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Osteoclastogenesis and osteoimmunology

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1. ABSTRACT

Bone is continuously renewed through a dynamic balance between bone resorption and formation and is the fundamental basis for maintaining normal bone mass and architecture by osteoclasts (bone resorption) and osteoblasts (bone formation). Bone resorption is an elementary cellular activity in the modeling of the skeleton during growth and development. Later in life, bone remodeling occurs, and is locally initiated by resorption. During remodeling, bone resorption is coupled to new bone formation, that ensures bone renewal with only minor and temporary local bone loss. Osteoclasts play a crucial role in both physiological and pathological bone resorption, and receptor activator of nuclear factor-κB ligand is the key cytokine that induces osteoclastogenesis. At the same time, various factors produced during immune responses are capable of profoundly affecting bone regulation; bone and immune systems share an abundance of molecules and regulatory mechanisms. We summarize the new insights on the link between osteoclastogenesis and osteoimmunology.

2. INTRODUCTION

As a living tissue, bone is continuously being created and destroyed in vertebrates by hematopoietic-derived osteoclasts and mesenchymal-derived osteoblasts, in order to maintain bone volume and calcium homeostasis (1). Bone cells are categorized into two main groups-osteoclasts and the osteoblast family (osteoblasts, osteocytes, and lining cells). Osteocytes, which help maintain bone, exist inside the bone and have long branches which allow them to contact each other as well as the lining cells on the bone surface (2). It has been reported that osteocytes sense mechanical strain and loading (3-5). Recent studies emphasize that osteocytes signal osteoblasts to induce bone remodeling (6). Bone remodeling is precisely regulated by the balance between bone formation and bone resorption, throughout life. Osteoclasts are derived from hematopoietic stem cells and share precursors with macrophages. Cells of osteoblast lineage, such as stromal cells, bone lining cells, osteoprogenitors, preosteoblasts, osteoblasts, and osteocytes are derived from

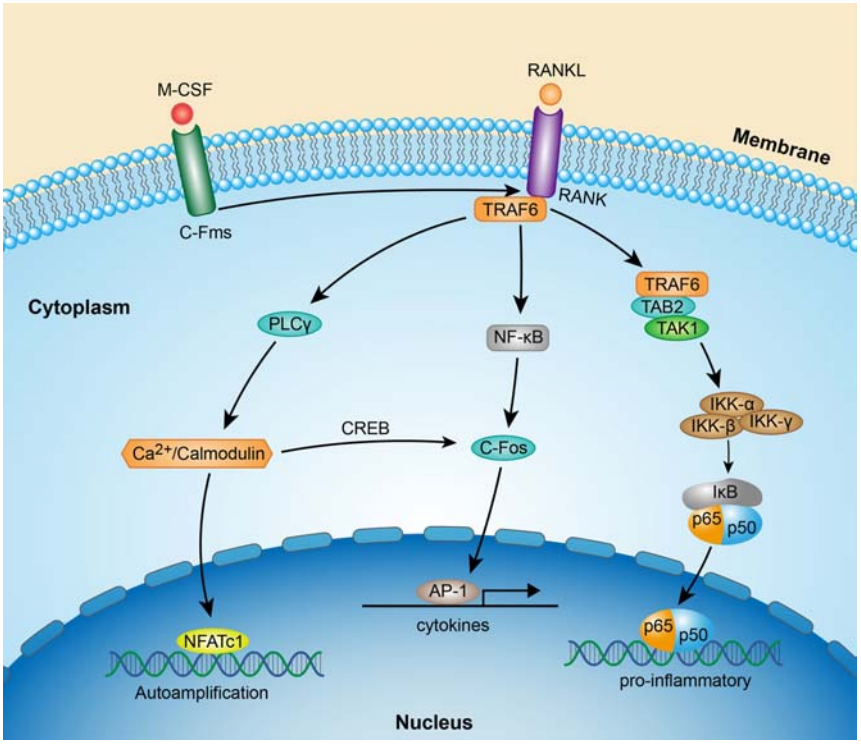

Figure 1. RANK signaling in osteoclastogenesis.

mesenchymal stem cells, which also differentiate into fibroblasts, chondrocytes, myoblasts, and adipocytes (7).

Osteoclast differentiation is regulated by macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL; also known as TRANCE), a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF)-superfamily and produced as a trimer by stromal cells (8, 9). A mutation in the M-CSF gene causes a defect in both macrophage and osteoclast formation, pointing to the fact that immune cells and bone cells are derived from the same origin (10). In addition, mice deficient in immunomodulatory molecules frequently develop an abnormal osteoclast phenotype.

Evidence for a relationship between immune and skeletal systems has further been recognized. Approximately forty years ago, antigen-stimulated immune cells were shown to produce soluble factors that increase the number of active osteoclasts, and stimulate osteoclastic bone resorption (11). Interleukin (IL)-1β was later confirmed to be one of these soluble factors (12). Since then, accumulating evidence has suggested that immune cells and osteoclasts share a number of regulatory molecules, including cytokines, receptors, signaling molecules, and transcription factors (13). Furthermore, immune cells are derived from bone marrow, and hematopoietic stem cells are maintained in the same microenvironment where they can interact with each other. Activated T cells, producing numerous pro-inflammatory cytokines such as IL-2, IL-4, IL-5, IL-6, and IL-8, TNF-α, TGF-β, granulocyte-macrophage colony stimulating factor, and interferon-γ (IFN-γ), are the most powerful regulatory factors (14). Bone and immune cells share the same progenitors in the bone marrow and the same regulatory molecules, including cytokines, receptors, signaling molecules, and transcription factors, that influence each other (13). Among these factors, RANKL is essential for osteoclast differentiation and activation.

We have summarized new insights with regards to osteoclast development in the newly established interdisciplinary field of osteoimmunology.

3. THE ESSENTIAL ROLE OF M-CSF AND RANKL

3.1. M-CSF

M-CSF, also known as CSF-1, is a four-alpha-helical-bundle cytokine that is the primary regulator of macrophage survival, proliferation, and differentiation. M-CSF was first identified as an essential factor for osteoclastogenesis. M-CSF transmits its signal to the cell through the sole receptor c-Fms, a member of the receptor tyrosine kinase superfamily. M-CSF is essential for the survival and proliferation of osteoclast progenitors and macrophages. It has been reported that *op/op* mice, which fail to express functional M-CSF as a result of the CSF1 gene mutation, are osteopetrotic (15). M-CSF also stimulates receptor activator of nuclear factor-κB (RANK) expression in monocyte-macrophage precursor cells, thereby rendering them able to efficiently respond to RANKL (Figure 1). The PU.1 gene, regulated by M-CSF,

encodes an ETS-domain transcription factor, activates gene expression during macrophage and osteoclast development (16). MITE, activted by M-CSF, induces B-cell lymphoma 2 (BCL-2), which has a pivotal role in cell survival (17). The transgenic expression of BCL-2 rescues cells from osteopetrosis found in *op/op* mice, indicating the critical role of M-CSF as a survival factor for osteoclast precursor cells (18).

3.2. RANKL

RANKL is an important protein of osteoclastogenic cytokines (19) (Figure 1). RANKL is essential for osteoclast formation thoughout life, and is viewed as a genetic marker of osteoclast support cells (20). In the late 1980s, an *in vitro* co-culture system for osteoclast formation was established. This system indicated the importance of the cell-to-cell contact of osteoblasts and hematopoietic cells for osteoclast differentiation (21). It was unexpected both that the transmembrane protein RANKL, a long sought after osteoclast-differentiation factor expressed by osteoblasts, would be the same molecule expressed by T cells to stimulate dendritic cells (22, 23). RANKL is a type II membrane protein belonging to the TNF superfamily, containing a C-terminal receptor-binding domain and a transmembrane domain.

RANKL functions as a membrane-anchored molecule, and is released from the cell surface as a soluble molecule following proteolytic cleavage by matrix metalloproteinases such as MMP-14 (24, 25). RANK is a type I membrane protein sharing high homology with CD40. The binding of RANKL to RANK is inhibited by the decoy receptor osteoprotegerin (OPG) (26). In bone, RANKL is expressed by osteoclastogenesis-supporting cells, including osteoblasts and synovial cells. RANKL expression can be upregulated by osteoclastogenic factors, such as 1α, 25-dihydroxyvitamin D3, prostaglandin E2, parathyroid hormone, IL-1, IL-6, IL-11, IL-17, and TNF-α (25, 27). In contrast, cytokines inhibiting osteoclastogenesis, such as IL-13, INF-γ, and TGF-β1 suppress RANKL expression and also augment OPG expression (25). OPG, produced by a variety of cells, including stromal cells and B lymphocytes, is a secreted TNF receptor superfamily member that acts as a decoy receptor molecule for RANKL by counteracting its osteoclastogenic activity (28).

Numerous osteoclastogenic factors, including hormones, cytokines, and growth factors, which are produced physiologically or excessively during inflammation, are now believed to exert their primary activity on osteoclasts by modulating the RANK/RANKL axis (29). Active T cells can produce RANKL to induce osteoclastogenesis from autologous peripheral monocytes (30-32). Thus, RANKL is a crucial determinant of bone resorption *in vivo*. T cells not only express RANKL on their membrane, but also release it as a soluble form, although the *in vivo* function of the soluble form remains to be elucidated (33).

4. IMMUNOLOGIC FUNCTION OF RANKL

Interestingly, RANKL-deficient mice have a defect in development and organization of secondary lymphoid tissue (34). Severe immunodeficiency is not observed in RANKL-deficient mice; loss of RANKL in T cells appears to be compensated by CD40L in mice (35). RANKL has been shown to play a role in a pathologic model of inflammatory bowel disease by stimulating dendritic cells (36, 37), suggesting that RANKL is distinctly involved in dendritic cell activation under certain autoimmune conditions. On the other hand, RANKL is involved in the generation of regulatory T cells (Tregs) in a diabetic model (38). In addition, keratinocyte expression of RANKL has a crucial role in Treg control, by modulating Langerhans cells' function, suppressing autoimmune reactions induced by CD40L (39). These reports clearly indicate that RANKL has an immunosuppressive role *in vivo*. In addition, a recent report indicated that RANKL is a chemotactic factor for RANK-expressing tumor cells as well as osteoclasts, indicating that RANKL also functions as a chemokine (40). Therefore, although RANKL's function in the immune system requires further clarification, it is clearly one of the most important molecules to link the bone and immune systems.

5. INTRACELLULAR SIGNAL TRANSDUCTION OF RANKL

RANK, a member of the tumor necrosis factor receptor (TNFR) family (22), is a transmembrane molecule expressed on osteoclast precursor cells and mature osteoclasts. Interaction between RANK and RANKL results in the commitment of monocyte and macrophage precursor cells to the osteoclast lineage and the activation of mature osteoclasts. Members of TNFR family lack intrinsic enzymatic activity in their intracellular domain, and transduce signals by recruiting adaptor proteins, primarily death domain (DD)-containing proteins and members of the TNF receptor-associated factor (TRAF) family (13). The DD-containing proteins include the Fas associated death domain and the TNFR associated death domain. These proteins link the DD-containing receptors to downstream proteases of the caspase family necessary for activation of apoptosis (41). The TRAF family contains six members (TRAFs 1-6), each containing a ring and zinc finger motif in their N-terminal and C-terminal domains that mediate self-association and protein interaction (41). The TRAFs link either the DD-containing receptors or the receptor lacking a DD to activation of various signaling pathways such as NF-κB, JNK, ERK, and p38 (41). As a member of the TNFR family lacking the DD, RANK may transduce intracellular signals by utilizing TRAF proteins. Among the TRAF proteins, TRAF6 has been shown to be the main adaptor molecule linking RANK to the differentiation and function of osteoclasts (42-44). RANK has three cytoplasmic motifs, $PFQEP^{369-373}$ (Motif 1), $PVQEET^{559-564}$ (Motif 2) and $PVQEQG^{604-609}$ (Motif 3), each with the ability to mediate osteoclast formation and function independently (45).

In response to RANKL stimulation, inducing trimerization of TRAF6, TAK1, and TAB2, Motif 1 actives NF-κB and three mitogen-actived protein kinase pathways (JNK, ERK, and p38) (46) (Figure 1). The essential role of NF-κB in osteoclastogenesis has been demonstrated (47,

48). NF-κB is a family of dimeric transcription factors that includes REL (cREL), RELA (p65), RELB, NF-κB1 (p50), and NF-κB2 (p52). p50 and p52 function by heterodimerization with any of the three REL proteins containing transcriptional activation domains. NF-κB p50 and p52 double-deficient mice develop severe osteopetrosis due to a defect in osteoclastogenesis. The upstream kinase complex that mediates inhibition of NF-κB (IκB) phosphorylation and degradation is comprised of catalytic subunits IκB kinase α (IKKα) and IKKβ, and non-catalytic subunit IKKγ, and are all important for RANK signaling and osteoclastogenesis. In mice, IKKβ is required for RANKL-induced osteoclastogenesis both *in vitro* and *in vivo*, whereas IKKα appears to be required only *in vitro* (49). In addition, activated TAK1 also actives the JNK pathway (50). p38 activation is mediated by TAB1, which has been shown to bind and recruit p38 to the TRAF6-TAK1 complex (51). RANKL activates the Akt/PKB pathway by formation of a signaling complex containing c-Src and TRAF6. Additional RANK-specific adaptor molecules might exit and enhance TRAF6 signaling (52). For instance, the molecular scaffold GRB2-associated binding protein 2 has been shown to be associated with RANK and has an important role in its signal transduction (53).

6. OSTEOCLASTOGENIC TRANSCRIPTION FACTOR NFATC1

Importantly, RANKL induces nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), the master regulator of osteoclast differentiation (54), and this induction is dependent on both the TRAF6-NF-κB and the c-FOS pathways. The NFAT family of transcription factors was originally discovered in T cells, but they are involved in the regulation of various biological systems (55, 56). NFATc1 is most potently induced by RANKL and is regulated by the serine/threonine phosphatase calcineurin, which is in turn activated by increased calcium levels. Dephosphorylation of the serine residues in NFAT by calcineurin leads to exposure of the nuclear-localization signal and subsequent translocation into the nucleus. During osteoclast differentiation, NFATc1 undergoes nuclear translocation in response to RANKL stimulation, suggesting the activation of calcium-calcineurin signals. NFATc1 activity is negatively regulated by specific transcription factors such as interferon regulatory factor-8, B cell lymphoma 6, and the v-Maf musculoaponeurotic fibrosarcoma oncogene family member protein B (57-59).

The role of the NFATc1 gene in osteoclastogenesis has been shown both *in vivo* and *in vitro* (54, 60, 61). The NFATc1 promoter contains NFAT binding sites and NFATc1 specifically autoregulates its own promoter during osteoclastogenesis, thus enabling the robust NFATc1 induction (60). The AP-1 transcription factor that is a dimeric complex composed of the FOS-family proteins, JUN-family proteins, and activating transcription factor-family proteins, together with continuous activation of calcium signaling, is crucial for this autoamplification (54, 62). NFATc1 regulates a number of osteoclast-specific genes in cooperation with other transcription factors such as AP-1, PI.1, and MITF (13). A recent study indicated that CREB, activated by CaMKIV, also cooperates with NFATc1 in the activation of osteoclast-specific genes (Figure 1). However, compared with the wealth of information on RANK signaling in osteoclasts, it is less well understood whether RANK uses the same signaling mechanisms in the immune system. Although regulation of NFATc1 by calcium-calcineurin is well documented, it is interesting to note that NFATc1 is also regulated by other factors, such as pH (63).

7. CALCIUM SIGNALING IN OSTEOCLAST DIFFERENTIATION

Osteoclasts are unique, multinucleated giant cells responsible for the decalcification and resorption of the bone matrix. Osteoclasts differentiate from the monocytemacrophage lineage (29). RANKL signaling is known to induce oscillatory changes in calcium, resulting in calcium/calcineurin-dependent dephosphorylation and activation of NFATc1, which translocates to the nucleus and induces osteoclast-specific gene transcription, allowing for the differentiation of osteoclasts (Figure 1). The differential activation of transcription factors is known to depend on the pattern of calcium signaling; NFATs are activated by low but sustained calcium activation (64), while calcium oscillation reduces the effective threshold for the activation of transcription factors (65, 66). Long-term calcium oscillation is thus thought to maintain NFATc1 in the nucleus and ensure a long-lasting transcriptional activation of NFATc1 required for terminal differentiation during osteoclastogenesis (67). Calcium oscillation is thus a critical feature of osteoclastogenic signaling. The change in calcium occurs through the sequential operation of two processes: calcium release from internal stores such as the endoplasmic reticulum and mitochondria, and calcium entry through permeable channels in the plasma membrane (68, 69). However, the molecular basis of the relationship between calcium oscillations and intra- and extracellular calcium channels remains largely unknown.

Phospholipase Cγ (PLCγ), which mediates calcium release from intracellular stores, is crucially important for the activation of the key transcription factor NFATc1 via calcineurin (54). However, it has been unclear until recently how RANKL specifically activates calcium signals. RANK belongs to the TND receptor family, which has yet to be directly connected to calcium signaling. RANKL stimulates PLCγ, resulting in calcium oscillations during the early stages of osteoclastogenesis. Inositol 1, 4, 5-triphosphate (IP_3) activates phospholipase C (PLC)-mediated calcium signaling by binding to IP_3 receptors (70, 71). IP_3R2 is predominantly expressed in mouse osteoclasts, and RANKL induced calcium oscillations were abolished in bone marrow macrophages derived from IP_3R2 and IP_3R3 knockout mice. The physiological role of IP_3-mediated signals in the osteoclast lineage has been investigated; calcium oscillations were impaired in osteoclast precursors derived from IP_3R2 and $IP_3R2/3$-deficient mice, resulting in osteoclastogenesis inhibition (72).

The activation of PLCγ by RANK requires the protein tyrosine-based activating protein 12 (DAP12) and the Fc receptor common γ chain (FcRγ) (73). In the osteoclast lineage, the immunoglobulin-like receptors (IgLR) associated with DAP12 include the triggering receptors expressed in myeloid cells 2 and signal-regulatory protein β1. Those associated with FcRγ include osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor. Although the ligands for the IgLR remain largely unknown, a recent finding suggests that OSCAR binds to specific motifs within collagens in the extracellular matrix that become uncovered on the nonquiescent bone surface during osteoclast differentiation (74). Because ITAM signals are essential for osteoclastogenesis, but by themselves cannot induce it, these signals should be considered to be co-stimulatory for RANK. The binding of M-CSF to its receptor c-Fms also generates a signaling complex composed of phosphorylated DAP12 and the nonreceptor tyrosine kinase Syk. Thus, RANKL and M-CSF signals appear to converge on the ITAM signaling pathway (75).

It is also conceivable that RANK activates a yet unknown pathway that specifically synergizes or upregulates ITAM signaling. Recently, it was shown that the Tec family tyrosine kinases (Btk, Tec) are activated by RANK, and are involved in PLCγ phosphorylation, which leads to calcium release from the endoplasmic reticulum through IP_3 generation (76). An osteopetrotic phenotype in Tec and Btk double-deficient mice revealed that these two kinases play an essential role in the regulation of osteoclastogenesis (76). Tec and Btk were previously reported to play a key role in proximal BCR (B cell antigen receptor) signaling, but this study established the crucial role in linking RANK and ITAM signals. This study also identified an osteoclastogenic signaling complex, composed of Tec kinases and scaffold proteins, which affords a new paradigm for the signal transduction mechanism of osteoclast differentiation.

8. REGULATION OF OSTEOCLASTOGENESIS BY T CELLS

As RANKL is expressed in activated T cells, T cells might have the ability to induce osteoclast differentiation by acting on precursor cells under pathologic conditions (30, 31). Further evidence implicating the role of RANKL in articular bone loss originates from the efficacy of denosumab (an anti-RANKL antibody) in attenuating erosions in rheumatoid arthritis (RA) (77, 78). It was reported that IFN-γ produced by T cells potently suppresses RANKL signaling through the rapid degradation of TRAF6 (79). Overexpression of IFN-α in an animal model of RA resulted in attenuation of osteoclast-mediated articular erosions (80). T cells also modulate osteoclastogenesis via cell-to-cell interactions. For example, Tregs suppress osteoclastogenesis via the expression of CTLA-4, in addition to the production of the anti-osteoclastogenic cytokines IL-4 and IL-10 (81, 82). There are two T-cell subsets, one subset producing IFN-γ (Th1) but not IL-4 (Th2); and the other producing IL-4 (Th2) but not IFN-γ (Th1). The Th1 phenotype is associated with inflammation, whereas the Th2 phenotype combats inflammation. The Th1 phenotype results from the expression of transcription factors such as T-bet and STAT4, whereas the Th2 phenotype results from the expression of STAT6 and GATA3. Previous studies have found that $CD4^+$ T cells, especially Th1 cells, are involved in the pathogenesis of RA (32, 83). However, other investigators showed that certain pro-inflammatory cytokines, mainly secreted by Th1 cells, are scarcely detectable in RA-affected joints (84). Therefore, additional mechanisms might be involved in RA development, other than the Th1-mediated response. In 2005, a new Th subset (Th17), produced by IL-17, was reported for the first time (85, 86). In RA, the site of T-cell differentiation (to Th17 or Th1 cells) remains unclear. There is some evidence that T-cell expansion and differentiation may occur within the synovial membrane as a result of a favorable cytokine environment, characterized by the presence of IL-1β, IL-6, IL-7, IL-12, IL-15, IL-18, IL-23p19, and TGF-β (87). IL-1β, IL-6, and IL-23, three well-known pro-inflammatory cytokines, promote Th17 differentiation in humans (88, 89). It is possible that Th17 may be attributable to inflammatory processes in RA. In contrary to Th17, Tregs are essential for the maintenance of peripheral tolerance and in controlling the immune response (90). In mice, differentiation to Th17 cells results from IL-6 and TGF-β signaling. However, the combination of TGF-β and IL-10 promotes differentiation of native T cells to Treg cells, which can inhibit Th17 cells, by production of TGF-β. Furthermore, defects in Treg function or reduced production lead to the development of RA (91). Thus, the reciprocal relationship between Treg and Th17 may provide a new perspective on therapies for autoimmune diseases by means of restoring the Th17/Treg balance.

9. BONE DESTRUCTION WITH ARTHRITIS AS A RANKL-DRIVEN DISEASE

Activation of the immune system is essential for host defense against pathogens, but aberrant or prolonged activation under pathologic conditions results in tissue damage, due to the activation of effector cells. The identification of osteoclast-like giant cells at the interface between the synovium and bone in rheumatoid joints dates back to the early 1980s. These multinucleated giant cells were subsequently characterized as being positive for TRAP and calcitonin receptor, features of authentic osteoclasts. TRAP-positive multinucleated cells are frequently observed in the synovium, which is not in contact with bone (92). These pathologic findings led investigators to hypothesize that osteoclasts play an important role in bone resorption in arthritis and that osteoclasts are formed in the synovium (92, 93). Osteoclasts were generated in primary synovial cell cultures obtained from RA patients, while high RANKL expression was detected in the synovium (93, 94). Specifically, inflammatory cytokines such as IL-1, IL-6, and TNF-α, which are abundant in the RA synovial fluid and synovium, have the potent capacity to induce RANKL on synovial fibroblasts and thus accelerate RANKL signaling, thereby directly contributing to bone destruction. RANKL was expressed by synovial and T cells, both of

which are found in inflamed synovium (31, 94, 95); however, at that point it was unclear which cell types were the major RANKL-expressing cells. Since then, a series of reports established that the pathologic bone damage associated with inflammation is caused by abnormal RANKL expression. In addition, osteoclast-deficient mice and ARO (autosomal recessive osteopetrosis) patients are protected from bone erosion in arthritis (96). In the absence of osteoclasts, bone destruction did not occur, despite a similar level of inflammation, indicating that RANKL and osteoclasts are indispensable in inflammation-associated bone loss (97). Blocking RANKL significantly prevents bone destruction in adjuvant arthritis (31). Anti-RANKL and anti-osteoclast therapies have been shown to be beneficial for the inhibition of bone loss without affecting the immune system in clinical trials.

10. CONCLUSION

The field of osteoimmunology has witnessed major advances in the past several years. Bone and immune systems share an abundance of molecules and regulatory mechanisms, thus leading to considerable attention to the field of osteoimmunology. Osteoclast biology has been the driving force in this trend. Most molecules involved in osteoclastogenic signaling were identified and studied from an immunologic perspective, and were demonstrated to be therapeutic targets for bone diseases. The use of genetically modified mice in animal models will lead to a deeper understanding of the molecular basis for cell lineage specifications, despite the similarity and evident overlap between skeletal and immune systems. Most importantly, cell type-specific treatments may become a realistic therapeutic option. In the end, both clinical and non-clinical researchers will derive benefit from the emerging insights obtained in osteoimmunology.

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Abbreviations: BCL-2, B-cell lymphoma 2; DD, death domain; ERK, extracellular signal-regulated kinase; FcRγ, Fc receptor common γ chain; IFN-γ, interferon-γ; IgLR, immunoglobulin-like receptor; IL, interleukin; IP3, inositol 1, 4, 5-triphosphate; JNK, c-jun N-terminal kinase; M-CSF, macrophage colony-stimulating factor; NFAT, nuclear factor of activated T cells; OPG, osteoprotegerin; OSCAR, osteoclast-associated receptor; PLCγ, phospholipase Cγ; RA, rheumatoid arthritis; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; TGF-β, transforming growth factor beta; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factor; Treg, regulatory T cell

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