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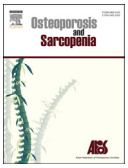

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Review article

Communication between the skeletal and immune systems

Seoung Hoon Lee [a], Yongwon Choi [b,*]

[a] Department of Oral Microbiology and Immunology, College of Dentistry, and Center for Metabolic Function Regulation (CMFR), Wonkwang University, Iksan, Republic of Korea

[b] Department of Pathology and Laboratory Medicine, The University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

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Abstract

In the last two decades, numerous researchers have focused on elucidating the relationship between the skeletal and immune systems with respect to their regulatory mechanisms. It has now become clear that osteoclasts are derived from the same myeloid precursor cells that can differentiate into macrophages and myeloid dendritic cells. In addition, bone and immune cells coexist in the common microenvironment of the bone marrow and are thus influenced by similar mediators. Discovery of a common regulatory mechanism via the receptor activator of nuclear factor kappa-B ligand (RANKL)–receptor activator of NF-κB (RANK)–osteoprotegerin (OPG) axis in both the bone and immune system has not only increased understanding of the fundamental processes of bone homeostasis but has further crystalized understanding of the definitive regulatory correlation between bone and immunity. Moreover, many of the soluble mediators produced by immune cells, including cytokines, chemokines, and growth factors, regulate the activities of osteoclasts and osteoblasts. This increased recognition of the complex interactions between the immune system and bone has led to the development of the interdisciplinary field of osteoimmunology. In this review, we summarize the characteristics of bone cells and the soluble mediators responsible for crosstalk between the skeletal and immune systems. A more complete appreciation of the interactions between immune and bone cells should lead to better therapeutic strategies for diseases that affect either or both systems.

Keywords: Osteoimmunology; Osteoclasts; Osteoblasts; RANKL/RANK/OPG; Pro-inflammatory cytokines

1. Introduction

The skeletal system has long been regarded as a metabolically inert organ; however, it is now well known that bone remodeling is continuously and dynamically carried out in order to maintain bone function and homeostasis. Recent reviews have described findings in the new field of osteoimmunology regarding the relationship between the skeletal and immune systems and their overlapping regulatory mechanisms [1,2]. Bone provides a microenvironment that is critical for the development of immune cells that are derived from hematopoietic stem cells (HSCs) and influence the various cytokines produced by immune cells to determine the fate of bone cells. Indeed, bone is an ideal anatomic microenvironment for HSC maintenance and differentiation, and recent data show that osteoblasts, which are bone matrix-producing cells, regulate the development of the HSC niche, from which all blood and immune cells are derived. The regulation of bone by hematopoietic and immune cells produces a variety of physiological and pathological effects. In pathological conditions such as rheumatoid arthritis (RA) and inflammatory bowel disease, infiltrating lymphocytes and other mononuclear cells provide several key factors that influence bone metabolism by altering the balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Whether or not these interactions also influence normal bone homeostasis had been unclear until the discovery of RANKL (also known as TNF-related

* Corresponding author. Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Room 308, BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, USA. Tel.: +1 215 746 6404; fax: +1 215 573 0888.
E-mail address: ychoi3@mail.med.upenn.edu (Y. Choi).
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activation-induced cytokine, TRANCE) expressed in activated T cells and subsequent elucidation of its role as a pivotal factor for osteoclastogenesis, which has provided critical evidence that a physiological relationship exists between the normal immune system and bone metabolism [3–5]. Here, we provide a brief description of the current understanding of bone cells (osteoclasts and osteoblasts) and the immune cell-derived factors that regulate these cells and affect bone metabolism.

2. Characterizations of bone cells

2.1. Osteoclasts

Osteoclasts originate from HSCs that can differentiate into macrophages and dendritic cells, and multinucleated giant cells that form via the fusion of mononuclear precursor cells [1,2], and have a unique capacity to efficiently resorb the bone matrix. It has been conclusively demonstrated that RANKL (also designated as TNFSF11) is an essential factor that controls osteoclastogenesis and bone resorption both *in vitro* and *in vivo*. Almost 20 years ago, four research groups independently discovered and alternatively named this factor as TRANCE, RANKL, osteoclast differentiation factor (ODF), and osteoprotegerin ligand (OPGL). The first two groups [3,6] demonstrated that TRANCE/RANKL and its receptor (TRANCE-R/receptor activator of NF-κB [RANK]) are expressed in activated T cells and dendritic cells, respectively, and that these receptor–ligand interactions promote dendritic cell function and survival. The second two groups [4,5] found that this cytokine is derived from bone marrow stromal cells (BMSCs) and is an essential factor for *in vitro* osteoclastogenesis. These findings demonstrated that both the immune and skeletal systems share the common RANKL/RANK/osteoprotegerin (OPG) signal axis, leading to the first concept establishing a functional connection between these systems. Since the discovery of RANKL, a number of reviews have been written that explain the molecular pathways regarding the maturation of osteoclasts from bone marrow precursors [1,5]. Therefore, we will here briefly describe recent progress on the identification of the bone marrow cell population as osteoclast precursors.

Mononuclear cells from the bone marrow, peripheral blood, and spleen can differentiate into osteoclast-like cells (OCLs) in various *in vitro* culture systems [1,3,4]. A portion of the cell population from the murine bone marrow that did not express Sca-1, but was positive for CD117/c-kit was identified as osteoclast progenitors [2]. These cells produced tartrate-resistant acid phosphatase (TRAP)-positive OCLs via culture in semi-solid media or in co-culture with ST2 stromal cells after treatment with 1α,25-$(OH)_2$ vitamin D_3. Arai et al. [7] found that when c-kit-positive murine bone marrow cells were cultured with ST2 stromal cells, RANKL, and macrophage colony-stimulating factor (M-CSF), this cell population could differentiate into OCLs. These authors concluded that bone marrow cells expressing c-kit, c-fms, and $CD11b^{low}$ contained multipotent progenitor cells that frequently produce osteoclasts. This cell population did not initially express RANK (the receptor for RANKL), but its expression was induced following M-CSF treatment. Interestingly, in methylcellulose culture, these progenitor cells differentiated into macrophages and mononuclear $TRAP^+$ cells, indicating their multipotent ability. Subsequently, osteoclast precursor cells were identified to be $CD3^-$, $CD45R^-$, and negative or low for CD11b expression, but positive for c-fms expression [2,8]. According to the expression of c-kit, this population of bone marrow cells was further separated into two categories. The first, c-kit^{high} cells rapidly formed OCLs *in vitro* when cultured with M-CSF and RANKL, and the second, c-kit ^{low} or c-kit^- cells slowly formed OCLs *in vitro*. This population of osteoclast precursors transiently expressed CD11b *in vitro*. Initially, the population of c-kit ^{low} or c-kit^- cells efficiently produced OCLs; however, this cell population transiently formed $CD11b^{high}$ mononuclear osteoclast precursors following M-CSF and RANKL induction, and the expression of this antigen disappeared in multinucleated cells [2].

Human and murine cells that express an early marker of the myeloid dendritic cell lineage were found to differentiate into osteoclasts *in vitro*, indicating that there is some connection between osteoclasts and professional antigen-presenting dendritic cells [9]. In addition, it was shown that murine bone marrow-derived and splenic $CD11c^+$ dendritic cells, which are activated by cytokines and can present antigens to T lymphocytes, developed into osteoclasts under M-CSF and RANKL treatment [10]. Moreover, immature dendritic cells were found to be able to differentiate into OCLs in response to M-CSF and RANKL *in vitro*; however, neither mature myeloid dendritic cells nor plasmacytoid dendritic cells formed OCLs in culture [11]. This transdifferentiation was stimulated by pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α), and was inhibited by the addition of interferon (IFN)-α as well as IL-2, IFN-γ, and IL-4. It was previously suggested that macrophages, osteoclasts, and myeloid dendritic cells can differentiate from common progenitor cells [9], and single-cell clones from mouse bone marrow progenitor cells that are specific for macrophages and resident spleen dendritic cells *in vivo* also differentiated into these cells following treatment with the cytokines M-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vitro* [12]. Recently, more conclusive results were reported with regards to the common progenitor cells for osteoclasts, macrophages, and antigen-presenting dendritic cells at the single-cell level [13]. Among the murine bone marrow cells expressing $B220^-CD3^-CD11b^{-/low}$ $CD115^+$ and either $CD117^{high}$, $CD117^{intermediate}$, or $CD117^{low}$, which have high osteoclastogenic potential, the population with a higher CD117 expression level was able to generate osteoclasts, macrophages, and dendritic cells *in vitro* with efficiencies of over 90%. In addition, cells with osteoclastogenic potential were also found in the blood and peripheral hematopoietic organs. However, the relationship between the bone marrow and peripheral monocyte progenitors remains unclear. In addition to the bone marrow, circulating osteoclast precursor cells expressing CD11b in the blood were identified, and proliferation of these precursors was stimulated by inducing an

inflammation condition, particularly in the presence of TNF-α [14]. Monocytes isolated from human peripheral blood expressing CD14 were also identified as osteoclast precursors [15]. Furthermore, several investigators have established the osteoclastogenic potential of splenocytes *in vitro*, although the specific condition for splenic osteoclastogenesis remains unknown [16]. However, recent findings showing that osteoclast differentiation and activation require various co-stimulatory molecules, which act in concert with M-CSF and RANKL, support the possibility of the important role of the splenic microenvironment for osteoclastogenesis [1,13]. Adaptor proteins function as co-stimulatory molecules for osteoclastogenesis, such as DNAX adaptor protein 12 (DAP12) and Fc receptor γ (FcRγ) containing an immunoreceptor tyrosine-based activation motif. These molecules have been found to be associated with triggering receptor expressed by myeloid cells-2 (TREM-2) and signal regulatory protein β1 (SIRPβ1), and with osteoclast-associated receptor (OSCAR) and paired Ig-like receptor A (PIR-A), respectively [17,18]. Recently, it was reported that OSCAR binds to specific motifs within fibrillar collagens in the extracellular matrix and promotes osteoclastogenesis *in vivo* [19]; however, the ligands for the other factors are still unclear.

2.2. Osteoblasts

Osteoblasts are differentiated from multipotent mesenchymal stem cells (MSCs) and can also differentiate into BMSCs, chondrocytes, and adipocytes [1,8]. In response to stimuli, these progenitor cells commit to the osteogenic lineage and differentiate into preosteoblasts, followed by differentiation into lining cells and mature osteoblasts, although the regulatory signals that drive the progenitor cells to an osteoblast fate have not been fully elucidated. However, a number of critical paracrine signals and cell-autonomous transcription factors, which are expressed at distinct time points during the differentiation processes, have been identified.

Runt domain-containing transcription factor (Runx2) is an essential transcription factor for osteoblast differentiation. *Runx2*$^{-/-}$ mice showed a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation [20]. In addition, *Runx2*$^{-/-}$ calvarial cells spontaneously differentiated into adipocytes and differentiated into chondrocytes in culture with bone morphogenetic protein 2 (BMP-2) *in vitro*, but they did not differentiate into osteoblasts *in vitro* or *in vivo*, even in the presence of BMP-2 [21]. Therefore, Runx2 plays a critical role in the differentiation of multipotent MSCs into osteoblasts and inhibits their differentiation into adipocytes and chondrocytes.

Osterix (Osx) is an essential factor for the commitment of preosteoblasts differentiation into mature osteoblasts. *Osx*$^{-/-}$ mice showed a completely lack of mineralized bone, whereas the cartilaginous tissue was essentially normal [22]. In addition, mesenchymal cells from *Osx*$^{-/-}$ mice expressed *Runx2*; however, *Osx* was not expressed in *Runx2*$^{-/-}$ mice. These results demonstrate that *Osx* is a downstream gene of *Runx2*. Consistently, Osx expression has been found to be positively regulated by direct binding of Runx2 to a responsive element in the promoter of the *Osx* gene. Moreover, *Osx*$^{-/-}$ mouse embryos did not express osteoblast differentiation markers such as osteocalcin, alkaline phosphatase (ALP), and others [22]. Therefore, Osx is an osteoblast-specific transcription factor that is required for bone formation and osteoblast differentiation.

BMPs were identified as members of the transforming growth factor-β (TGF-β) superfamily and have been shown to induce ectopic bone formation in mice. Upon BMP binding to BMPR-II (a subtype of BMP receptors), another receptor, BMPR-I, is recruited to form an activated quaternary complex, and then phosphorylates and activates intracellular Smad protein. The Smad receptor binds to co-Smad and translocates to the nucleus to serve as a transcription factor [23]. One of the main target genes of the BMP-Smad complex is *Runx2*. In a human BMSC line, BMP-2 treatment enhanced osteoblast commitment via increasing the *RUNX2* gene expression and ALP protein levels [8]. Recent studies have shown that deletion of the genes encoding BMP ligands or BMP receptors from the limb bud mesenchyme resulted in severe impairment of osteogenesis or chondrogenesis, respectively [24].

Wnt signaling is also a major molecular pathway involved in osteoblastogenesis. Wnt signaling is categorized in two pathways: the canonical pathway (through β-catenin) and the non-canonical pathway (not requiring β-catenin). In the canonical pathway, Wnt binds to the transmembrane receptor Frizzed and its co-receptor low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6). This process induces the accumulation and stabilization of cytosolic β-catenin, which enters the nucleus and stimulates the transcription of Wnt target genes such as *Runx2*, *Osx*, and *Fra-1/2* [2]. Promotion of osteoblastogenesis via the non-canonical Wnt signaling pathway was also identified in bone marrow mesenchymal progenitors [25]. Wnt5A repressed PPAR-gamma transactivation and induced *Runx2* expression through chromatin inactivation, leading to the formation of osteoblasts from MSCs. Therefore, both the canonical and non-canonical Wnt signaling pathways are involved in regulation of the differentiation of osteoblast progenitor cells into mature osteoblasts.

3. Cytokines and immune cell-derived factors that regulate bone cells and bone metabolism

Many cytokines, including pro-inflammatory cytokine produced by immune cells such as macrophage and lymphocytes, regulate bone cell and bone metabolism as well as immune response. In this section, we first describe the current understanding of the roles of RANKL/RANK/OPG and M-CSF, key regulators of bone metabolism, and next summarize pro-inflammatory cytokines, which were categorized into three groups (osteoclastogenic, anti-osteoclastogenic, and both) depending upon their influence on osteoclast differentiation, activation, and survival.

3.1. RANKL/RANL/OPG, and M-CSF

Identification of the functions of the interaction of RANKL and its receptors (RANK and OPG) has led to the emergence of the field of osteoimmunology, which is concerned with the relationship between the immune and bone systems. RANKL (TNFSF11), a member of the TNF superfamily (TNFSF), is a type II membrane protein showing close homology to other TNFSF members such as TRAIL, FasL, and TNF-α. Both murine and human RANKL consists of a C-terminal extracellular receptor-interacting domain and a transmembrane domain, but is found in both membrane-bound and soluble forms [26]. It has been reported that various cells and tissues, including T lymphocytes, osteoblasts, bone marrow stromal cells, and the lung, express RANKL [4,12,26]. In addition, osteocytes were recently identified as a major source of RANKL production via observation of mice lacking RANKL specifically in osteocytes [27]. Expression of RANKL is induced and regulated by various cytokines and growth hormones, including glucocorticoid, 1α,25-$(OH)_2$ vitamin D_3, IL-1, TNF-α, parathyroid hormone (PTH), prostaglandin E2 (PGE_2), TGF-β, Wnt ligands, and lipopolysaccharide [28]. RANKL-deficient mice showed severe osteopetrosis and defective tooth eruption owing to the complete lack of osteoclasts. In addition, these mice exhibited an inability of the early differentiation of T and B lymphocytes, and showed deficiency of all lymph nodes, even though the splenic structure and Peyer's patches were normal [29].

RANK (TNFRSF11A) is the signaling receptor for RANKL and is specifically expressed on osteoclast precursor cells upon M-CSF induction. Upon the RANKL-RANK interaction, tumor necrosis factor receptor-associated factors (TRAFs) are recruited and bind to the cytoplasmic domain of RANK. TRAF6, a major adaptor molecule in RANKL-RANK signaling, mediates the activation of the mitogen-activated protein kinases (MAPKs) p38 and JNK, as well as the canonical NF-κB pathway in response to RANK signaling [30]. Similar to RANKL knockout (KO) mice, RANK-deficient mice exhibited severe osteopetrosis via impairment of osteoclastogenesis and the complete lack of all peripheral lymph nodes, except for mucosal-associated lymphoid tissues [31]. Thus, RANKL and RANK are critical factors for osteoclastogenesis and are essential regulators of lymph-node organogenesis *in vivo*.

OPG, also designated as TNFRSF11B, is known primarily as a soluble decoy receptor that modulates interactions between ligands and signaling receptors, especially RANKL and RANK. BMSCs and osteoblasts are the main producers of OPG, but it can also be induced in B lymphocytes, dendritic cells, and follicular dendritic cells. In contrast to RANKL and RANK KO mice, OPG-deficient mice exhibited severe osteoporosis accompanied with a high incidence of fractures, and also showed calcification of the aorta and renal arteries [32]. A number of recent reviews have been published that have elucidated the diverse physiological functions of the RANKL–RANK–OPG signaling axis in the skeletal and immune systems [1,20,28]. A brief summary of the RANKL–RANK–OPG axis is presented in Fig. 1.

M-CSF is expressed by osteoblasts and BMSCs and regulates the development, maintenance, and function of numerous myeloid cells, including osteoclasts. M-CSF binds to its receptor (c-Fms) expressed on monocyte/macrophage lineage cells. The importance of this cytokine in osteoclast differentiation was demonstrated from experiments in spontaneous *op/op* mutant mice. This mouse model exhibited defective macrophage/monocyte formation via deficiency in M-CSF expression, and showed congenital osteopetrosis due to a severe deficiency of osteoclasts, thereby underlying the importance of M-CSF for osteoclast development [1,2]. M-CSF injections or osteoblast-specific expression of M-CSF in *op/op* mice restored the defect in osteoclast formation and bone resorption [8]. In addition, M-CSF induced the proliferation and differentiation of osteoclast precursor cells, and also regulated osteoclast apoptosis, resulting in prolonged mature osteoclast survival [33]. In osteoclast precursors, M-CSF is also a potent stimulator of RANK expression, and is essential for expansion of the osteoclast precursor pool.

3.2. Pro-inflammatory cytokines having osteoclastogenic effects

TNF-α was reported to induce the formation of osteoclasts from bone marrow macrophages *in vitro*, and to enhance osteoclast formation and bone resorption *in vivo* [1]. In the presence of TNF-α, low concentrations of RANKL showed a synergistic effect to promote osteoclast formation *in vitro* and *in vivo*; however, TNF-α alone did not induce osteoclast differentiation [34]. The osteoclastogenic effect of TNF-α in a co-culture of osteoclast precursors and stromal cells was found to be mediated by IL-1 via enhancing RANKL expression on stromal cells and through direct stimulation of osteoclast differentiation precursors [35]. Thus, TNF-α is considered to increase RANKL signaling. Although *in vivo* administration of TNF-α to RANK-deficient mice produced only a few osteoclasts, TNF-α directly stimulated *in vitro* osteoclast formation in a RANK-independent manner in cultures of cells from RANK-deficient mice [36]. From the perspective of osteoblastogenesis, TNF-α is classically known to have an inhibitory effect on osteoblast differentiation and collagen synthesis, which is caused by the repression of insulin-like growth factor-1 (IGF-1), Osx, and Runx2 [37]. In human MSCs, TNF-α also inhibited Runx2 and collagen synthesis; however, ALP activity and matrix mineralization were increased [38]. In addition, TNF-α induced apoptosis of osteoblasts, presumably through Fas–Fas ligand signaling [28]. Interestingly, both TNF receptor 1 and TNF receptor 2-deficient mice show normal bone phenotypes, suggesting that TNF-α affects the bone only in inflammatory states, rather than during normal development. Conversely, TNF-α has exhibited a dual effect on the osteogenic differentiation of MSCs from rodents and humans. At low concentrations, TNF-α increased osteogenic differentiation via promotion of Runx2, Osx, osteocalcin, ALP, and BMP-2 levels, while higher concentrations of TNF-α down-

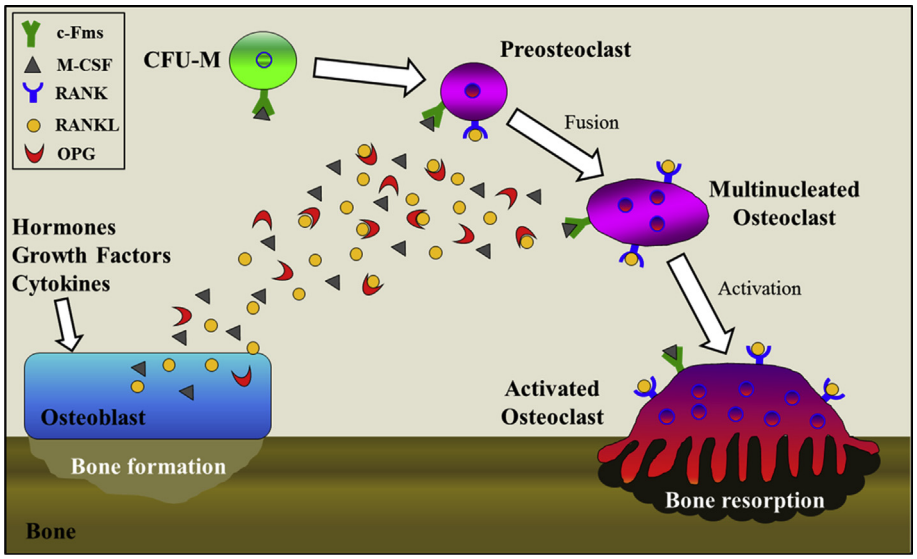

Fig. 1. A schematic diagram of the RANKL–RANK–OPG axis in osteoclastogenesis. Osteoblasts, which are responsible for bone matrix formation, produce RANKL in response to many inflammatory cytokines and calcitropic factors such as 1α,25-(OH)2 vitamin D3, PGE2, TNF-α, IL-1, IL-6, and PTH. Induced RANKL may bind to its receptor, RANK, which is expressed on osteoclast precursor cells. This binding evokes the differentiation of osteoclast precursor cells to preosteoclasts expressing tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor. Mononuclear preosteoclasts then fuse together to become nonfunctional multinucleated osteoclasts that are polykaryons lacking ruffled borders, and thus cannot resorb bone. These cells are activated into functional mature osteoclasts with the capacity to resorb bone. RANKL participates in all steps of osteoclastogenesis, differentiation, fusion, and activation *in vitro* and *in vivo*. Osteoblasts as well as bone marrow stromal cells also produce M-CSF, which binds to its receptor c-Fms, expressed on monocyte/macrophage lineage cells (osteoclast precursor cells). M-CSF has important roles in the proliferation and survival of osteoclasts as well as their differentiation. M-CSF is also a potent stimulator of RANK expression. Bone marrow stromal cells and osteoblasts produce OPG, a decoy receptor for RANKL, to modulate osteoclastogenesis. OPG can bind to RANKL and inhibits the interaction between RANKL and RANK. Initially, the RANKL–RANK–OPG signaling axis was identified from the immune system. A delicate balance of the RANKL–RANK–OPG signaling axis is necessary for regulation of both bone metabolism and the immune system.

regulated these factors [39]. This dual effect of TNF-α on osteoblastogenesis is directly dependent on the TNF-α concentration, cell type, and exposure time. The paradoxical effects of TNF-α occur depending on the differentiation stage of the responding cells [37].

IL-1α and IL-1β, two distinct *IL-1* gene products, show identical activity and are members of the IL-1 family, which consists of 11 members and plays a central role in the regulation of immunity and inflammation. IL-1 is a potent stimulator of *in vitro* and *in vivo* bone resorption. IL-1, produced in the bone, affects osteoclasts both directly and indirectly via enhancing RANKL production and its activity, resulting in stimulation of osteoclastogenesis [1,2,8]. In addition, IL-1 partially mediates osteoclast formation by RANKL and 1α,25-$(OH)_2$ vitamin D3 stimulation from *in vitro* co-culture [1,8,28]. IL-1 also enhances the production of OPG, a decoy receptor of RANKL, and prostaglandin synthesis in the bone, which may explain some of its resorptive activity [40]. In fact, PGE2 enhances the production of RANKL by inflammatory stimuli, and thus regulates osteoclastogenesis and resorptive activity [8]. Furthermore, IL-1 also stimulates osteoblastic cells to produce M-CSF in a dose-dependent manner and inhibits osteoclast apoptosis [40]. IL-1-induced osteoclastogenesis in co-culture of murine stromal cells and hematopoietic cells was found to be dependent on RANKL but not TNF-α [41]. The effects of IL-1 on stimulation of RANKL production and prolongation of osteoclast survival were found to be dependent on the expression of myeloid differentiation factor 88 (MyD88), PI3K/AKT, and ERK, but not Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF) [8,28]. Furthermore, IL-1 also inhibits osteoblastogenesis via modulation of MAPK, causing down-regulation of new bone formation. IL-1 also stimulates Dickkopf-related protein 1 (DKK1) and sclerostin (SOST), which may suppress osteoblast differentiation via inhibition of Wnt signaling [42].

IL-6 family cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), and oncostatin M (OSM), which share the same receptor subunit and often have similar functions, are known to regulate both osteoclast and osteoblast differentiation, although IL-6-mediated bone resorption varies depending on the *in vitro* assay system employed [2]. IL-6 is a multifunctional cytokine with a wide variety of activities and is produced by osteoblastic cells and BMSCs. The receptor for IL-6 (IL-6R) has two subunits, a ligand-binding and a common signal-transducing subunit, glycoprotein 130 (gp130), which are required for the tyrosine phosphorylation of STAT3 [8]. An *in vitro* study with dominant-negative STAT3 and gp130, the activation of the signaling pathway through IL-6/soluble IL-6R/STAT3 induced the expression of RANKL on osteoblasts, indicating stimulation of osteoclastogenesis [43]. However, in an *in vivo* experiment with *IL-6* transgenic mice, osteoclast number and bone resorption decreased due to the absence of soluble IL-6 receptor under physiological conditions [44]. In addition, IL-6 with soluble IL-6R significantly stimulated bone resorption via enhancement of RANKL and

OPG expression on osteoblasts as well as down-regulation of RANK expression on osteoclast progenitors [45]. These results demonstrate the complexity of the function of IL-6 for bone metabolism. Furthermore, the expression of IL-6 decreased following estrogen treatment in osteoblasts, and ovariectomy (OVX)-induced bone loss was protected by treatment of a neutralizing antibody against IL-6 or by delection of the *IL-6* gene [46]. These results strongly suggested that IL-6 is an essential factor for the bone loss caused by estrogen deficiency leading to postmenopausal osteoporosis. In addition, IL-6 appears to mediate pathological conditions characterized by excessive bone loss, including Paget's disease, hypercalcemia associated with malignancy, fibrous dysplasia, multiple myeloma, and Gorham–Stout disease [8]. IL-11, which is produced by bone cells in response to resorption stimuli, stimulates osteoclast formation and bone resorption *in vitro*, but has no effect on isolated mature osteoclasts. IL-11 receptor-deficient mice exhibited increased trabecular bone mass associated with down-regulated osteoclast numbers and bone resorption. This result was reproduced in *ex vivo* cultures, which showed decreased bone turnover, osteoclast formation, and resorption activity [47]. Like IL-6, LIF is produced by osteoblasts in response to resorption stimuli such as PTH, resulting in a variable effect on bone resorption. LIF was found to stimulate osteoclastogenesis and bone resorption via a prostaglandin-independent or dependent manner *in vivo* and *in vitro* [48], whereas it has also been shown to have a potent inhibitory effect on bone resorption [49]. LIF also enhanced both RANKL and OPG expression in murine calvaria cultures [45]. However, mice with targeted deletion of the LIF receptor gene exhibited a reduced bone volume and increased osteoclast numbers, resulting in severe osteopenia [50]. OSM is produced within the bone microenvironment by osteocytes, osteoblasts, macrophages, and T lymphocytes. In co-culture of bone marrow cells with osteoblasts, OSM stimulates osteoclast formation via enhancement of RANKL expression by osteoblasts. In addition, OSM can inhibit the production of sclerostin, a bone formation inhibitor [51]. In contrast, OSM inhibited 1,25-$(OH)_2$ vitamin D3-stimulated OCL formation in human bone marrow cultures, and decreased bone resorption rates in fetal mouse long bone cultures. Moreover, overexpression of OSM in transgenic mice induced an osteopetrotic phenotype [52]. Hence, the effect of OSM in bone metabolism remains controversial.

Chemokines are a family of small cytokines, i.e., signaling proteins that direct the recruitment and homing of myeloid cells expressing their receptors. Chemokines can be classified into four main subfamilies, CXC, CC, CX3C, and XC, depending on the sequence motif containing the first cysteine (C) residue. Chemokines exert their biological effects by interacting with G protein-coupled transmembrane receptors to initiate cytoskeletal rearrangement, adhesion, and directional migration [53]. IL-8 is a CXC chemokine produced by macrophages and other cell types, including osteoclasts. IL-8 directly stimulates the differentiation of human peripheral blood mononuclear cells (PBMCs) into osteoclasts and bone resorption through the RANKL-RANK-independent pathway [54]. In addition, IL-8 has been implicated in the lytic bone lesions associated with metastatic cancers, and also found to be partially involved in modulation of bone resorption by enhanced expression of inducible nitric oxide synthase (iNOS) in osteoclasts [55]. CXCL12 (also termed stromal-derived factor-1 or B cell-stimulating factor) produced by BMSCs and osteoblasts, and its receptor CXCR4 play crucial roles in the regulation of hematopoietic cell homeostasis and the immune response [56]. CXCR4 is markedly expressed in osteoclast precursor cells and its expression is downregulated during differentiation to the osteoclast linage. CXCL12, as a regulator of bone resorption, has also been shown to be involved in the migration of osteoclast precursors to resorption sites, their maintenance within the bone microenvironment, and enhanced RANKL-induced osteoclastogenesis [56]. In addition, the expression of CXCL12 was significantly increased in osteoclasts differentiating on calcium phosphate-coated slides [57]. Recent studies have also implicated CXCL12 in the pathogenesis of several diseases, including RA and multiple myeloma [58]. CCL2 (also termed monocyte chemoattractant protein-1), the ligand for CCR2, is a chemokine known to recruit monocytes and macrophages to sites of inflammation, and is highly expressed in osteoblasts associated with inflammatory lesions induced by proinflammatory cytokines [59]. In the presence of RANKL, CCL2 significantly enhanced OCL formation, and PTH-treated osteoblasts induced CCL2 expression and enhanced the recruitment and fusion of preosteoclasts [60]. In addition, CCL2 was found to be specifically expressed in osteoclasts, and CCL2-deficient mice exhibited down-regulated osteoclast differentiation and NFATc1 expression, in which treatment of CCR2 in the presence of RANKL restored osteoclastogenesis [61]. Recently, CCL2 has been implicated in tumor progression and bone metastasis through recruitment of macrophages and osteoclasts to the tumor site [62]. CCL3 (also known as macrophage inflammatory protein-1α) is expressed in the bone and bone marrow cells, and directly stimulates osteoclast formation through its receptors CCR1 and CCR5 [8]. It was also shown to mediate the osteolytic lesions in multiple myeloma through induction of RANKL expression by stromal cells [63]. Recently, in an experiment with CCL3-deficient mice, CCL3 was found to partially participate in decreased bone remodeling induced by mechanical loading during orthodontic tooth movement [64]. RANKL dramatically induced the expression of CCL9 (also termed macrophage inflammatory peptide-1γ) and its receptor CCR1 on osteoclasts. In addition, CCL9 stimulated osteoclast differentiation, survival, and activation for bone resorption [65]. Treatment of M-CSF to *Csf1*-null toothless (*tl/tl*) rats was found to enhance osteoclastogenesis and bone resorption, and strongly induced the expression of CCL9 and its receptor CCR1 in the bone. Moreover, anti-CCL9 antibody inhibited osteoclast differentiation in cultures and suppressed the osteoclast response in M-CSF-treated *tl/tl* rats [66]. A more recent study showed that locally inhibiting CCL9 expression by small hairpin RNA treatment ameliorated osteoarthritis progression in mice; treated mice showed less severe osteoarthritis through

inhibition of osteoclast formation and matrix metalloproteinase-13 expression than control mice [67]. CCL20 (also known as macrophage inflammatory protein-3α) was significantly increased in RA synovial fluid, and proinflammatory cytokines induced the expression of CCL20 in isolated synovial fibroblasts. In addition, CCL20 directly induced both osteoblast proliferation and osteoclast differentiation [68].

IL-15 is a member of the IL-2 superfamily and shows similar activity to IL-2 with respect to the stimulation of many cell types, including lymphocytes and hematopoietic progenitor cells. IL-15 increased the formation of OCLs through enhancing the number of osteoclast progenitor cells, but IL-2 could not replace the effect of IL-15 on osteoclastogenesis [2]. In addition, IL-15 was produced on T cells and monocytes from the peripheral blood and synovial fluid of RA patients, and was associated with enhanced osteoclast differentiation and bone destruction of RA patients. A recent study suggested that IL-15 promotes osteoclast formation, and significantly upregulates the expression of RANKL and phospholipase D-1 (PLD1) via activation of the MAPKs and NF-κB pathways in rheumatoid synovial fibroblasts. Interestingly, the treatment of short interfering RNA against PLD1 markedly repressed IL-15-stimulated RANKL and PLD1 expression [69].

IL-17 consists of at least six members, IL-7A–F, that play central roles in adaptive immune responses and in the products of the T_H17 population, a subset of CD4 T lymphocytes with a unique cytokine expression pattern [2]. IL-17A was initially known to stimulate osteoclast differentiation in a co-culture of bone marrow cells and osteoblasts via stimulation of PGE2 and RANKL expression in osteoblasts [70]. In addition, IL-17 levels were significantly increased in the synovial fluids, indicating that it might be involved in the activation of osteoclasts and joint destruction of RA patients. Blocking IL-17A with its specific antibody inhibited bone erosion by suppressing inflammation in an arthritis model, and decreased the synovial expression of both IL-1β and TNF-α [71]. IL-17 also directly induced osteoclastogenesis from human monocytes alone without osteoblasts, and this effect was potently repressed by treatment with an anti-TNFα antibody [72]. On the other hand, the functions of IL-17 were also demonstrated in human mesenchymal stem cells (hMSCs). IL-17 stimulated the proliferation of hMSCs and their differentiation into osteoblasts in a manner dependent on the generation of reactive oxygen species, as well as osteoclastogenesis by induction of M-CSF and RANKL expression [73].

3.3. Pro-inflammatory cytokines having anti-osteoclastogenic effects

IL-10, which is produced by activated T and B lymphocytes, inhibited bone resorption in both bone marrow cultures and in a co-culture of BMSCs with spleen cells via repression of osteoclast differentiation resulting from down-regulation of NFATc1 expression and nuclear translocation, as well as suppression of c-Fos and c-Jun expression [8]. In addition, IL-10 also suppressed osteoblastogenesis via down-regulation of bone proteins, including ALP and collagen type 1, and via inhibition of the formation of mineralized bone matrix [74]. Like IL-8, IL-10 also upregulated the production of iNOS and nitric oxide (NO) in osteoclasts, causing modulation of bone resorption [55]. After dental pulps were infected with endodontic pathogens, IL-10-deficient mice showed significantly increased infection-induced bone resorption through regulation of IL-1α production by macrophages [75]. A recent study demonstrated that IL-10 also suppressed the induction of co-stimulatory signals by ITAM-coupled receptor, especially inhibition of TREM-2 expression, during RANKL-mediated human osteoclastogenesis [76].

IL-18, also known as IFN-γ-inducing factor and a member of the IL-1 superfamily, is produced by osteoblasts and inhibits osteoclast formation due to its action upon T cells to stimulate the expression of GM-CSF, but in a manner independent of IFN-γ production [8]. However, a recent study showed that IL-18 inhibited TNF-α-mediated osteoclastogenesis *in vivo* in a T cell-independent manner [77]. IL-18 has been shown to upregulate the expression of OPG, but not RANKL, in stromal/osteoblastic cells. In addition, IL-18 was identified as a mitogen for osteogenic cells *in vitro* and stimulated INF-γ production in the bone. In IL-18-overexpressing transgenic mice, the number of osteoclasts decreased, although so did bone mass, suggesting that IL-18 may also affect bone growth [2]. Surprisingly, IL-18 has also been shown to indirectly stimulate osteoclastogenesis through its effects on T lymphocytes [78]. The expression level of IL-18 increased at inflammation sites, including in RA and periodontitis. Recently, IL-18 was found to enhance RANKL expression and also increased the RANKL/OPG ratio in human fibroblast-like synoviocytes in RA. Therefore, the net effect of IL-18 may promote osteoclast formation and bone resorption [79].

IFNs were originally recognized for their potent antiviral activity; however, they are now known to also profoundly affect many other cellular and body functions, including differentiation of bone cells and bone metabolism. INFs have been classified into three major types: type I (INF-α, β, ε, κ, and ω), type II (INF-γ), and type III. First, IFN-γ inhibited IL-1-stimulated bone resorption, and also partially suppressed osteoclast formation induced by 1,25-$(OH)_2$ vitamin D3, PTH, and IL-1, resulting in inhibition of bone resorption [31]. In addition, INF-γ significantly inhibited osteoclastogenesis via RANKL-RANK signaling by accelerating the degradation of TRAF6 via activation of the ubiquitin-proteasome system [80]. Conversely, INF-γ was also reported to indirectly stimulate osteoclast formation and promote bone resorption by stimulating antigen-dependent T cell activation and secretion of RANKL and TNF-α in T cells [81]. From the perspective of osteogenics, IFN-γ inhibited proliferation of osteoblasts and 1,25-$(OH)_2$ vitamin D3-stimulated osteocalcin production, and showed variable effects on osteoblast differentiation. Recently, treatment of IFN-γ in primary osteoblast cultures was shown to increase NO and PGE2, and to decrease cell viability [82]. In addition, nodule formation was also found to be reduced by IFN-γ treatment. *In vivo* experiments involving intraperitoneal INF-γ injections induced osteopenia in rats, and long-term

administration of IFN-γ in patients with osteopetrosis increased bone resorption, which may be due to its effect in stimulating osteoclast formation [2,83]. With respect to type I IFNs (especially IFN-α and IFN-β), RANKL was found to induce INF-β expression in osteoclast precursor cells, and IFN-β inhibited osteoclast differentiation via suppression of RANKL-mediated c-Fos expression. These effects were confirmed in mice deficient in IFNAR1, a receptor component of IFN-α/β, which exhibited severe osteopenia and enhanced osteoclastogenesis [84]. Interestingly, RANKL also induced the expression of SOCS-1 and -3, suppressors of IFN signaling [85]. Therefore, although IFN-β induced by RANKL inhibits osteoclastogenesis properties, the induction of signaling suppressors by RANKL may rescue the inhibitory effects of IFN-β. In addition, IFN-α modulated the proliferation and differentiation of human osteoprogenitor cells through upregulation of BMP-2, estrogen receptor-α, and osteocalcin mRNA expression in ascorbate/dexamethasone cultures [86]. The treatment of IFN-α in RANKL-mediated osteoclastogenesis with human PBMCs also resulted in reduced osteoclast differentiation and bone resorption via downregulation of c-Fos [87]. However, IFN-α did not have an effect on bone turnover *in vivo*, in contrast to IFN-γ [88].

3.4. Pro-inflammatory cytokines having dual effects on osteoclatogenesis

IL-7 is a multifunctional cytokine involved in the regulation of hematopoiesis, and plays non-redundant roles in supporting B- and T-cell lymphopoiesis. Studies have demonstrated that IL-7 also regulates bone homeostasis [89]; however, IL-7 shows controversial effects on osteoclasts and osteoblasts because of its various target cell-dependent actions. *In vivo* and *in vitro* neutralization studies with anti-IL-7 showed that IL-7 may inhibit OVX-induced bone loss as well as act as a critical factor in early B cell development [90]. In addition, systemic administration of IL-7 induced inflammatory bone loss and osteoclast formation via the production of soluble osteoclastogenic cytokines by T cells [91]. Importantly, IL-7 did not induce secretion of RANKL and TNF-α by splenic T cells or bone loss in T cell-deficient mice [92]. In addition, the disruption of T cell development and bone homeostasis by OVX was mediated by IL-7. Although the effect of IL-7 on bone homeostasis was elucidated, the secondary effects of IL-7 resulting from the production of bone-resorbing cytokines by T cells complicate the interpretation of results obtained from *in vivo* IL-7 treatment studies [91,92]. Furthermore, the direct inhibitory effect of IL-7 on RANKL-induced osteoclastogenesis in a culture of bone marrow cells was reported [93]. IL-7-deficient mice also exhibited significantly increased osteoclast numbers and decreased trabecular bone mass [94]. In addition, the OVX-induced trabecular bone loss of IL-7-deficient mice was comparable to that of wild-type mice. Addition of IL-7 to neonatal calvaria *in vitro* inhibited new bone formation, and injection of IL-7 into mice *in vivo* blocked bone formation via down-regulation of *Cbfa1/Runx2* [89]. Recently, mice specifically overexpressing human IL-7 in osteoblasts exhibited augmented trabecular bone mass *in vivo* and reduced osteoclast formation *in vitro* [95]. Moreover, targeted overexpression of IL-7 in osteoblasts rescued the osteoporotic bone phenotype and B-cell development of IL-7 KO mice, although these effects were observed only in females. These results demonstrate that IL-17 shows both direct inhibitory effects on osteoclastogenesis *in vivo* and a differential response between the sexes.

In general, IL-12 is considered to inhibit osteoclastogenesis; however, the underlying mechanism of its effects is under debate. Although IL-12 did not inhibit osteoclast formation in RANKL-pretreated cells, a direct inhibitory effect of IL-12 on RANKL-mediated osteoclastogenesis in osteoclast precursors and RAW 264.7 cells via down-regulation of NFATc1 was reported [96]. In contrast, previous studies demonstrated an indirect inhibitory effect of IL-12 on osteoclastogenesis. The inhibitory effect of IL-12 on osteoclastogenesis in a co-culture of osteoblasts and spleen cells was mediated by T cells, and this effect was not affected by INF-γ and GM-CSF stimulation [8]. Conversely, another group demonstrated that inhibition of RANKL-mediated osteoclastogenesis by IL-12 was caused by INF-γ, which was possibly induced from a non-T cell population [8]. In addition, IL-12 inhibited TNF-α-stimulated osteoclast differentiation and induced apoptotic changes by interaction of Fas–FasL in a T cell-independent manner [97].

IL-23, which is a critical factor for T_H17 differentiation and proliferation, stimulated osteoclast formation in cultures of hPBMCs without osteoclasts or RANKL treatment. IL-7, TNF-α, and RANKL are considered to be involved in IL-23-induced osteoclastogenesis [98]. Moreover, blocking of IL-23 by its specific antibody delayed bone destruction in rats with collagen-induced arthritis. IL-23 indirectly stimulated osteoclastogenesis by upregulation of IL-17 release and RANKL expression from CD4 T lymphocytes. In addition, recent data showed that systemic IL-23 exposure induced chronic arthritis, osteoclast differentiation, and severe bone loss via expansion and activation of myeloid cells [99]. Conversely, RANKL-induced osteoclastogenesis from bone marrow cells was partially inhibited by IL-23 through activated T cells [100].

4. Conclusions

To date, several studies conducted under physiological and pathological conditions have contributed to remarkable advancements in understanding the relationship between the immune system and bone metabolism. However, despite the extensive crosstalk between these systems, the detailed mechanisms underlying their cross-regulation remain poorly understood. Recently, interesting data emerging from human studies as well as animal models have illustrated the specific interplay between the skeletal and immune systems. Further investigations of intersystem crosstalk will provide valuable knowledge as to how bone and the immune system are physiologically regulated. Moreover, this effort may lead to

better therapeutic treatments for pathological conditions, including inflammatory and metabolic bone diseases as well as tumor-induced bone lysis.

Conflict of interests

The authors declare that they have no conflict of interests.

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