabecular bone in both the tibia and femur of 15-d-old mice (47). Bone volume (tibia) was reduced by 67%, and osteoid volume was reduced by 55%. Observed increases in bone resorption may be accounted for, at least in part, by a greater than 100% increase in the number of osteoclasts in

Pax5-deficient (−/−) bone. These data demonstrate a marked increase in the number of osteoclasts in *Pax5*^{−/−} mice and suggest that they are functional. The number of osteoblasts in the mutant mice was reduced, although not significantly. These results imply that osteopenia in *Pax5*^{−/−} mice was due, in large part, to an increase in osteoclasts. However, we cannot rule out the possibility that a delay in the development of osteoblasts contributes to the bone phenotype. In fact, a delay in osteoblast development may be responsible, at least in part, for the runting of these mice. Therefore, we propose that loss of *Pax5* causes a bone phenotype by deregulating certain genes that enhance osteoclastogenesis and delay formation.

VI. Role of Megakaryocytes in Bone Turnover

Similar to their role in hematopoiesis, cells of the osteoblast lineage support megakaryopoiesis. Studies by Ahmed *et al.* (145) demonstrated that culture of CD34⁺ cells on osteoblast lineage cells resulted in expansion of CD34⁺ and CD34⁺CD41⁺ (early MK) cells. When various combinations of cytokines were added to the cultures, it was determined that SCF, IL-3, IL-11, and thrombopoietin (TPO) were most effective in increasing CD34⁺CD41⁺ and CD41⁺ (late MK) cell number. Similarly, it was determined that BMSC were able to support MK differentiation and platelet formation (71). In other experiments, it was shown that culture of human MK on BMSC, which express SCF, resulted in adhesion of the MK to the BMSC and proliferation of the MK through SCF-c-kit interaction (146). Separation of the MK from the BMSC by a cell-impermeable membrane blocked proliferation, indicating that a cell-cell interaction was required.

MK arise from pluripotent hematopoietic progenitor cells that pass through a series of identifiable stages of differentiation that culminate in the production of terminally differentiated MK and the release of platelets. As with B cell differentiation, the molecular dissection of the MK differentiation pathway has been greatly facilitated by the identification of transcription factors that are required for the successful advance of cells from stage to stage. Loss of these specific factors precludes the cells from continued maturation and results in the accumulation of cells at the latest stage of differentiation, before the arrest. The selective loss of two different transcription factors, GATA-1 and NF-E2, which were originally thought to be required exclusively for erythroid lineage development, has now been shown to play a critical role in MK differentiation. GATA-1 knockdown mice and NF-E2-deficient mice exhibit a phenotype characterized by marked megakaryocytosis and thrombocytopenia (147, 148).

The GATA family of zinc-finger transcription factors in vertebrates is presently composed of six members, GATA-1 through GATA-6. GATA is a single polypeptide chain with DNA binding activity in the C-terminal zinc finger (149). GATA-1 is almost solely restricted to hematopoietic lineage cells and is a critical factor for erythroid cell development. GATA-1 is expressed in MK, multipotent hematopoietic progenitors, and mast cells (150). In GATA-1-deficient mice, MK number is increased approximately 10-fold in the bone

marrow and spleen of adult mice, and platelet numbers in the peripheral blood are markedly reduced (15% of normal) (151). It has been documented that MK from GATA-1-deficient mice express lower levels of TGF β -1, platelet-derived growth factor, and vascular endothelial growth factor than do wild-type control MK (152). TGF β -1 levels are increased in the spleen and bone (including bone marrow) of GATA-1-deficient mice but not in the plasma. The animals develop myelofibrosis after 1 yr of age (152, 153), which is preceded by a high bone mass phenotype (detected after 3–4 months), which is associated with a greater than 3-fold increase in bone volume and bone formation indices (154).

NF-E2 is a heterodimeric nuclear protein comprised of two polypeptide chains, a hematopoietic-specific 45-kDa subunit and a widely expressed p18 subunit. Both proteins belong to the basic leucine zipper family of transcription factors (155, 156). Expression of p45 is restricted to erythroid precursors, MK, mast cells, and multipotential progenitors. Mice lacking p45 NF-E2 exhibit profound thrombocytopenia, which results from a maturational arrest of MK and a lack of platelets in the peripheral blood (148). MK number is increased 2- to 5-fold in the bone marrow and spleen of adult p45NF-E2-deficient mice. These mice respond to exogenous TPO with a marked increase in bone marrow cell proliferation but no detectable increase in platelet production. Although MK number is markedly elevated in p45 NF-E2-deficient mice, TPO levels are normal (148, 157, 158). Interestingly, these mice also develop a high bone mass phenotype with up to a 5-fold increase in bone volume and bone formation parameters (154, 159).

The strikingly similar bone phenotype, along with the elevated osteoblast number and MK number in both NF-E2 and GATA-1-deficient animal models, led us to examine the potential interaction between osteoblast lineage cells and MK. We showed that when osteoblast lineage cells were cocultured with MK, osteoblast proliferation was increased 3- to 6-fold by a mechanism that required direct cell-to-cell contact (154). Miao *et al.* (160) also demonstrated that direct cell-to-cell contact of BMSC with MK enhanced osteoblastogenesis. In other studies, MK have also been reported to stimulate the differentiation of osteoblasts as defined by enhanced expression of procollagen (161). Thus, MK acts to stimulate both osteoblast proliferation and differentiation *in vitro*.

MK may also play a role in osteoclastogenesis as documented by the expression of OPG and RANKL in MK (161–166). The fact that MK expresses RANKL suggests that they may be an additional vector for osteoclast induction, particularly during inflammatory responses.

In contrast, MK expression of OPG suggests that MK may also play a role in inhibiting osteoclastogenesis. Recent data by our laboratory demonstrated that, *in vitro*, MK and MK conditioned media (CM) inhibited osteoclast development by up to 10-fold (98). We examined MK CM for known inhibitors of osteoclastogenesis and could demonstrate by ELISA that low levels of OPG were present (167). However, Chagraoui *et al.* (164) did not find OPG in MK CM, suggesting that the OPG, if secreted, was not detectable because it was bound to MK-expressed RANKL (161, 164, 165). Importantly, in our work, the addition of anti-OPG antibody failed

to neutralize the ability of MK CM to inhibit osteoclast formation, suggesting that MK-secreted OPG was not responsible for the inhibition of osteoclast development.

We confirmed that OPG was not responsible for the MK-mediated inhibition of osteoclast development by testing MK derived from OPG-deficient (–/–) mice. These experiments demonstrated that MK from OPG–/– and control mice inhibited osteoclast formation equivalently. Finally, using tandem mass spectrophotometry, we demonstrated that there exists a factor or factors in MK CM that inhibit osteoclast development, and while the identity of this inhibitory factor remains to be determined, it was not any of the major factors known to inhibit osteoclast formation including OPG, IL-4, IL-10, IL-12, IL-13, IL-18, interferon γ (IFN- γ), TGF β , GM-CSF, osteoclast inhibitory lectin, calcitonin, amylin, or calcitonin-gene-related peptide (167).

Taken together, these data suggest that MK play a dual role in regulating skeletal mass. They secrete a factor(s) that inhibits osteoclast formation while directly stimulating osteoblast proliferation and differentiation. Both of these MK-mediated actions may contribute to the osteosclerosis seen in GATA-1-deficient and NF-E2-deficient mice.

VII. Cytokines and Local Immune Cell Factors as Regulators of Bone Cell Functions

A. Receptor activator of nuclear factor- κ B ligand (RANKL), receptor activator of nuclear factor- κ B (RANK), and osteoprotegerin (OPG)

Characterization of the functions of RANKL and its receptors (RANK and OPG) (Fig. 2) have contributed significantly to the emergence of osteoimmunology, specifically with respect to examination of the interplay between active immunity and maintenance of bone homeostasis (16, 168, 169). Because there are a number of recent reviews on the diverse physiological function of the RANKL-RANK-OPG axis (169–171), we will focus here on its role in the context of osteoimmunology.

The discovery of RANKL, a TNF superfamily member that has potent activity as a stimulator of both the formation of osteoclasts from precursor cells and bone-resorbing activity in mature osteoclasts, clarified our understanding of how stromal and osteoblastic cells regulate bone resorption (172, 173). RANKL is the critical cytokine that directs the terminal differentiation of osteoclast precursor cells and stimulates and maintains resorption activity in mature cells. Importantly, this activity occurs *in vitro* in the absence of BMSC (172–174).

In vivo RANKL-deficient mice have significant osteopetrosis and no osteoclasts, but a normal number of monocyte/macrophages (175). These mice also exhibit failed tooth eruption, which is a common defect associated with developmental osteopetrosis, and diversion of hematopoiesis to the spleen and liver because a functional bone marrow cavity fails to form in the absence of osteoclasts (175, 176). Marrow stromal and osteoblastic cells produce RANKL, and regulation of its mRNA expression in murine marrow cell cultures correlates with activation of osteoclastogenesis (177). Many well-known osteotropic factors, including cyto-

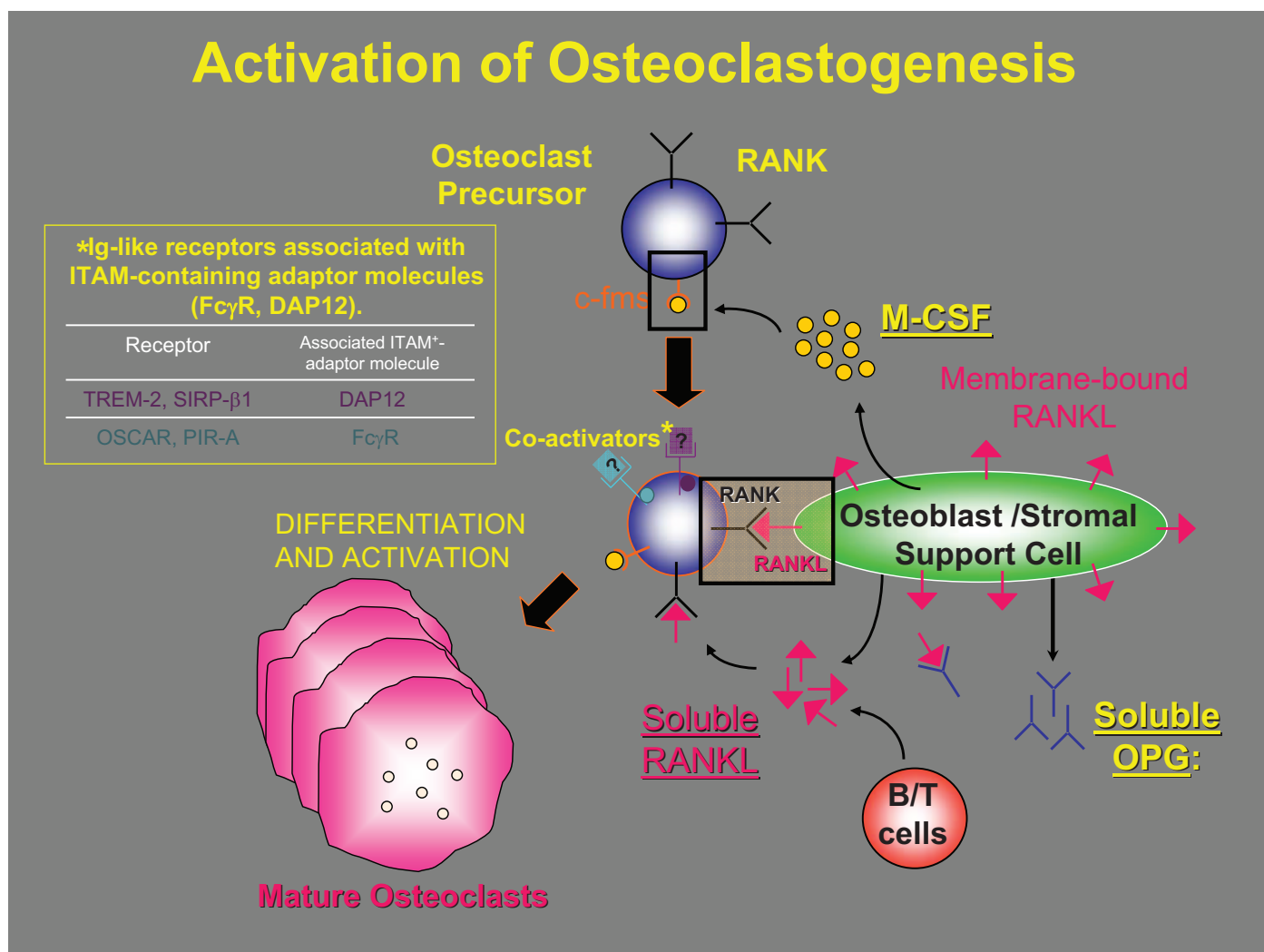


FIG. 2. Activation of osteoclastogenesis. Osteoclast precursor cells replicate and are induced to express RANK when stimulated by the binding of M-CSF to its receptor c-fms. In states in which osteoclastogenesis is stimulated, osteoblast or stromal support cells express relatively more RANKL than OPG. This facilitates the binding of RANKL to RANK, which is the critical signal for the differentiation of mature osteoclasts from precursor cells. However, the formation of mature osteoclasts is significantly enhanced by costimulatory molecules on osteoclast precursor cells. It is critical that the ITAM proteins DAP12 and FcγR on the surface of osteoclast precursor cells interact with their respective Ig-like receptors (TREM-2 and SIRP-β1 with DAP12; OSCAR and PIR-A with FcγR) for costimulation to occur. In addition, in inflammatory states and, possibly, in normal physiology, B and T lymphocytes also produce RANKL, which can influence osteoclastogenesis.

kines and hormones, are now believed to exert their primary osteoclastogenic activity by inducing RANKL expression in osteoblast lineage cells (16, 170). Conversely, the shedding of membrane-bound RANKL appears to be a mechanism for inhibiting osteoblast-mediated osteoclast formation by removing RANKL from the osteoblast surface. The process appears mediated by expression of matrix metalloproteinase (MMP) 14 (178) because osteoclasts were increased in mice deficient in this enzyme.

OPG is a novel secreted TNF receptor superfamily member (TNFRSF-11B) and a potent inhibitor of osteoclast formation that acts as a decoy receptor for RANKL (173, 174, 179). It was initially identified as a soluble factor that was capable of inhibiting osteoclastogenesis *in vitro* (179, 180) and inducing osteopetrosis when transgenically overexpressed in mice (179). In marrow, it is produced by a variety of cells, including stromal cells, B lymphocytes, and dendritic cells

(181). In addition to RANKL, OPG also binds the TNF-like ligand TRAIL (TNF-related apoptosis inducing ligand) (182). Mice that lack OPG were shown to have severe osteoporosis, increased numbers of osteoclasts, and arterial calcification (183, 184). The latter finding highlights a potential genetic link between osteoporosis and vascular calcification (170). Overexpression of OPG in transgenic mice caused osteopetrosis, decreased osteoclast numbers, and extramedullary hematopoiesis (179).

The biologically active receptor for RANKL is RANK. Like OPG, RANK is a TNF receptor superfamily member (TNFRSF-11A). It was first identified on dendritic cells (185), but it is also present on osteoclast precursors and mature osteoclasts (186). RANK expression at the RNA level is detected in a variety of cell types and tissues (185). RANK-deficient mice were demonstrated to phenocopy the defect in osteoclast development that was observed in the RANKL-knock-

out mouse, confirming the exclusive specificity of RANKL for osteoclast-expressed RANK (186). In humans, gain-of-function mutations in RANK were found to be associated with familial expansile osteolysis and expansile skeletal hyperphosphatasia (17–21, 187–191).

Although OCL can form *in vitro* in the absence of RANK or TNF receptor-associated factor (TRAF) 6 signaling when exposed to a cocktail of cytokines and growth factors (192–194), the significance of this *in vitro* finding is questionable because osteoclasts are not detected in RANK-deficient animals (186, 195). More likely, additional cytokines and growth factors produced at sites of inflammation or physiologically during bone turnover act as cofactors that enhance or modulate the response of osteoclasts and their precursors to RANKL-RANK stimulation (196–198).

B. RANKL-RANK mediated signals for osteoclast differentiation

Efforts aimed at elucidating the signaling mechanisms involved in RANKL-mediated osteoclastogenesis have been informative (16, 170, 199). RANK signal transduction is mediated by adapter proteins called TRAFs (174, 200–203). Of the six known TRAFs, RANK interacts with TRAF1, -2, -3, and -5 in a membrane-distal region of the cytoplasmic tail and with TRAF6 at a distinct membrane-proximal Pro-X-Glu-X-X-(aromatic/acid residue) binding motif (200–203). Genetic experiments show that TRAF6-deficient mice have severe osteopetrosis, implying that the key signals sent through RANK in osteoclast precursors are mediated by the adapter molecule TRAF6 (204, 205) (Y. Choi, unpublished data).

Downstream of TRAF6, RANKL signaling in osteoclasts has been shown to activate phosphatidylinositol-3-kinase (PI3K), TAK1, c-Src, JNK1, p44/42 ERK, p38 MAPK, Akt/PKB, and mTOR, and subsequently a series of transcription factors including NF- κ B, c-Fos, Fra-1, and NFATc1. This aspect of RANKL signaling has been recently reviewed elsewhere (16, 168–171, 206–208). In addition to the signaling pathways mentioned above, RANKL stimulation also triggers reactive oxygen species (ROS) production (209). ROS, like superoxide anions, hydroxyl radicals, and H₂O₂, have been associated with many cellular responses, including metabolic bone diseases found in aged osteoporotic women (210). Recent reports suggest that ROS act as a key second messenger during osteoclastogenesis (209), such that RANKL stimulation induces the production of ROS in osteoclast precursors via the small GTPase Rac1 and the ROS-inducing factor nicotinamide adenine dinucleotide phosphate oxidase 1. It is not clear how ROS cross-regulates the signaling pathways necessary for osteoclast differentiation, although one interesting hypothesis is that ROS may potentiate MAPK activation by inactivating protein tyrosine phosphatase activity in a manner similar to mechanisms recently described in B cells (211).

C. Costimulation in RANKL-induced osteoclast differentiation

The formation and activation of osteoclasts are processes that are tightly regulated by osteoblast lineage cells, which

provide at least two known essential factors for osteoclastogenesis, RANKL and M-CSF (Fig. 2). In addition, stromal cells produce various osteotropic factors that influence osteoclastogenesis. These factors can be divided into two groups: those that influence the activity of osteoblasts (*e.g.*, TNF α , which induces RANKL on osteoblasts), and those that affect the osteoclast precursors or osteoclasts *per se*. A series of experiments showed that M-CSF and RANKL together appear to be sufficient to induce the differentiation of bone marrow precursors, spleen cells, or blood monocytes to become mature osteoclasts *in vitro*. However, the expression of M-CSF, RANKL, and their receptors is not limited to bone cells. For example, M-CSF and RANKL are important cytokines for the activity/viability of macrophages and dendritic cells. Despite this pleiotropy, osteoclasts are not found in soft tissues, raising the question of why the same set of signaling receptors leads to different functional outcomes in different anatomical environments. One possibility is the existence of costimulatory molecule(s) present only in bone. Alternatively, there could be powerful inhibitors of osteoclastogenesis in soft tissues that are not found in bone.

To address this question, we proposed the hypothesis a few years ago that there exists a mechanism in preosteoclasts analogous to the costimulation requirement for T cell activation (212). Hence, our hypothesis proposed that osteoclast differentiation is controlled not only by two “essential factors,” M-CSF and RANKL (analogous to major histocompatibility complex/antigen complexes interacting with T-cell receptor (TCR)/CD4 or TCR/CD8), but also by other “non-essential but critical costimulatory molecules” (analogous to B7 family proteins interacting with CD28) (213). Because the *in vivo* concentrations of M-CSF and RANKL produced by osteoblasts in response to bone-resorbing hormones are likely to be much lower than that used in *in vitro* experiments, costimulatory molecules are likely to influence physiological differentiation of osteoclasts in a manner analogous to T cell activation, whereby signals from the costimulatory receptor CD28 amplify requisite signals from the TCR complex (212, 213). In addition, as with T cells, the requirement for a particular set of costimulatory factors/receptors for osteoclasts should vary depending on the microenvironment. Cells expressing ligands for costimulatory receptors expressed on osteoclasts also vary, but those interacting with osteoclasts themselves, such as osteoblasts, most often provide costimulation (analogous to dendritic cell providing B7 family proteins or TNF family proteins like 4–1BBL to T cells) (212). The signals resulting from the interaction of costimulatory factors and their receptors on osteoclast precursors determine the efficacy of the signals from the essential osteoclastogenic receptor, RANK (similar to TCR/CD4 or TCR/CD8 for T cells), and the sum of the two will determine the quality of osteoclast differentiation and activation.

In support of the costimulation hypothesis, we have identified a novel cell surface receptor, OSCAR, which is preferentially expressed on osteoclasts, and have shown that in addition to normal RANKL-RANK signaling, interaction of OSCAR with its putative ligand (OSCAR-L) is important for osteoblast-induced osteoclast differentiation (213). Moreover, it appears that OSCAR-L expression is most prevalent on osteoblastic cells (213). Therefore, the OSCAR receptor/

ligand pairing could be characterized as a putative costimulation receptor/factor for efficient osteoclast differentiation and may provide bone-specific costimulation required for the differentiation of osteoclasts in conjunction with the essential factors M-CSF and RANKL. This signaling combination may provide a mechanistic explanation of why osteoclasts are found only on bone surfaces *in vivo*.

Although the nature of bone-specific costimulatory molecules, such as OSCAR-L, requires further study, a series of recent experiments have supported our costimulation hypothesis (41, 214). For osteoclast development *in vivo*, it appears that some surface receptors on osteoclast precursors, such as PIR-A, OSCAR, TREM2, and SIRP β 1, associate with ITAM-containing molecules, DAP12 and FcR γ , and provide necessary costimulation and activation of Ca²⁺ signaling (41, 214). Hence, whereas a single deficiency for either DAP12 or FcR γ results in only minor osteoclast defects, double deficiency results in severe osteopetrosis (41, 214). Additional analysis of mutant mice suggests that these receptors activate calcineurin via Syk and phospholipase-C (PLC) γ (41, 214, 215). More signaling proteins have been identified in lymphocytes that bridge Syk (or ZAP-70) and PLC γ , and lead to Ca²⁺ activation (216, 217). Indeed, Gab2 and PLC γ 2 have recently been shown to be critical for generation of functional osteoclasts (218, 219). In addition, after the implication that Tec family kinases are likely to be involved in ITAM-mediated signaling (220), we have obtained data that Bruton's tyrosine kinase-deficient cells from X-linked immunodeficiency mice have defects in the multinucleation of preosteoclasts (Y. Choi, unpublished data). It will not be surprising if additional molecules (or family members) that were previously implicated in the ITAM-mediated signaling in immunocytes (e.g., lymphocytes or monocytes) are identified as playing an equivalent role in osteoclast differentiation.

However, it is important to point out that the osteopetrosis observed in the mice with defects in the costimulation pathway (e.g., DAP12/FcR γ double-deficient mice) is much less severe than that in RANKL or RANK knockout mice, and that, in contrast to RANKL or RANK knockout mice, these animals exhibit significant numbers of osteoclasts. This is consistent with the hypothesis that costimulatory receptors for osteoclast differentiation are not essential and that multiple redundancies probably exist (213).

Sustained Ca²⁺ mobilization is necessary for osteoclast differentiation because NFATc1 activation is absolutely required for the process (221). The NFAT family of transcription factors was originally identified as a set of regulators of gene transcription in activated T cells (222). Recently, it was found that RANK signaling induces expression of the NFAT family member NFATc1 (NFAT2) and that this factor is critical for osteoclast development because NFATc1-deficient precursor cells exhibit an absolute failure to differentiate into osteoclasts (221). Like other NFAT family members, the induction and activation of NFATc1 relies on the calcium-regulated phosphatase, calcineurin, thereby explaining negative effects of calcineurin inhibitors like FK506 and cyclosporine on osteoclastogenesis. The ability of NFATc1 to regulate its own expression points to the existence of an autonomic feedback loop. This likely triggers NFATc1 induction through a TRAF6 and c-fos-mediated mechanism

that is initiated by RANKL/RANK stimulation (223). Thus, Ca²⁺ signaling via costimulatory receptors on preosteoclasts is critical for amplification of NFATc1 activity to a level sufficient for osteoclast differentiation. Interestingly, NFATc1, in conjunction with microphthalmia-associated transcription factor (MITF) and PU.1, transactivates OSCAR expression during RANKL-induced osteoclast differentiation (Y. Choi, unpublished data). This suggests that there is a positive feedback circuit from RANKL to NFATc1 via costimulatory receptors such as OSCAR during osteoclast differentiation, which ensures a high level of NFATc1 activity. Recent data suggest that costimulatory receptors also activate another transcription factor, cAMP response element-binding protein, via CaMKIV, that cooperates with NFATc1 to activate osteoclast-specific genes (224).

Key to the analogy with lymphocyte costimulation, RANK, like TCR, is still the primary, requisite receptor, the absence of which renders the secondary receptors inconsequential for osteoclastogenesis. However, we still do not fully understand why this system evolved and whether there exists a state in osteoclast development that mimics anergy (induced tolerance) in lymphocytes.

D. Macrophage colony-stimulating factor

In addition to RANKL, M-CSF is important for normal osteoclast formation (Fig. 2). This cytokine was originally identified as a regulator of macrophage formation (225); however, it was subsequently shown that a spontaneous mouse mutant (*op/op*) with a phenotype of absent osteoclasts and defective macrophage/monocyte formation was deficient in M-CSF (226–228). Injection of M-CSF into *op/op* mice corrected the defect in osteoclast formation and bone resorption (229), as did expression of the protein specifically in osteoblastic cells (230).

Stimulators of bone resorption were shown to increase the production of M-CSF in bone (231–233), and multiple transcripts of M-CSF are produced by alternative splicing (234, 235). The membrane-bound form of M-CSF is regulated by stimulators of resorption and facilitates the differentiation of osteoclasts from precursor cells (232, 236). This may be significant because in marrow cultures soluble M-CSF inhibited OCL formation that was stimulated by 1,25-dihydroxyvitamin D₃ (237, 238).

The role of M-CSF in regulating osteoclast apoptosis has also been examined. Addition of M-CSF to mature osteoclast cultures prolongs their survival (239, 240). This response may be important for the development of the osteopetrotic phenotype in *op/op* mice because transgenic expression in myeloid cells of Bcl-2, which blocks apoptosis, partially reversed the defects in osteoclast and macrophage development in these animals (241). The effects of M-CSF on osteoclasts has been linked to activation of a Na/HCO₃ cotransporter (242). M-CSF also is a potent stimulator of RANK expression in osteoclast precursor cells (22), and it is critical for the expansion of the osteoclast precursor pool size (24).

E. Additional colony stimulating factors

Like M-CSF, GM-CSF and IL-3 affect osteoclast differentiation (238, 243, 244). Both appear to inhibit RANKL-medi-

ated osteoclastogenesis (245, 246). In contrast, these factors enhance the expansion of osteoclast precursor cells (247, 248). It is now known that these CSF drive a common myeloid precursor cell toward lineages other than osteoclasts (3, 245). Both also inhibit expression of TNF receptors on myeloid precursor cells (249). IL-3 also inhibits osteoblast differentiation, which may be one mechanism for how multiple myeloma influences bone because this malignancy can produce IL-3 (250).

G-CSF decreases bone mass in rodents when injected systemically (251, 252), and this response appeared to result from increased osteoclast formation and decreased osteoblast function. G-CSF also mobilizes hematopoietic precursor cells from bone marrow into the circulation (253) and increases the number of circulating osteoclast precursor cells (254), which is likely related to its ability to increase osteoclast resorptive activity. In mice, overexpression of G-CSF inhibited the ability of osteoblasts to respond to bone morphogenetic protein (255). In addition, mice overexpressing G-CSF had increased bone resorption, which was not increased with ovariectomy, as occurred in wild-type mice (256).

F. Interleukin-1

There are two separate IL-1 gene products, IL-1 α and IL-1 β , which have identical activities (257). IL-1 is a potent peptide stimulator of *in vitro* bone resorption (258), and it also has potent *in vivo* effects (259). Its effects on resorption appear to be both direct on osteoclasts (260) and indirect through its ability to stimulate RANKL production (261). In addition, both RANKL- and 1,25-dihydroxyvitamin D₃-stimulated osteoclast formation *in vitro* are mediated, in part, by effects on IL-1 (197, 262). IL-1 enhances the activity of RANKL to stimulate osteoclastogenesis (263) and also increases prostaglandin synthesis in bone (258, 264), which may account for some of its resorptive activity because prostaglandins are also potent resorption stimuli (265). Direct stimulation of osteoclastogenesis by IL-1 in mixed murine stromal and hematopoietic cell cultures is dependent on RANKL expression in the stromal/osteoblastic cells but not TNF (266).

IL-1 is produced in bone (267), and its activity is present in bone marrow serum (268, 269). A natural inhibitor of IL-1, IL-1 receptor antagonist, is an analog of IL-1 that binds but does not activate the biologically important type I IL-1 receptors (270–272).

There are two known receptors for IL-1: type I and type II (273). All known biological responses to IL-1 appear to be mediated exclusively through the type I receptor (274). IL-1 receptor type I requires interaction with a second protein, IL-1 receptor accessory protein, to generate postreceptor signals (275–277). Signaling through type I receptors involves activation of specific TRAFs and NF- κ B (278, 279). IL-1 receptor type II is a decoy receptor that prevents activation of type I receptors (280). One recent report found a decrease in the bone mass of mice that were deficient in the bioactive type I IL-1 receptor (281); however, this has not been our experience (282).

Expression of myeloid differentiation factor 88 (MyD88) but not Toll/IL-1 receptor domain-containing adapter in-

ducing IFN- β (TRIF) was necessary for IL-1 to stimulate RANKL production in osteoblasts and prolong the survival of osteoclasts (283). Survival of osteoclasts by treatment with IL-1 appears to require PI3K/AKT and ERK (284).

G. Tumor necrosis factor

Like IL-1, TNF represents a family of two related polypeptides (α and β) that are the products of separate genes (285–289). TNF α and TNF β have similar biological activities and are both potent stimulators of bone resorption (258, 290, 291).

In vivo administration of TNF α was shown to increase the serum calcium of mice (291) and to stimulate new osteoclast formation and bone resorption (292). Like IL-1, TNF also enhances the formation of OCL in bone marrow culture (291). The ability of TNF to stimulate osteoclast formation in mixed stromal cell/osteoclast precursor cell cultures was dependent on the production of IL-1 (293). In addition, TNF-induced osteolysis was found to be dependent on M-CSF production (294).

TNF was shown to directly stimulate osteoclast formation in an *in vitro* culture system by a mechanism that was independent of RANK because it occurred in cells from RANK-deficient mice (192, 193, 295). The significance of this *in vitro* finding is questionable, however, because *in vivo* administration of TNF to RANK-deficient mice caused only an occasional osteoclast to form (195).

Like IL-1, TNF binds to two cell surface receptors, TNF receptor 1 or p55 and TNF receptor 2 or p75 (296). In contrast to IL-1, however, both receptors transmit biological responses. Mice deficient in TNF receptor 1 and TNF receptor 2 have been produced (297–299). These animals appear healthy and are not reported to have an abnormal bone phenotype. TNF may also regulate c-fms expression in osteoclast precursor cells (34).

TNF also appears to regulate the abundance of osteoclast precursor cells in the bone marrow by increasing expression of c-fms, the receptor for M-CSF (300). It also enhances RANK signaling mechanisms, which activate osteoclasts and their precursor cells (196), and it enhances expression of the costimulatory molecule PIR-A leading to activation of NFATc1 (301).

H. Additional TNF superfamily members

Fas ligand (FasL), which binds its receptor Fas on responsive cells, regulates apoptosis and other cellular processes in multiple cell types (302). In osteoblasts, FasL inhibits differentiation through a caspase 8-mediated mechanism (303). In osteoclasts, addition of FasL to cultures of osteoclast precursor cells, which were also treated with M-CSF and RANKL, increased osteoclast formation. Osteoclast precursors and mature osteoclasts express Fas and FasL (304). Expression of Fas was up-regulated by RANKL treatment in the RAW 264.7 osteoclast precursor cell line and treatment of mature osteoclasts with Fas-induced apoptosis (305). However, in contrast to their similar effects on osteoclastogenesis in cultures of precursor cells, there appears to be counterregulatory roles of RANKL and FasL on mature osteoclast apoptosis because at high concentrations, RANKL inhibited the ability of FasL

to induce this response (306). The effect of FasL deficiency on bone mass is controversial. One group has found that this index is decreased in FasL-deficient mice (305), whereas another found it to be increased (307). However, the significance of studying bone mass in Fas- or FasL-deficient mice is probably minimal because these models have a generalized lymphoproliferative disorder, which activates a wide variety of immune responses affecting bone and makes interpreting the results of these studies difficult. Most recently, it was shown that estrogen receptor α in osteoclasts regulates FasL production by these cells, which, in turn, mediates bone loss induced by estrogen withdrawal in mice (308).

TRAIL is another TNF-superfamily member that has a wide variety of activities. Treatment of osteoclasts with TRAIL induced apoptosis (309), and this effect may be mediated through up-regulation of the death receptor DR5 (310). In bone, injection of TRAIL for 8 d in 4-wk-old mice induced an increase in bone mass. *In vitro* this effect was associated with an increase in the cyclin-dependent kinase inhibitor, p27^{Kip1}, through effects of TRAIL on the ubiquitin-proteasome pathway (311). TRAIL may also be a factor in the effects of myeloma on osteoblasts (312).

CD40 ligand (CD40L) is involved in the differentiation of naive T lymphocytes into T-helper (T_H) 1 effector cells (313). In humans, deficiency of CD40L causes X-linked hyper IgM (XHIM) syndrome. Bones of XHIM patients develop spontaneous fractures and are osteopenic (314). Activated T lymphocytes from XHIM patients have normal RANKL and deficient IFN- γ production, which may contribute to decreased bone mass in these patients (314). In addition, expression of CD40L in rheumatoid arthritis synovial cells induced RANKL expression in these cells and enhanced their ability to stimulate osteoclastogenesis, which suggests that this mechanism is involved in the effects of rheumatoid arthritis on bone (315).

1. Interleukin-6

IL-6, like IL-1 and TNF, has a wide variety of activities related to immune cell function and to the replication and differentiation of a number of cell types (316, 317). Osteoblastic cells (both rodent and human) produce IL-6 and IL-6 receptors (318, 319). Another source of IL-6 in the bone microenvironment is BMSC, which can produce IL-6 after they are stimulated with IL-1 and TNF (320). The receptor for IL-6 is composed of two parts: a specific IL-6 binding protein (IL-6 receptor), which can be either membrane-bound or soluble, and gp130, an activator protein that is common to a number of cytokine receptors (321). Soluble IL-6 receptor binds IL-6, and this complex can then activate cells that contain the gp130 signal peptide (321, 322). The shedding of IL-6 receptor from osteoblasts is stimulated by IL-1 and TNF α (323).

The ability of IL-6 to stimulate bone resorption *in vitro* is variable and depends on the assay system that is used (319, 324–326). It appears that a major effect of IL-6 is to regulate osteoclast progenitor cell differentiation into mature osteoclasts (327, 328). IL-6 also directly stimulates both RANKL and OPG mRNA production in bone (329), and it enhances production of prostaglandins (330). There is also one publication that suggests that IL-6 can stimulate osteoclastogen-

esis *in vitro* by a RANKL-independent mechanism (331). IL-6 appears to mediate some of the increased resorption and bone pathology that is seen in the clinical syndromes of Paget's disease (185), hypercalcemia of malignancy (332), fibrous dysplasia (333), giant cell tumors of bone (334), and Gorham-Stout disease (335). There have been conflicting data on the role of IL-6 in PTH-mediated responses in bone because some investigators have found it critical (336) whereas others have not (337).

J. Additional interleukin-6 family members

IL-6 is a member of a group of cytokines that share the gp130 activator protein in their receptor complex (338, 339). Each family member utilizes unique ligand receptors to generate specific binding. Signal transduction through these receptors utilizes the JAK/STAT (Janus kinase/signal transduction and activators of transcription) pathway (321).

1. Interleukin-11

IL-11 is produced by bone cells in response to a variety of resorptive stimuli (340). It stimulates osteoclast formation in murine bone marrow cultures (341) and bone resorption in a variety of *in vitro* assays (342, 343). Interestingly, it has no effect on isolated mature osteoclasts. In mice deficient in the specific IL-11 receptor, trabecular bone mass is increased, and this appears to result from decreased bone turnover, which is associated with decreased *in vitro* osteoclast formation and resorption (344).

2. Leukemia inhibitory factor

LIF is produced by bone cells in response to a number of resorption stimuli (86, 345, 346). The effects of LIF on bone resorption are variable. In a number of *in vitro* model systems, LIF stimulated resorption by a prostaglandin-dependent mechanism (347), whereas in other *in vitro* assays, it had inhibitory effects (348, 349). In neonatal murine calvaria cultures, LIF stimulated both RANKL and OPG (329).

Local injections of LIF *in vivo* were shown to increase both resorption and formation parameters, as well as the thickness of the treated bones (350). In mice that lacked the specific LIF receptor and hence, could not respond to LIF, bone volume was reduced, and osteoclast number was increased 6-fold (351).

3. Oncostatin M

Oncostatin M was demonstrated to stimulate multinuclear cell formation in murine and human bone marrow cultures (322, 352). These cells appeared to be macrophage polykaryons, however, and not osteoclasts (352). In contrast, oncostatin M inhibited OCL formation that was stimulated by 1,25-dihydroxyvitamin D₃ in human marrow cultures (352), and it decreased bone resorption rates in fetal mouse long bone cultures (353). *In vivo*, overexpression of oncostatin M in transgenic mice induced a phenotype of osteopetrosis (354). Hence, it appears that oncostatin M is an inhibitor of osteoclast formation and bone resorption (355).

The role of all IL-6 family members in osteoclast formation has to be examined in the light of data demonstrating that mice lacking the gp130 activator protein have an increased number of osteoclasts in their bones compared with normal

animals (356). Because gp130 is an activator of signal transduction for all members of the IL-6 family, this result argues that at least some IL-6 family members have a predominantly inhibitory effect on osteoclast formation and bone resorption. Available data implicate oncostatin M (353) and possibly LIF (348, 349) in this role.

K. Interleukin-7

IL-7 is a cytokine that has diverse effects on the hematopoietic and immunological systems (357) and is best known for its nonredundant role in supporting B and T lymphopoiesis. Studies have demonstrated that IL-7 also plays an important role in the regulation of bone homeostasis (358, 359). 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 up-regulated osteoclast formation in human peripheral blood cells by increasing osteoclastogenic cytokine production in T cells (360). Significantly, IL-7 did not induce bone resorption and bone loss in T cell-deficient nude mice *in vivo* (361). In addition, treatment of mice with a neutralizing anti-IL-7 antibody inhibited ovariectomy-induced proliferation of early T cell precursors in the thymus, demonstrating that ovariectomy up-regulates T cell development through IL-7. This latter effect may be a mechanism by which IL-7 regulates ovariectomy-induced bone loss (362). 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 (360, 361).

In contrast with previously reported studies (358, 360, 361), we found differential effects of IL-7 on osteoclastogenesis (363). IL-7 inhibited osteoclast formation in murine bone marrow cells that were cultured for 5 d with M-CSF and RANKL (363). In IL-7-deficient mice, osteoclast number was markedly increased and trabecular bone mass was decreased compared with wild-type controls (364). In addition, we found that trabecular bone loss after ovariectomy was similar in wild-type and IL-7-deficient mice (364). Curiously, IL-7 mRNA levels in bone increase with ovariectomy, and this effect may be linked to alterations in osteoblast function with estrogen withdrawal (359, 365). Addition of IL-7 to the medium of newborn murine calvaria cultures inhibited bone formation, as did injection of IL-7 above the calvaria of mice *in vivo* (359). When IL-7 was overexpressed locally by osteoblasts, trabecular bone mass was increased compared with wild-type mice (366). Furthermore, targeted overexpression of IL-7 in IL-7-deficient mice rescued the osteoporotic bone phenotype of the IL-7-deficient mice (367). These studies indicated that the actions of IL-7 on bone cells are dependent on whether IL-7 is delivered systemically or locally.

L. Interleukin-8 and other chemokines

Recruitment and homing of myeloid cells often occur under the direction of chemokines and their receptors. This superfamily of relatively small proteins induce interactions

through cognate G protein-coupled receptors to initiate cytoskeletal rearrangement, adhesion, and directional migration (368, 369). Chemokines can be divided into four branches, depending on the spacing and sequence motifs of their first cysteine (C) residues. These are CXC, CC, C, and CX₃C, where X is any other amino acid (370, 371). The majority of chemokine receptor interactions occur through the CC and CXC chemokines, which are referred to as major, whereas C and CX₃C chemokines are referred to as minor.

Many cells produce chemokines that bind specific G protein-coupled receptors. IL-8, a CXC chemokine, is produced by osteoclasts (372) and stimulates osteoclastogenesis and bone resorption by a mechanism that is reported to be independent of the RANKL pathway (373–375). IL-8 may also be produced by certain cancers and stimulate lytic bone lesions in metastatic disease (373–375). Effects of IL-8 on bone may be in part mediated by up-regulation of nitric oxide synthase expression in osteoclasts (376).

CCL3 (macrophage inflammatory protein-1 α) is a direct stimulator of osteoclastogenesis that is expressed in bone and bone marrow cells (377–380). This response is proposed to be independent of RANK activation (381). CCL3 is also a mediator of the osteolytic activity of multiple myeloma (382–384). Activation of osteoclastogenesis by CCL3 is mediated by the receptors CCR1 and CCR5 (385). Interestingly, CCL3 and IL-8 stimulate motility but suppress resorption in mature osteoclasts (386).

CCL9 (macrophage inflammatory peptide γ) and its receptor, CCR1, are also an important chemokine ligand receptor interaction that regulates osteoclasts (387). Injection of M-CSF to induce osteoclastogenesis and bone resorption in osteopetrotic *tl/tl* rats, which lack M-CSF, caused a rapid (within 2 d) up-regulation of CCR1 as well as its ligand CCL9 in the bones of *tl/tl* mice and a rapid increase in osteoclastogenesis (388). Furthermore, antibodies to CCL9 ameliorated the ability of M-CSF injections to stimulate osteoclastogenesis in this model.

RANKL appears to be a major inducer of CCL9 and CCR1 in osteoclasts (389), and induction of CCR1 by RANKL is dependent on NFATc1 expression (390). CCL9 and other chemokines that bind CCR1 (CCL3, CCL5, and CCL7) are produced by osteoclasts, osteoblasts, and their precursors in bone. In addition, expression of these chemokines in differentiating osteoblasts is induced by proinflammatory cytokines (IL-1 and TNF) (391). Additional chemokine receptors that are produced on osteoclasts include CCR3, CCR5, and CX₃CR1 (385, 387).

Inhibition of CCR1 expression with small interfering RNA or by blocking NFATc1 activation with cyclosporin A inhibited migration of RAW 264.7 cells (a model for osteoclast precursors) and murine bone marrow cells in Boyden chambers (390). Furthermore, inhibition of CCR1 signaling with a mutated form of CCL5, which blocks the binding of CCR1 to its ligands, prevented OCL formation in murine bone marrow cultures (390). In addition, antibody neutralization of CCL9 inhibited RANKL-induced osteoclastogenesis by 60–70% in murine bone marrow cultures (389).

CXCL12 (stromal cell derived factor-1) and its receptor CXCR4 are involved in a variety of cellular processes including hematopoietic cell homeostasis and immune re-

sponses (392). Osteoclast precursor cells express CXCR4 (393), and expression of this receptor is down-regulated by differentiation of these cells toward the osteoclast lineage (394, 395). Treatment of the cell line RAW 264.7 with CXCL12 induced expression of MMP9, which may be a mechanism for the migration of precursor cells toward bone (393). In human osteoclast precursor cells, CXCL12 stimulated migration and enhanced osteoclastogenesis in response to RANKL and M-CSF (393, 394). Expression of CXCL12 is up-regulated in osteoclasts when they differentiate on a calcium phosphate matrix (394). Production of CXCL12 may also be involved in the recruitment of precursor cells, which form giant cell tumors of bone (396), and in the increased osteolysis that is seen in multiple myeloma (397).

CCL2 (monocyte chemoattractant protein-1) is a potent chemokine for monocytes and a variety of other immune cells. Its receptor is CCR2, which is expressed at high levels on monocytes (398). In bone with an induced inflammatory lesion, CCL2 was expressed at high levels in osteoblasts (399). Induction of CCL2 in these lesions was mediated by proinflammatory cytokines (400). CCL2 also may be involved in tooth eruption because it is expressed by dental follicle cells (401–403). Among the factors that stimulate CCL2 in the dental follicle are PTHrP (404), platelet-derived growth factor-BB, and fibroblast growth factor-2 (405). However, CCL2 is not critical for tooth eruption because there were only minor changes in the temporal pattern of this process in CCL2-deficient mice (406). CCL2 is induced by RANKL in mononuclear precursor cells (407) and enhances OCL formation in these cells (408). However, cells induced by treatment of cultures with CCL2 alone, while multinucleated and calcitonin receptor positive, did not resorb bone unless they were also exposed to RANKL (408). Most recently, it was shown that treatment of osteoblasts with PTH increased CCL2 expression and enhanced the fusion of preosteoclasts (409).

M. Interleukin-10

IL-10 is produced by activated T and B lymphocytes (410). It is a direct inhibitor of osteoclastogenesis (411, 412) and osteoblastogenesis (413). This effect is associated with increased tyrosine phosphorylation of a variety of proteins in osteoclast precursor cells (414). The direct effects of IL-10 on RANKL-stimulated osteoclastogenesis are associated with decreases in NFATc1 expression and reduced translocation of this transcription factor into the nucleus (415) as well as suppressed c-Fos and c-Jun expression (416). Administration of IL-10 may have utility as a mechanism to control wear-induced osteolysis (417). In the dental follicle cells, which function to regulate tooth eruption, treatment *in vitro* with IL-10 inhibited RANKL production and enhanced OPG (418). Hence, there appears to also be an indirect effect of IL-10 on osteoclastogenesis that is mediated by its ability to regulate RANKL and OPG production.

Treatment of bone marrow cell cultures with IL-10 suppressed the production of osteoblastic proteins and prevented the onset of mineralization (413). IL-10 also inhibited the formation of OCL in bone marrow cultures without affecting macrophage formation or the resorptive activity of

mature osteoclasts (419). This effect appears to involve the production of novel phosphotyrosine proteins in osteoclast precursor cells (414). IL-10 also stimulates a novel inducible nitric oxide synthase (376).

4-1BB is an inducible T cell costimulatory molecule that interacts with 4-1BB ligand. Treatment of RANKL-stimulated osteoclast precursor cells *in vitro* with 4-1BB ligand enhanced IL-10 production. In addition, expression of IL-10 was greater in RANKL-stimulated wild-type osteoclast precursor cell cultures than in cultured cells from 4-1BB-deficient mice (420). These results imply that some effects of IL-10 on osteoclasts may be mediated through interactions of 4-1BB with 4-1BB ligand.

N. Interleukin-12

IL-12 is a cytokine that is produced by myeloid and other cell types. It induces T_H1 differentiation in T lymphocytes and the subsequent expression of IFN- γ (421). IL-12 has an inhibitory effect on osteoclastogenesis. However, the mechanism by which this effect occurs *in vitro* is controversial. Some authors have demonstrated direct inhibitory effects of IL-12 on RANKL-stimulated osteoclastogenesis in purified primary osteoclast precursors and RAW 264.7 cells (422). This effect was associated with inhibition of the expression of NFATc1 in the osteoclast precursor cells. Interestingly, the inhibitory effects of IL-12 on osteoclastogenesis were absent in cells that were pretreated with RANKL (422). In contrast, others have found that the inhibitory effects of IL-12 on osteoclastogenesis are indirect. One group demonstrated that the inhibitory effects of IL-12 are mediated by T lymphocytes and do not involve production of IFN- γ (423). A second group disputes this result and found inhibition of osteoclastogenesis by IL-12 in cells from T lymphocyte depleted cultures and cells from T lymphocyte-deficient nude mice (424). The latter authors also demonstrated that antibodies to IFN- γ blocked some of the inhibitory effect of IL-12 on RANKL-stimulated osteoclast formation.

O. Interleukin-15

IL-15, like IL-7, is a member of the IL-2 superfamily and shares many activities with IL-2 including the ability to stimulate lymphocytes. It has been shown to enhance osteoclast progenitor cell number in culture (425). Production of IL-15 by T lymphocytes has been linked to the increased osteoclastogenesis and bone destruction seen in the bone lesions of rheumatoid arthritis (426).

P. Interleukin-17 and interleukin-23

IL-17 is a family of related cytokines that are unique and contain at least six members (A–F) (427). IL-17E is also called IL-25 (428). These cytokines are central for the development of the adaptive immune response and the products of a subset of CD4 T lymphocytes with a unique cytokine expression profile, termed T_H17. This is in contrast to the more established T lymphocyte cytokine-expressing subsets T_H1 and T_H2.

IL-17A was initially identified as a stimulator of osteoclastogenesis in mixed cultures of mouse hematopoietic cells and

osteoblasts (429). It stimulated osteoclastogenesis by inducing prostaglandin synthesis and RANKL. Production of IL-17A in rheumatoid arthritis appears involved in the production of activated osteoclasts and bone destruction in involved joints (429–431). Effects of IL-17 on osteoclastogenesis and bone resorption are enhanced by TNF α , which is also produced in the inflamed joints of patients with rheumatoid arthritis (432). Inhibition of IL-17A in an antigen-induced arthritis model inhibited the joint and bone destruction that is typically seen and decreased production of RANKL, IL-1 β , and TNF α in the involved lesions (433).

IL-23 is an IL-12-related cytokine composed of one subunit of p40, which it shares with IL-12, and one subunit of p19, which is unique (434). It is critical for the differentiation of the T_H17 subset of T lymphocytes along with TGF β and IL-6 (435). IL-23 appears most important for expanding the population of T_H17 T lymphocytes. This is a subset of T lymphocytes that produce RANKL and have a high osteoclastogenic potential that is mediated by their production of IL-17 (436). Using a LPS-induced model of inflammatory bone destruction, Sato *et al.* (436) found markedly decreased loss of bone in mice that were deficient in either IL-17 or IL-23. Hence, production of both IL-23 and IL-17 is involved in the bone loss in this model. These authors also demonstrated IL-23 mRNA expression in the synovial tissue of involved joints from patients with rheumatoid arthritis, which suggests that similar mechanisms are involved in the bone loss that occurs in this condition in humans.

Q. Interleukin-18

IL-18 is similar to IL-1 in its structure and is a member of the IL-1 superfamily (437). IL-18 synergizes with IL-12 to induce IFN- γ production (438), and its levels are increased at sites of inflammation such as rheumatoid arthritis (439). It is expressed by osteoblastic cells and inhibits osteoclast formation through a variety of mechanisms. These include its ability to stimulate GM-CSF (100), which is produced by T cells in response to IL-18 treatment (440). It also stimulates IFN- γ production *in vivo* in bone (441), and its inhibitory effects on osteoclastogenesis and bone resorption are enhanced by cotreatment with IL-12 (442). Finally, it has been shown to increase production of OPG (443). In IL-18 overexpressing transgenic mice, osteoclasts were decreased; although, curiously, so was bone mass. These results indicate that there also may be effects of IL-18 on bone growth (441). Interestingly, IL-18 has been shown to indirectly stimulate osteoclastogenesis through its effects on T lymphocytes (444). IL-18 is also a mitogen for osteoblastic cells *in vitro* (445).

R. Interferons

IFN- γ is a type II IFN with a wide variety of biological activities. *In vitro*, IFN- γ has inhibitory actions on bone resorption (446, 447). These appear to be direct and are mediated by its effects on osteoclast progenitor cells. IFN- γ inhibits the ability of 1,25-dihydroxyvitamin D₃, PTH, and IL-1 to stimulate the formation of OCL in cultures of human bone marrow (448). IFN- γ also inhibits RANK signaling by accel-

erating the degradation of TRAF6 through activation of the ubiquitin/proteasome system (449); however, it does not directly inhibit resorption in mature osteoclasts (450). IFN- γ is also reported to have stimulatory effects on resorption through its ability to stimulate RANKL and TNF α production in T lymphocytes (451). It is an inhibitor of osteoblast proliferation (445, 452, 453) and has variable effects on osteoblast differentiation (452, 454, 455).

The effects of IFN- γ on bone *in vivo* are variable because both inhibitory and stimulatory effects have been reported. In mice with collagen-induced arthritis, loss of the IFN- γ receptor leads to increased bone destruction (456, 457). Similarly, in mice that are injected with bacterial endotoxin over their calvaria, loss of IFN- γ receptor resulted in an enhanced resorptive response (449).

In contrast in rats, ip injection of IFN- γ for 8 d induced osteopenia (458). In patients who have osteopetrosis, because they produce defective osteoclasts, administration of IFN- γ stimulated bone resorption and appeared to partially reverse the disease. The latter effects are possibly due to the ability of IFN- γ to stimulate osteoclast superoxide synthesis (459, 460), osteoclast formation *in vivo* (461), or a generalized immune response (462).

Type I IFNs (IFN- α and IFN- β) are typically produced in response to invading pathogens (463). Mice deficient in the IFN- α/β receptor component IFNAR1 have a reduction in trabecular bone mass and an increase in osteoclasts (464). RANKL induces IFN- β in osteoclasts, and IFN- β , in turn, inhibits RANKL-mediated osteoclastogenesis by decreasing c-fos expression (464). IFN- α has also been shown to inhibit bone resorption *in vitro* although its mechanism of action is not as well studied as that of IFN- γ and - β (465). *In vivo*, IFN- α had no effect on bone turnover (466).

S. Additional cytokines

IL-4 and IL-13 are members of a group of locally acting factors that have been termed “inhibitory cytokines.” The effects of IL-4 and IL-13 seem related and appear to affect both osteoblasts and osteoclasts. Transgenic mice that overexpress IL-4 had a phenotype of osteoporosis (467). This effect may result from both an inhibition of osteoclast formation and activity (468, 469) and an inhibition of bone formation (470). IL-13 and IL-4 inhibited IL-1-stimulated bone resorption by decreasing the production of prostaglandins and the activity of cyclooxygenase-2 (471). IL-4 and IL-13 have also been demonstrated to induce cell migration (chemotaxis) in osteoblastic cells (472). IL-4 and IL-13 influence the ability of osteoblasts to regulate osteoclast formation and activity through their ability to increase OPG and inhibit RANKL production (473, 474). The direct inhibitory actions of IL-4 on osteoclast precursor cell maturation into osteoclasts are stronger than that of IL-13 and involve effects on STAT6, NF- κ B, peroxisome proliferator-activated receptor γ 1, MAPK signaling, Ca²⁺ signaling, NFATc1, and c-Fos (474–479).

Macrophage migration inhibitory factor (MIF) was initially identified as an activity in conditioned medium from activated T lymphocytes that inhibited macrophage migration in capillary tube assays (480). Once purified and cloned

(481), it became available for functional studies and was shown to have a variety of activities. In addition to T lymphocytes, it is produced by pituitary cells and activated macrophages. Mice that overexpress MIF globally were found to have high turnover osteoporosis (482). In contrast, MIF-deficient mice failed to lose bone mass or increase osteoblast or osteoclast number in bone with ovariectomy (483). Hence, MIF appears to be another mediator of the effects of estrogen withdrawal on bone. Estrogen down-regulates MIF expression in activated macrophages (484), and a similar response may occur in bone or bone marrow to mediate some of the effects of ovariectomy on bone mass. MIF is made by osteoblasts (485), and its production by these cells was up-regulated by a variety of growth factors including TGF β , FGF-2, IGF-II, and fetal calf serum (486). *In vitro*, MIF increased MMP9 and MMP13 expression in osteoblasts (487) and inhibited RANKL-stimulated osteoclastogenesis (488).

VIII. Regulation of Osteoblasts by Immune Cells and Cytokines

A variety of cytokines are known to regulate osteoblastic cells. TNF α inhibits the differentiation of osteoblasts (489–492). IL-1, TNF α , and IFN- γ inhibits collagen synthesis in osteoblasts (452, 455, 493–495). IL-4 and IL-13 suppress prostaglandin synthesis in bone and are reported to be chemoattractants for osteoblasts (471, 472). IL-4 has been shown to act as a direct stimulator of proliferation and inhibitor of differentiation in an osteoblastic cell line (496). Similarly, IL-4-overexpressing mice exhibited a decrease in bone formation and decreased differentiated osteoblasts on their bone surface (467). The role of cytokines in osteoblast apoptosis has also been studied. TNF α is potentially proapoptotic for osteoblasts (497), possibly through induction of the Fas-FasL system (498). Activated T lymphocytes also produce products that drive differentiation of human BMSC toward an osteoblastic phenotype (499). B7-H3 is an Ig superfamily member that is expressed on the surface of antigen-presenting cells. Recently, B7-H3 was found to be expressed on developing osteoblasts, and its expression was increased during cell maturation (500). Furthermore, B7-H3-deficient mice had decreased cortical bone mineral density compared with littermate controls (500).

IX. Role of Osteoclasts in Regulating Osteoblasts

It is generally believed that, in addition to their *bona fide* function as mediators of bone resorption, osteoclasts can influence osteoblast differentiation and function through a process termed “coupling” (501–504). It has been postulated that, during remodeling of adult bone, bone formation occurs via the coupling process in response to bone resorption. Failure of such coupling was suggested to cause unbalanced bone remodeling, resulting in osteopetrosis or osteoporosis (502, 503). In support of the coupling hypothesis, a variety of osteopetrotic mouse models with defective osteoclast formation or function demonstrate decreased bone formation. For example, c-Fos and RANKL-deficient mice, which lack osteoclasts, also have reduced bone formation although these

mice have no known osteoblast-intrinsic defects (175, 213). In addition to these genetically altered mice, clinical trials also support the concept of coupling. Humans who are treated with bisphosphonates to inhibit bone resorption in combination with daily PTH injections, which increase bone mass, have a diminished anabolic response to PTH compared with patients given daily PTH without bisphosphonates (505). One interpretation of these data is that anabolic regimens of PTH require osteoclasts (and the coupling factors produced by them) to increase bone formation. Not all osteopetrotic mice demonstrate altered bone formation. For example, blockade of chloride channel-7 prevents bone resorption in ovariectomized rats, whereas bone formation in this rodent model is unaltered (506). In addition, mice lacking c-Src also show osteopetrosis with increased bone formation (507, 508). However, interpretation of this model is more complicated because c-Src also has a role in osteoblasts and its absence causes increased osteoblast differentiation *in vitro* (509).

Many of the correlative studies supporting the notion of coupling have previously been extensively reviewed (503, 504). However, we know of no direct evidence supporting the hypothesis that osteoclasts *per se* trigger enhanced bone formation during remodeling. Thus, we would like to discuss several outstanding issues that need to be clarified to validate our understand of coupling. For example, it is critical to clarify genetically whether coupling does indeed require bone resorption by mature osteoclasts and, hence, the release of byproducts from bone matrix (502, 503). Although it cannot be mutually excluded, it is possible that coupling simply requires the presence of mature osteoclasts because their encoded gene products are sufficient to carry out the coupling mission.

It is unknown whether coupling requires contact between osteoclasts and osteoblasts. Many soluble factors (e.g., IGF-I) have been implicated in the coupling process (502, 503), but these hypotheses still require genetic confirmation. As mentioned above, a membrane-associated factor (EphrinB2), which is produced by osteoclast-lineage cells independent of their resorptive action, has been suggested to influence bone formation by osteoblasts (105). However, the question remains whether EphrinB2-expressing mature osteoclast-lineage cells are the cells mediating the coupling of bone formation and resorption because the recruitment of bone-forming cells occurs after resorption is completed. Along this line, it is necessary to first define, molecularly, the differentiation status of the tentatively identified osteoclasts and osteoblasts in the coupling process. For example, one may have to define what the differentiation state of osteoblast lineage cells that potentially interact with osteoclasts is. Are they bone matrix-producing cells or RANKL-expressing cells? Conversely, it needs to be determined whether the osteoclasts that are involved in the coupling process are bone-resorbing multinucleated cells or osteoclast lineage cells that are committed but not necessarily fully differentiated. Our recent data show that an increased number of gene-mutated mononuclear osteoclasts can induce increased bone formation *in vivo* (510). This result suggests that even mononuclear osteoclast lineage cells can potentially interact with osteoblast lineage cells to regulate coupling.

In summary, the coupling hypothesis needs further veri-

fication because it is of great importance both physiologically and clinically to identify the factor(s) involved in this phenomenon and, more importantly, to show whether osteoclasts do indeed influence bone formation during bone remodeling.

X. Role of the Immune System in Bone Disease: the Birth of Osteoimmunology

Production of proinflammatory cytokines by immune cells and the subsequent induction of RANKL-mediated osteoclast formation and bone resorption have been linked to human diseases. Perhaps the most extensive studies have been on the role of cytokines in the development of the osteolytic lesions observed in rheumatoid arthritis and other inflammatory joint diseases (Fig. 3).

RANKL expression on T lymphocytes is induced upon T cell receptor engagement and depends on Ca^{+2} mobilization (208, 511). Initial experiments demonstrated that activated T lymphocytes, or even supernatants from activated T lymphocyte cultures, were capable of supporting osteoclastogenesis *in vitro* (512). It was subsequently observed that mice lacking CTLA4 (*ctla4*^{-/-}), in which T lymphocytes are systemically activated, exhibit an osteoporotic phenotype associated with increased osteoclast numbers. Transfer of *ctla4*^{-/-} T lymphocytes into *rag2*^{-/-} mice, which lack lymphocytes, leads to decreased bone density over time, which can be prevented by OPG treatment. This finding indicated that activated T cells can disrupt bone homeostasis by modulating RANKL expression (512), although it is not clear whether T cell-derived RANKL *per se* is responsible for the aberrant bone metabolism in this model. In a complementary study, transgenic overexpression of RANKL restricted to T or T/B lymphocytes was sufficient to partially correct the osteopetrotic phenotype observed in RANKL-deficient mice (213) (Y. Choi, unpublished data). Together these data definitively showed the ability of lymphocytes to regulate bone homeostasis *in vivo* through expression of RANKL and confirmed a *bona fide* interplay between the adaptive immune system and bone metabolism.

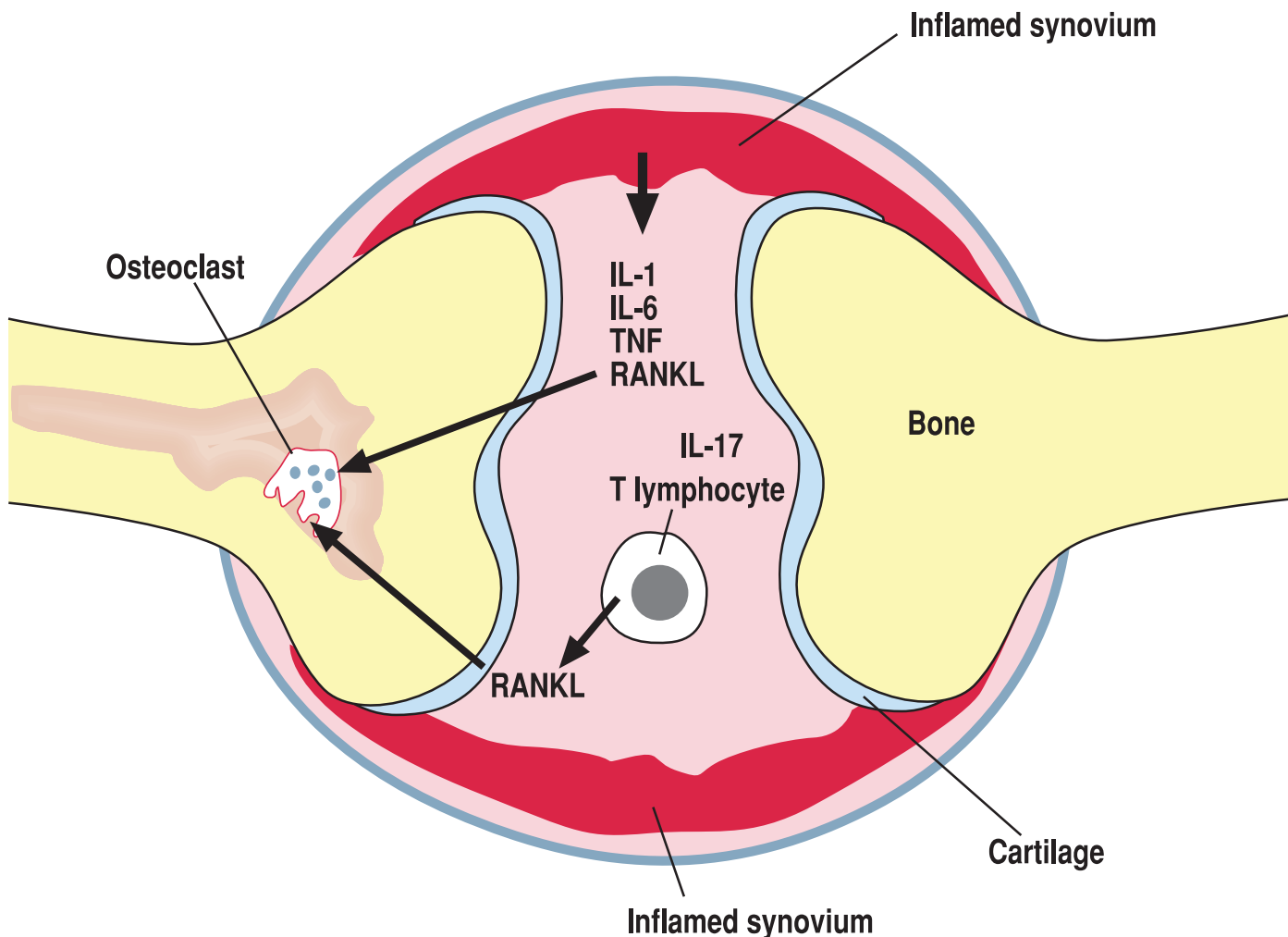


FIG. 3. Regulation of osteoclastogenesis in inflammation. In inflammatory states such as inflammatory arthritis, local production of proinflammatory cytokines (IL-1, IL-6, and TNF) as well as RANKL by inflamed tissues such as the synovium leads to stimulation of osteoclastogenesis and bone destruction. In addition, IL-17-producing $\text{T}_{\text{H}}17$ T lymphocytes stimulate local production of RANKL by inflamed tissues and produce RANKL themselves, which enhances resorptive destruction of bone at sites adjacent to the inflammation.

In human arthritis, inflammation of the synovial joints is accompanied by bone and cartilage destruction. Various animal models have been established for the study of arthritis, and the role of RANKL in their pathogenesis has been investigated. Treating adjuvant-induced arthritis in Lewis rats with OPG had no discernible effect on inflammation but prevented bone loss and cartilage destruction (512). These experiments could not resolve whether preservation of cartilage was an indirect benefit of inhibiting bone erosion or was due to independent mechanisms. A subsequent study demonstrated that bone loss and cartilage destruction were independent in an arthritis model induced by transfer of serum from K/BxN transgenic mice, where T cell activity is not required for the onset of disease (513). When K/BxN serum was transferred into RANKL-deficient mice, inflammation and cartilage destruction were comparable to control recipients, but bone erosion was greatly reduced (513). These findings reinforced the notion that RANKL *per se* mediates induction of bone destruction by osteoclasts in animal models of autoimmune arthritis. Examination of the cellular constituents of synovial fluid, collected from human arthritis patients, revealed that all local T lymphocytes expressed RANKL, establishing the clinical relevance of the connection between arthritis and immunologically derived RANKL (512). Recently, it has been demonstrated that RANKL, in combination with M-CSF, can induce transdifferentiation of immature dendritic cells to the osteoclast lineage and that this process is significantly enhanced by rheumatoid arthritis synovial fluid, potentially identifying another mechanism for disease-related bone destruction (27).

Periodontitis, induced by infection with various subgingival bacteria, is a major cause of tooth loss and is associated with increased risk for heart failure and stroke (514, 515). To examine the etiology of the disease, peripheral blood leukocytes from patients with localized juvenile periodontitis were transferred into *rag2*^{-/-} mice, which were then orally inoculated with the Gram-negative bacterium *Actinobacillus actinomycetemcomitans* (515). Localized juvenile periodontitis was recapitulated in the recipient animals and was accompanied by accumulation of osteoclasts at the alveolar sockets (515). It was demonstrated that treatment with OPG inhibited the osteoclast infiltration and bone damage (515). *In vitro* stimulation of peripheral blood lymphocytes showed that RANKL was induced on CD4⁺ T lymphocytes that were activated with *A. actinomycetemcomitans* antigens and that disease was attenuated when the same cells were specifically depleted from recipient mice (515). This study demonstrated the importance of CD4⁺ T lymphocytes in the pathogenesis of periodontitis, specifically with regard to disease-related bone destruction.

Bone loss has long been recognized as an extraintestinal complication of inflammatory bowel disorders, like Crohn's disease and ulcerative colitis (516). One recent study found that patients with these diseases have elevated levels of serum OPG, which derive from the site of inflammation, and inversely correlate with severity of bone loss (517), whereas another study found that Crohn's disease patients have elevated levels of both OPG and soluble RANKL (518). Mechanistic insight into this link is provided by a study demonstrating that OPG treatment of mice suffering from IL-2

deficiency-induced ulcerative colitis results not only in reduced osteopenia, but also mitigation of colitis due to reduced colonic dendritic cell survival (519).

In addition to arthritis, periodontal disease, and inflammatory bowel disorders, pathological bone loss is observed in patients suffering from other autoimmune diseases (diabetes mellitus and lupus erythematosus), chronic viral infections (HIV), allergic diseases (asthma), and metastatic breast and lung cancers (520–522). The contribution to pathogenesis by osteoimmunological factors merits further investigation and may provide viable therapeutic options for alleviating painful sequella associated with a variety of conditions.

Although autoimmunity is, in some cases, associated with bone loss, all T cell responses do not necessarily have such a deleterious outcome. T Cells also secrete cytokines, like IFN- γ , IL-4, and TGF β , that have been shown to inhibit the proosteoclastogenic effects of RANKL (520, 521, 523). In particular, the role of the T_H1 cytokine, IFN- γ , appears to be crucial in preventing T lymphocyte-mediated osteoclastogenesis (449). TGF β is characterized as both an osteotropic and immunosuppressive cytokine. Although the largest repository of latent TGF β is in bone, its role in osteoclast formation is complex and insufficiently understood (168). TGF β down-modulates RANKL expression in osteoblasts, thereby negatively impacting their ability to mediate osteoclastogenesis in culture (524). However, TGF β has also been shown to potentiate RANKL expression in activated T lymphocytes (208) and enhance osteoclastogenesis in cultures supplemented with soluble RANKL (524). Additional studies will be necessary to determine whether TGF β utilizes multiple regulatory mechanisms, and if so, what disparate purposes they might serve. If most of the cytokines produced by activated T cells are antiosteoclastogenic, the question becomes how T cells induce bone loss by activating osteoclasts in, for example, inflammatory joints. The answer is recently provided by the discovery of T_H17 cells (Fig. 3), which produce IL-17 and have been shown to be critical mediators of many inflammatory autoimmune diseases such as multiple sclerosis or rheumatoid arthritis (525). T_H17 cells do not produce large amounts of IFN- γ or IL-4 that are antiosteoclastogenic, rather they produce IL-17 (525). Recent data show that IL-17-producing T_H17 cells can induce monocytes to become osteoclasts via RANKL. In addition, IL-17 can further increase the level of RANKL in stromal cells, thereby enhancing overall osteoclastogenesis in the pathogenic joint (436). Given the variety of T lymphocyte-associated cytokines with osteotropic function, it will also be useful to clarify the correlation between T_H1/T_H2/T_H17 cytokine polarization and any attendant osteoimmunological bone destruction.

XI. Role of Immune Cells in Estrogen-Withdrawal-Induced Bone Loss

Estrogen withdrawal after menopause is associated with a rapid and sustained increase in the rate at which bone is lost. This phenomenon seems to result from an increase in bone resorption that is not met by an equivalent increase in bone formation. Production of cytokines is likely involved

because in mice deficient in the receptor for IL-1 (526), TNF α (527), and IL-6 (528), estrogen withdrawal induced by ovariectomy did not cause bone loss. Production of IL-1 (529), IL-1 receptor (530), TNF α (531), M-CSF (532), IL-6, and IL-6 receptor (533–535) is regulated by estrogen in bone or hematopoietic cells, which may link cytokines to estrogen withdrawal-induced bone loss. Production of IL-7 has also been linked to the bone loss that occurs with estrogen deficiency (359). However, this result is controversial because trabecular bone loss after ovariectomy was found to be similar in wild-type and IL-7-deficient mice (364). Responses of osteoclast precursor cells to RANKL are inhibited by estrogen, and this effect is mediated, in part, by a down-regulation of JNK activation in these cells (536, 537).

T cells have also been proposed to influence the rapid bone loss that occurs after acute estrogen deficiency. This response was postulated to be mediated by enhanced TNF α production (528, 538). In a series of experiments involving ovariectomy (OVX)-induced bone loss in mice, an animal model for postmenopausal bone disease, it was reported that nude mice, which lack T lymphocytes, did not lose bone mass after OVX. This result suggested that T cells are critical for this response (528, 538). However, this hypothesis is controversial because similar experiments using nude rats (539) and nude RAG2- or TCR- α -deficient mice (all of which lack functional T lymphocytes) demonstrated that OVX-induced trabecular bone loss in these models was equivalent to that seen in wild-type mice (540). Curiously, loss of cortical bone with OVX was different between T cell-deficient and wild-type models and dependent on the bone that was examined (540). These results suggest that there may be compartmental and bone-specific effects of T cell depletion on OVX-induced bone loss. Additional experiments will be required to determine how T cells are involved in this response of bone. These studies will likely require mutant mouse models that are deficient in specific immunoregulatory molecules to mechanistically examine the causes of OVX-induced bone loss.

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 (44, 46, 48, 541). In addition, production of osteoclastogenic activity in these populations was increased after ovariectomy. However, when isolated to very high purity, purified B lymphocytes failed to differentiate into osteoclasts *in vitro* (24). These results demonstrate that the osteoclastogenic potential of B lymphocyte 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 that were deficient in the majority of their B lymphocytes (364, 540, 542).

XII. Modulation of Immunity by the RANKL-RANK-OPG Axis

The significance of RANKL-RANK-OPG signaling in regulating the immune system continues to emerge. Initial studies of RANKL- and RANK-deficient mice demonstrated the importance of these signals for secondary lymphoid organ development because these animals display a lack of pe-

ripheral lymph nodes and abnormalities in B cell follicle formation and marginal zone integrity in the spleen (171, 199). In this section, however, we will focus on the role that RANKL-RANK plays in shaping the immune response in the adult immune system.

To date, most reported data indicate that RANKL modulates immunity through dendritic cells. Dendritic cells are the most potent professional antigen-presenting cells and are required to initiate T cell-mediated immunity *in vivo* (543). Dendritic cells differentiate from the hematopoietic monocyte/macrophage progenitor cell lineage and, as close relatives of osteoclasts, can be generated *in vitro* by treating a common precursor cell with GM-CSF. Such treatment has been shown to suppress c-Fos and Fra-1 (26, 544), which are key transcription factors for osteoclast differentiation. These results highlight a mechanism of developmental divergence between these two cell types. Upon receipt of inflammatory or activating stimuli, dendritic cells home to the T cell areas of the lymph nodes to activate antigen-specific T cells. Productive activation relies on numerous dendritic cell-specific factors, including alteration of the chemokine receptor repertoire, up-regulation of costimulatory molecules, and cytokine production. These modifications are induced by exogenous inflammatory stimuli, as well as signals transmitted by the TNF family members TNF α and CD40L.

RANKL signaling has also been implicated in dendritic cell function, particularly with regard to regulation of dendritic cell survival. Activated dendritic cells are relatively short-lived cells, with a half-life as low as 1–2 d upon arrival in the lymph node (545), yet *in situ* imaging studies suggest that individual T dendritic cell couplings may last 37 h or longer (546–548). RANKL-prolonged dendritic cell survival is attributed to up-regulation of the antiapoptotic protein Bcl-xL (549), through a pathway requiring the NF- κ B components p50 and c-Rel (550). Treatment of dendritic cells with RANKL also activates the antiapoptotic serine/threonine kinase, Akt/PKB, through recruitment of PI3K by TRAF6 and Cbl-b to RANK, in a mechanism dependent on the kinase activity of c-Src (201, 551). RANKL-prolonged dendritic cell survival also has *in vivo* relevance because pretreatment of peptide-pulsed dendritic cells with RANKL before sc injection into recipient mice results in significantly elevated dendritic cell persistence in draining lymph nodes and enhanced T_H-1 cytokine production and T cell memory formation (552). Dendritic cell vectors intended for use in immunotherapy have been shown to persist longer when pretreated with RANKL (553), and enforced autocrine RANKL-RANK, but not CD40L-CD40, signaling on dendritic cells has been shown to enhance antitumor immunity (554). *Opg*^{−/−} dendritic cells potentiate *in vitro* mixed lymphocyte reactions, despite CD86, MHCII, and antigen presentation levels identical to syngeneic *opg*^{+/−} dendritic cells (555).

Blockade of RANKL signaling *in vivo* results in a slightly reduced CD4⁺ T cell response to lymphocytic choriomeningitis virus infection, although the response is severely inhibited in the absence of CD40 signaling (556). These experiments highlight the requirement for TNF family member signaling in the generation of antiviral immunity, as well as the degree to which the functions of RANK-RANKL and CD40-CD40L interactions overlap. However, physiological

signaling through RANK is more limited in scope than CD40, in that treatment of immature dendritic cells with RANKL cannot initiate activation, and RANKL signaling does not complement the *cd40*^{-/-} defect in germinal center formation and B cell affinity maturation (549, 555). This disconnect is likely not explained by intrinsic signaling differences because RANK and CD40 activate the same set of signaling cascades, but instead by differential expression patterns and kinetics. For example, on T cells CD40L is rapidly and transiently expressed and is limited only to the CD4⁺ T cell subset (557). In contrast, RANKL is expressed on both CD4⁺ and CD8⁺ T cells (552) and is capable of binding both its functional (RANK) and decoy (OPG) receptors. These interactions are also likely to succeed CD40-CD40L signaling because CD40L is a key inducer of RANK and OPG expression by dendritic cells (181). The physiological role of CD40-CD40L *vs.* RANK-RANKL signaling in dendritic cell function may, therefore, depend on the phase of the immune response. CD40-CD40L signaling may be more prominent during the initiation and effector phases, when many cellular components of the immune system are strongly activated. By contrast, RANK-RANKL signaling may be more important during the waning phases, to ensure that T memory formation is established, and then to wind down remaining T dendritic cell interactions, possibly through OPG interference with RANKL signaling. The severe phenotype of RANKL and RANK-deficient mice has thus far not allowed a thorough examination of the role of RANKL in memory T cell formation.

Evidence also suggests that RANKL may be important for survival of interstitial dendritic cells engaged in antigen surveillance during the interim period separating immune responses. Human CD34⁺ immature dendritic cells have been shown to express both RANK and RANKL and are therefore capable of providing an autocrine survival signal. Peripheral maturation of these dendritic cells leads to a down-regulation of RANKL, suggesting a requirement for an independent source of RANKL to validate dendritic cell activation (558).

RANKL may also be involved in actively inducing tolerance. RANKL signaling has been directly implicated in the induction of oral tolerance in mice. Feeding low-dose ovalbumin to mice concomitant with *iv* RANKL treatment produced T cells that were refractory to rechallenge and correlates with *in vitro* production of the suppressive cytokine IL-10 by mucosal dendritic cells (559). Another study has demonstrated that RANKL-mediated signaling is required to prevent the onset of autoimmune disease in a TNF α -inducible mouse model of diabetes and that blockade of RANKL-RANK interactions parallel a diminution of CD4⁺CD25⁺ regulatory lymphocytes, which are necessary to prevent cytotoxic T lymphocyte-mediated islet cell destruction (560). In a recent study of murine cardiac allograft tolerance, RANKL-RANK signaling was shown to be important for the generation of regulatory T cells via intratracheal delivery of alloantigen (561). It remains unclear, however, whether RANKL directly triggers T lymphocyte suppression or, alternatively, acts through dendritic cell intermediaries. RANKL has also been shown to be induced preferentially among key costimulatory molecules on T cells activated by

tolerogenic dendritic cells (562). In addition to systemic action of RANKL, a recent report suggests a potential role of RANKL-RANK interactions in UV-induced immunosuppression in the skin. In the study, Loser *et al.* showed that UV-activated keratinocytes, by expressing RANKL, activated nearby Langerhans cells, which in turn preferentially expanded regulatory T cells (563).

In addition to modulating T cell tolerance via dendritic cells, RANKL may mediate its action during thymic selection. Although earlier studies using RANKL or RANK null mice did not reveal any significant defect in thymocyte development, recent studies nonetheless indicate that the RANKL-RANK interaction and its signaling molecule TRAF6 are required for the development of autoimmune regulator (AIRE)-expressing medullary thymic epithelial cells (564, 565). Aire-expressing medullary thymic epithelial cells play a critical role in preventing autoimmune diseases by expressing tissue-restricted antigens and thus deleting potentially self-reactive thymocytes during development (566–568). Whether the RANKL-RANK interaction is continuously required for the maintenance of Aire-positive thymic epithelia cells during adult life is an important question because interference with this pathway may limit the therapeutic use of anti-RANKL blockers to treat various chronic bone diseases like osteoporosis. Further studies are necessary to yield molecular insights into the generation and maintenance of T lymphocyte tolerance that critically requires the interaction of RANKL-RANK.

Study of the role of RANKL-RANK-OPG signaling in the immune system continues to emerge, although progress is slower than that produced by the extensive analysis of this pathway in bone (169, 206). Future studies will provide more insight into how much the RANKL axis controls immune responses during normal homeostasis, infection, or inflammatory challenges. The outcome of these studies will have a major impact on the feasibility of using anti-RANKL therapy to treat chronic diseases of bone such as osteoporosis.

XIII. Toll-Like Receptors, Inflammation, and Osteoimmunology

Toll-like receptors (TLRs) are members of an ancient receptor family that share homology with IL-1R and are critical activators of the innate immune response (569). They are most highly expressed on antigen-presenting cells like dendritic cells, macrophages, and B cells, but some members are expressed on a diverse array of tissues. Ligand of these receptors by conserved microbial molecules or endogenous “danger” factors results in the up-regulation of costimulatory molecules and the elaboration of inflammatory cytokines in preparation for an adaptive immune response. TLR signaling is mediated by the adapters MyD88, TRAF6, and TRIF, which activate various downstream signaling pathways, including inhibitory κ B kinase-NF- κ B, MAPK, and IFN regulatory factor-1 (569).

Because macrophages and dendritic cells share a common progenitor with osteoclasts, it is not surprising that TLR expression is also detected on bone cells (74, 570, 571). Direct signaling of various TLRs (including TLR4) on osteoclast

precursors inhibits RANKL-mediated osteoclastogenesis (74). The data that microbial products inhibit osteoclast differentiation via TLRs is counterintuitive because bacterial infection can cause inflammatory bone diseases such as periodontitis, osteomyelitis, and bacterial arthritis (572). Bone mineral density is reduced in such diseases because of excessive bone resorption by osteoclasts. In addition, LPS has been suggested to be a potent stimulator of bone loss by causing an increase in the number of osteoclasts in mice. Moreover, TLR activation can enhance osteoblast-mediated osteoclast differentiation by inducing RANKL and TNF α on osteoblasts (570, 571, 573). Our recent data suggest that TLR inhibits RANKL-induced osteoclast differentiation in part by inducing the expression of type I IFNs. IFN- β receptor-deficient monocytes are resistant to TLR-mediated suppression during RANKL-induced osteoclast differentiation (Y. Choi, unpublished data). A negative feedback regulatory mechanism via type I IFN has been previously described (464). Activation of the *fos* gene by RANKL leads to up-regulation of IFN- β , which mediates a feedback mechanism blocking further c-Fos-dependent activity (464). As such, it was shown that mice deficient for the IFN- α/β receptor (IFNAR1) suffer from an osteoporotic phenotype that is characterized by an increase in osteoclasts (464). Promoter characterization showed that RANKL-mediated up-regulation of IFN- β utilizes activator protein-1 binding sites, and that c-fos-deficient osteoclast precursors are incapable of inducing IFN- β production (464). To facilitate osteoclast development, therefore, osteoclast precursors need to up-regulate the cytokine signaling regulator suppressors of cytokine signaling 3 to inhibit IFN-mediated suppression (483, 574, 575). Additional studies are needed to determine whether type 1 IFN production or its action is different when osteoblast lineage cells are present with osteoclast precursors during bacterial infection.

The basis for the apparent discrepancy between TLR stimulation as a potent negative regulator of osteoclastogenesis and the association of bacterial infection with excessive bone resorption by osteoclasts remains unclear. As described earlier, alveolar bone destruction in periodontitis caused by infection of Gram-negative bacteria is mediated by enhanced osteoclastogenesis, which results in T cell activation and subsequent up-regulation of RANKL (515). In the same study, bacterial infection of severe combined immunodeficient mice did not lead to significant levels of alveolar bone loss. This result implies that bacterial products do not have a direct role in osteoclastogenesis because severe combined immunodeficiency mice have no known defect in osteoclast precursors or osteoblasts (515). Therefore, it is likely that bone loss associated with bacterial infection may be an indirect outcome of exacerbated T cell responses.

Similar to macrophages or dendritic cells, osteoclast precursors also produce proinflammatory cytokines, such as TNF α , in response to various TLR ligands (74). Moreover, whereas TLR stimulation inhibits osteoclast differentiation, osteoclast precursors treated with TLR ligands still retain high levels of phagocytic activity, which is a major host-defense mechanism for the clearance of bacterial infection. Therefore, the net outcome of TLR stimulation in osteoclast precursors is likely the enhancement of immune responses

toward achieving bacterial clearance. This enhancement of immune responses can be achieved by promoting cytokine production from precursor cells and by inhibiting their differentiation into nonphagocytic, nonimmune cells, such as mature osteoclasts. Thus, interaction of these microbial products with TLRs on osteoclast precursors appears to favor the role of osteoclast precursors as part of the proinflammatory system by inhibiting their differentiation into mature osteoclasts and promoting the production of inflammatory cytokines. However, because these cells can differentiate into mature osteoclasts if TLR ligands are removed (74), it appears that after a microbial infection is cleared the presence of residual activated T cells can lead to the differentiation of phagocytic precursors into mature, bone-resorbing osteoclasts. In addition, TNF α produced by osteoclast precursors upon TLR stimulation can enhance osteoclastic bone resorption.

Conversely, the RANKL axis may regulate the inflammatory action of TLR stimulation. For example, a recent report suggests that LPS-induced production of proinflammatory cytokines via TLR4 was reduced in OPG-deficient mice, whereas it was increased in RANKL null mice, which demonstrated increased lethality after LPS injection. Moreover, if mice were pretreated with RANKL, they were somewhat protected from LPS-induced death (576). These results suggest that RANKL may suppress cytokine responsiveness to LPS (or other TLR ligands) *in vivo*.

TLRs are thus likely to regulate the balance of immune responses and bone metabolism during acute attacks of vertebrate hosts by various microbes. However, physiological *in vivo* stimulation of TLRs, which are expressed on various cells, may result in different effects on bone metabolism depending on the nature of the given immune responses. In addition, ongoing stimulation of TLRs by commensal bacteria might affect bone metabolism. In support of this idea, recent data show that mice deficient in mediators of the TLR/IL-1R signaling pathway (MyD88 or IL-1 receptor-associated kinase-M) exhibit an altered bone metabolism, although it is not clear whether the defects are due to the signals from TLRs or IL-1R (283, 577).

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