Review

New insights into osteoclastogenic signaling mechanisms

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Bone is continuously renewed through a dynamic balance between bone resorption and formation. This process is the fundamental basis for the maintenance of normal bone mass and architecture. Osteoclasts play a crucial role in both physiological and pathological bone resorption, and receptor activator of nuclear factor-κB ligand (RANKL) is the key cytokine that induces osteoclastogenesis. Here we summarize the recent advances in the understanding of osteoclastogenic signaling by focusing on the investigation of RANKL signaling and RANKL-expressing cells in the context of osteoimmunology. The context afforded by osteoimmunology will provide a scientific basis for future therapeutic approaches to diseases related to the skeletal and immune systems.

Introduction

The bony skeleton enables weight-bearing locomotive activity, the storage of calcium, and the harboring of the hematopoietic stem cells from which blood and immune cells are derived. Although bone appears to be metabolically inert, it is actually a dynamic organ [1]. It is now well accepted that increased weight-loading on bone leads to increased bone mass, and the reduced loading which is associated with conditions of disuse, such as bed-rest and microgravity, inevitably induces bone loss [2]. Bone is constantly renewed by the balanced activities of osteoblastic bone formation and osteoclastic bone resorption, both of which occur mainly at the bone surface. This restructuring process, termed 'bone remodeling', is important not only for achieving normal bone mass and strength, but also for mineral homeostasis [2]. Excessive osteoclast activity leads to pathological bone resorption, as seen in a variety of local or generalized osteopenic conditions, such as rheumatoid arthritis (RA), bone metastasis and osteoporosis [1]. Therefore, the elucidation of the regulatory mechanisms involved in osteoclastogenesis is crucial for a deeper understanding of the skeletal system in health as well as disease.

Traditionally, the hormonal regulatory system has been understood to be the mainstay controller of the osteoclast lineage; however, in fact, osteoclasts and immune cells share several regulatory molecules, including cytokines, receptors, signaling molecules and transcription factors that mutually influence each other [1]. Among these, RANKL (see Glossary) is the most important cytokine because it is essential for osteoclast differentiation/activation as well as immune regulation. Patients with excessive activation of the immune system, as in the case of RA, are at increased risk of experiencing concomitant osteoporosis as well as localized bone destruction [1,3]. In addition, mice deficient in immunomodulatory molecules have been found

Glossary

Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1): an inducible protein component of the nuclear factor of activated T cells DNA-binding transcription complex. NFATc1 is the major molecular target for the immunosuppressive drugs such as cyclosporin A.
Osteoblast: cell that produces a bone matrix; also responsible for mineralization. This process is known as bone formation.
Osteoclast: cell that removes mineralized bone matrix by HCl and several proteases. This process is known as bone resorption.
Osteocyte: derived from osteoblasts; embedded within bone matrix. Osteocytes are networked to each other and osteoclasts/osteoblasts on bone surface via long cytoplasmic extensions.
Osteoimmunology: the study of the 'osteo-immune system', the interface between the skeletal system and the immune system. The study of osteoimmunology is particularly relevant to osteoporosis, osteopetrosis, bone metastases and rheumatoid arthritis. Shared components and mechanisms between the two systems include receptors and their ligands, signaling molecules and transcription factors.
Osteopetrosis: a syndrome characterized by the failure of osteoclastogenesis or osteoclastic bone resorption. In consequence, the defect in bone turnover characteristically results in skeletal fragility despite increased bone mass, and it may also cause hematopoietic insufficiency, disturbed tooth eruption, and growth impairment.
Osteoporosis: a syndrome characterized by decreased bone mass and loss of normal skeletal microarchitecture, leading to increased susceptibility to fractures. Osteoporosis may be classified as primary and secondary. Postmenopausal osteoporosis after menopause is referred to as primary. Senile osteoporosis occurs after age 75 and is seen in both females and males. Secondary osteoporosis results from predisposing chronic disease, or prolonged use of drugs such as glucocorticoids.
Osteoprotegerin (OPG): also known as osteoclastogenesis inhibitory factor (OCIF), or tumor necrosis factor receptor superfamily member 11B, is a cytokine receptor and a member of the tumor necrosis factor (TNF) receptor superfamily. Osteoprotegerin is a decoy receptor for RANKL. By binding to RANKL, OPG inhibits NF-κB and activation of immune-related genes.
Receptor activator of nuclear factor κ-B ligand (RANKL): a member of the tumor necrosis factor (TNF) cytokine family and a protein crucial for adequate bone metabolism. RANKL, a ligand for osteoprotegerin and a key factor for osteoclast differentiation and activation, and in T helper cells is involved in dendritic cell maturation.
Rheumatoid arthritis (RA): a chronic systemic inflammatory disorder that may affect many tissues and organs, but principally attacks flexible joints. Synovitis, arthritis of joints, is inflammation of the synovial membrane and always affects multiple joints. Synovitis can lead to tethering of tissue with loss of movement and bone erosion of the joint surface, causing deformity and loss of function.

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to develop frequently an abnormal osteoclast phenotype [1], and these animals have provided important insights into osteoclast biology. We summarize here recent progress in the understanding of RANKL signaling and RANKL-related diseases in the context of the newly established interdisciplinary field of osteoimmunology [1,3].

The crucial role of RANKL in bone remodeling

Since the early 1980s it has been thought that the osteoblasts/stromal cells derived from mesenchymal cells participate actively in osteoclast differentiation from hematopoietic cells in the bone marrow microenvironment, and that osteoclastogenesis-supporting mesenchymal cells provide specific factors essential for osteoclast differentiation [4]. In 1984, Burger *et al.* found that osteoclasts could be developed using an *in vitro* coculture of embryonic bone rudiments and hematopoietic cells. Because embryonic bone rudiments contain chondrocytes as well as osteoblasts and osteocytes, the result suggested that these cells are necessary for osteoclastogenesis [5]. Another *in vitro* coculture system for osteoclast formation was established in 1988 [6]. This osteoclast differentiation system required cell-to-cell contact between osteoblast lineage cells derived from calvarial bone and hematopoietic cells. Based on these observations, it was proposed that osteoclastogenesis-supporting mesenchymal cells must express an osteoclast differentiation factor (ODF) as a membrane-associated protein [7].

In 1997, a potential inhibitor of osteoclastogenesis was cloned, osteoprotegerin (OPG; encoded by the *Tnfrsf11b* gene) [8]. In 1998, OPG ligand (OPGL), a transmembrane protein of the tumor necrosis factor (TNF) superfamily, was identified as a molecule associated with OPG [9,10]. This molecule was essential for osteoclastogenesis and was shown to be the long-sought ODF. Interestingly, immunologists cloned the same molecule as a stimulator of the dendritic cells expressed by T cells, and named it receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL; encoded by the *Tnfsf11* gene), or TNF-related activation-induced cytokine (TRANCE) [11,12]. The receptor for RANKL is RANK (encoded by the *Tnfrsf11a* gene), a type I transmembrane protein, which, similarly to other members of the TNF receptor superfamily, assembles into a functional trimer upon stimulation [11,13]. The binding of RANKL to RANK is inhibited by OPG functioning as a soluble decoy receptor [9,10].

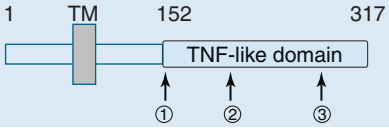
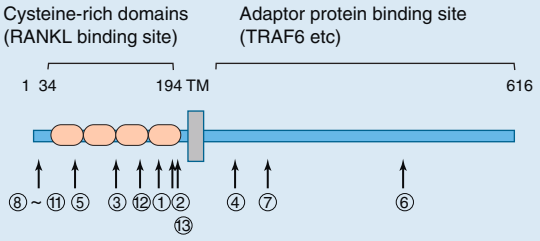
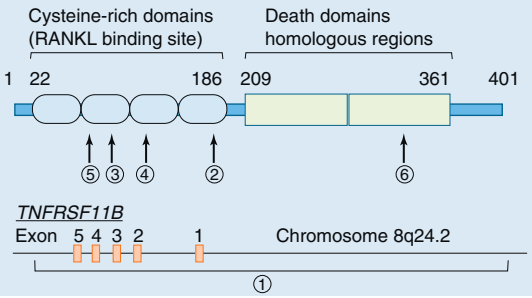
Mice lacking the *Tnfsf11* or *Tnfrsf11a* gene exhibited severe osteopetrosis accompanied by a defect in tooth eruption owing to a complete lack of osteoclasts [14–16]. RANKL transgenic mice showed a marked decrease in bone mass and increase in the number of osteoclasts [17]. By contrast, mice lacking the *Tnfrsf11b* gene exhibited severe osteoporosis with spontaneous fractures, resulting from both increased number and enhanced activity of osteoclasts [18,19]. Interestingly, the number and activity of osteoblasts in the mice were also elevated, indicating the systemic nature of the increased bone turnover [20]. OPG transgenic mice showed a severe osteopetrotic phenotype without failed tooth eruption and the development of osteoclasts was inhibited [20]. These findings clearly demonstrate that

Box 1. Phenotypes of mouse mutants in the RANKL/RANK/OPG axis

Phenotype	RANKL KO	RANKL Tg	RANK KO	RANK Tg	OPG KO	OPG Tg
Skeletal system						
	Osteopetrosis No tooth eruption	Osteoporosis Partial rescue of osteopetrosis	Osteopetrosis No tooth eruption	N.D.	Osteoporosis Bone fracture	Osteopetrosis
Immune system						
T lymphocytes	$CD4^+/CD8^+$ ratio normal T-cell activation impaired	N.D.	$CD4^+/CD8^+$ ratio normal T-cell activation N.D.	N.D.	N.D.	Normal
B lymphocytes	Development impaired	N.D.	Development impaired	N.D.	N.D.	Normal
Thymus	Size/development impaired mTECs impaired	N.D.	Size/development normal mTECs impaired	N.D.	Size/development N.D. mTECs increased	N.D.
Lymph nodes	Peripheral lymph node defect Peyer's patch small	N.D.	Peripheral lymph node defect Peyer's patch small	N.D.	N.D.	Normal
Spleen	Normal architecture Extramedullary hematopoiesis	N.D.	Normal architecture Extramedullary hematopoiesis	N.D.	N.D.	Normal
Dendritic cells	Normal	N.D.	Normal	N.D.	N.D.	N.D.
Others						
Mammary gland	Development impaired	N.D.	Development impaired Inhibition of tumorigenesis	Acceleration of tumorigenesis	N.D.	N.D.
Vascular system	N.D.	N.D.	N.D.	N.D.	Arterial calcification	Rescue of calcification
Behavior	N.D.	N.D.	N.D.	N.D.	Hearing loss	N.D.
Inflammation	Osteopetrosis No osteoclasts No joint erosion	Inhibition of skin inflammation	Osteopetrosis No osteoclasts No joint erosion	N.D.	N.D.	N.D.
Refs	[14,39,68–71]	[17,39,72]	[15,16,73–75]	[76]	[18,19,69,70,77]	[20]

Abbreviations: KO, knockout; N.D., not determined; Tg, transgenic.

Table 1. Genetic inheritance of *RANKL, RANK* and *OPG* in human

Gene (Product)	Allelic variants		Type of mutation (genetic inheritance)	Feature	Refs
TNFSF11 (RANKL)	OMIM (Online Mendelian Inheritance in Man) 602642 	(1) A145delS177 (532+4_532+8del) (2) M199K (596T→A) (3) V277WfsX5 (828_829delGC)	Loss-of-function (ARO; autosomal recessive osteopetrosis)	Severe osteopetrosis Osteoclasts absent RANKL rescues osteoclastogenesis *in vitro* Hematopoietic stem cell transplant (HSCT); no alternative therapy	[40]
TNFRSF11A (RANK)	OMIM 603499 	(1) R170G (508A→G) (2) C175R (523T→C) (3) R129C (385C→T) (4) A244S (730G→T) (5) G53R (157G→C) (6)W434X (1301G→A) (7) G280X (838G→T)	Loss-of-function (ARO; autosomal recessive osteopetrosis)	Severe osteopetrosis Osteoclasts absent/few RANKL does not induce multinuclear osteoclasts *in vitro* Hypogammaglobulinemia associated with B cell impairment HSCT a possible therapy	[78]
		(8) 84dup18 (9) 83dup18	Gain-of-function (FEO; familial expansile osteolysis)	Osteolytic lesions, hearing loss, tooth loss Anti-osteoclast therapy possible	[79,80]
		(10) 84dup15	Gain-of-function (ESH; expansile skeletal hyperphosphatasia)	Osteolytic lesions, hypercalcemia Hearing loss, tooth loss Anti-osteoclast therapy possible	[81]
		(11) 75dup27 (12) H141Y (421C→T) (13) V192A (575T→C)	Gain-of-function (PDB; Paget's disease of bone)	Osteolytic lesions, hypercalcemia Anti-osteoclast therapy possible	[79,82]
TNFRSF11B (OPG)	OMIM 602643 	(1) 100 kb deletion (includes the gene) (2) D182del (638_640delGAC) (3) C87Y (354G→A) (4) F117L (443T→C) (5) C65R (287T→C) (6) D323SfsX3 (965_967delTGA969_970insTT)	Loss-of-function (JPD; juvenile Paget's disease)	Osteolytic lesions, hyperphosphatasia Hearing loss, tooth loss Ant-osteoclast therapy possible	[83–85]

the RANKL/RANK/OPG system is essential for osteoclastogenesis *in vivo*. The phenotypes of these genetically modified mice are summarized in Box 1. Mutations in RANK, RANKL and OPG have been identified in patients with bone disorders such as familial expansile osteolysis, autosomal recessive osteopetrosis, and Juvenile Paget's disease, respectively [21] (Table 1).

RANKL is a membrane-anchored molecule which is released from the cell surface as a soluble molecule upon proteolytic cleavage by matrix metalloproteinases (MMPs) such as MMP-14 [22,23]. Both the soluble and membrane-bound RANKL forms function as agonistic ligands for RANK. However, the membrane-bound RANKL functions significantly more efficiently [22–24]. RANKL has been shown to be expressed in mesenchymal cells, such as osteoblasts and bone marrow stromal cells (BMSCs). RANKL expression on calvarial cells, including osteoblasts, is upregulated by osteoclastogenic factors such as vitamin D_3, prostaglandin E_2, parathyroid hormone, interleukin (IL)-1, IL-6, IL-11, IL-17, and TNF-α [22,25].

RANKL expression in osteocytes

Osteoclastogenesis is induced by the cell–cell contact between osteoclast precursor cells of the monocyte/macrophage lineage and mesenchymal cells in bone [4,7]. Osteoblast linage cells and BMSCs are thought to be the major cell types that express RANKL in support of osteoclastogenesis [1,7]. However, because RANKL is expressed by other cell types in both bone and bone marrow, including osteocytes and lymphocytes, the actual major source of RANKL *in vivo* was, until recently, unclear.

Osteocytes, the most numerous and least well studied bone cells, are stellate-shaped cells enclosed within the bone lacuno-canalicular network [26]. Osteocytes are descended from mesenchymal cells which underwent osteoblast differentiation [26]. Based on the osteocyte location within the bone matrix and cellular morphology, it is proposed that osteocytes potentially contribute to the regulation of bone remodeling in response to mechanical and endocrine stimuli [2,26]. Importantly, in the course of an *in vivo* bone graft experiment, osteoclast differentiation and activation were induced in grafted bone containing living osteocytes. As a result, the grafted bone came to be replaced by newly formed bone. However, osteoclastic bone resorption and osteoblastic bone formation were not observed on the surface of grafted bone which contained only dead osteocytes [27]. In an organ culture of calverial bone, the bone resorption activity of osteoclasts was thus supported by living but not dead osteocytes [28]. In addition, the osteocyte support of osteoclastogenesis was suggested by the observation that both isolated avian osteocytes and the osteocyte-like cell line MLO-Y4 induced osteoclastogenesis [26]. Previous studies demonstrated that the targeted ablation of osteoblasts in mice harboring a thymidine kinase transgene under the control of osteocalcin or type I collagen promoter [29,30]. These mice did not affect the number of osteoclasts, bone resorption, or the expression level of RANKL in bone tissue. Thus, it appears that osteoblasts are not essential for osteoclastogenesis and osteocytes may play an important role in the initiation of bone remodeling through their role in the differentiation and activation of osteoclasts.

To identify the most physiologically relevant osteoclastogenesis-supporting cells among the mesenchymal lineage cells in bone, the expression of RANKL in osteoblasts and osteocytes was analyzed. The conventional method for isolation and purification by the enzymatic digestion of bone to obtain an osteocyte-rich fraction [31] was followed, and the cells exhibited a high expression level of osteocyte marker genes such as *Dmp1*(encoding dentin matrix protein 1) and *Sost* (encoding sclerostin), but not the osteoblast marker *Kera* (encoding keratocan) [32]. Interestingly, *Tnfsf11* was found to be more highly expressed in the osteocyte-rich fraction than the osteoblast-rich fraction, although the percentage of osteocytes is reported to be only ~60–70% using this conventional method [31]. Generation of an osteocyte-specific enhanced green fluorescence protein (EGFP) reporter mouse line, in which osteocytes could be identified by the expression of EGFP driven by the *Dmp1* promoter, and by using fluorescence-activated cell sorting, facilitated the isolation of high-purity osteocytes from fractions obtained by enzymatic digestion of the neonatal calvariae or adult long bones [33]. The isolated osteocytes morphologically exhibited dendritic processes and exclusively expressed *Dmp1*, *Sost*, *Reln* (encoding reelin) and *Npy* (encoding neuropeptide Y), which are well-known osteocyte-specific genes. By contrast, osteoblast-specific genes such as *Kera* and *Fmod* (encoding fibromodulin) were strongly expressed in isolated osteoblasts. Notably, osteocytes expressed a much higher amount of RANKL and had a much greater capacity to support osteoclastogenesis than either osteoblasts or BMSCs [33].

Bone remodeling induced by osteocyte-derived RANKL

To determine the physiological role of RANKL expressed by osteocytes, osteocyte-specific RANKL-deficient mice were generated (RANKL-floxed mice crossed to *Dmp1–Cre* mice) [33]. These mice did not exhibit any gross abnormalities such as a defect in tooth eruption or growth retardation, but the bone volume was greatly increased and the bone marrow cavity was abnormally filled with trabecular bone in the adults [33]. The number of osteoclasts and the parameters of osteoclastic bone resorption were markedly reduced. These results show that osteocyte-specific RANKL-deficient mice develop severe osteopetrosis due to a lack of osteoclasts. Interestingly, no unusual phenotype was evident in the osteocyte-specific RANKL-deficient mice at birth, but the postnatal osteopetrotic phenotype became increasingly obvious with age [33]. Thus, RANKL expressed by osteocytes contributes more significantly to physiological bone remodeling after birth than to skeletal development in the embryo (Figure 1).

Although the relative contribution of the membrane-bound and soluble forms of RANKL expressed by osteocytes remains to be elucidated, cell–cell contact was required for osteoclastogenesis in a coculture of osteocytes and osteoclast precursor cells [33]. Osteocyte conditioned medium alone did not potently induce osteoclastogenesis [33]. These results suggest that in osteocytes membrane-bound RANKL has a powerful physiological role. Anatomically, osteocytes can contact osteoclast precursor cells and mature osteoclasts through their long processes which

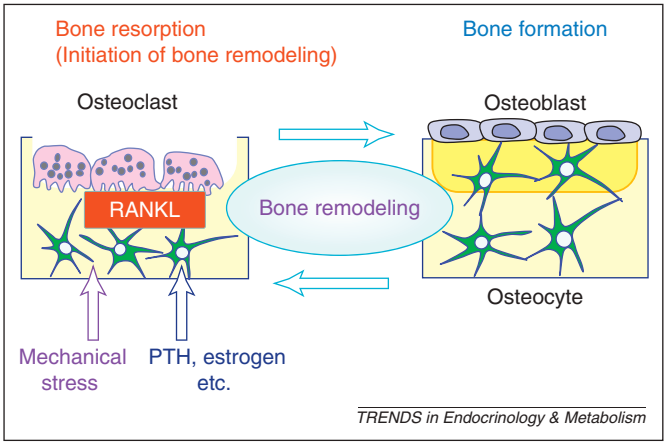


Figure 1. Osteocytes are the command cell at the time of the initiation of bone remodeling through RANKL expression. Osteocytes, the most numerous but also the least studied cell type in bone, are embedded within the lacuno-canalicular network. Based on the site of localization, osteocytes are thought to orchestrate bone homeostasis by regulating both bone-forming osteoblasts and bone-resorbing osteoclasts. Recent studies have provided *in vivo* evidence for the key role of osteocyte-derived RANKL in bone remodeling in response to mechanical loading, thus establishing a molecular basis for osteocyte regulation of bone resorption.

reach the bone surface and vascular space, thus directly communicating through membrane-bound factors [34,35].

Dr O'Brien's group has also generated mice in which RANKL was conditionally deleted in osteoblasts, osteocytes and chondrocytes [36]. These mice carrying the *Cre* transgene under the control of the type X collagen, osterix, or osteocalcin promoters exhibited severe osteopetrosis. These results demonstrate that the RANKL expressed by osteoblasts and chondrocytes has a crucial role in the regulation of osteoclastogenesis in skeletal development. Consistent with another finding, osteocyte-specific RANKL-deficient mice exhibited an increased bone mass with age. Furthermore, to evaluate the role of RANKL expressed on committed osteoblast progenitors, doxycycline was administered to conditional RANKL knockout mice from *in utero* to four months of age, to suppress Cre recombinase of the osterix–*Cre* transgene [36]. When maintained under doxycycline-free conditions for a further two months, the mice did not exhibit abnormal bone phenotype or osteoclast numbers, and the expression of osteoclast-related genes was also normal. These findings suggest that RANKL on committed osteoblasts in adult mice does not contribute to osteoclastogenesis in bone remodeling.

Osteocytes are mechanosensory cells, suggesting that RANKL on osteocytes may be active in response to physiological mechanical stress, the importance of which increases after birth [33]. Consistent with this notion, *Tnfsf11* expression was induced by mechanical stress inflicted on osteocyte cell line MLO-Y4 cells [33]. In addition, osteocyte-specific RANKL-deficient mice were shown to be resistant to unloading-induced bone loss, a model of pathological bone remodeling [36]. These findings suggest that osteocytes sense the mechanical stress and modify RANKL expression. However, it is unclear how or why osteocytes express RANKL to such a greater degree than other cells such as osteoblasts in bone microenvironments, but the difference in mechanical stress response may be one of the reasons because these cells express receptors for parathyroid hormone and vitamin D_3 which are thought to be physiological inducers of RANKL [37,38].

Despite T-cell expression of RANKL, the osteoclastogenic T-cell subset is limited to autoimmune T cells [1], and RANKL expression in bone marrow cells, including T cells, is much lower than that in bone cells (including osteoblasts and osteocytes) [33]. However, it has also been reported that osteopetrosis in RANKL-null mice was partly rescued when these mice were crossed with transgenic mice expressing RANKL under the control of a lymphocyte-specific promoter [39]. However, T cell-specific RANKL-deficient mice did not exhibit any discernible osteopetrotic phenotype [33]. In addition, osteopetrosis in humans with a mutation affecting RANKL does not recover upon bone marrow transfer [40]. These findings strongly suggest that the RANKL expressed on T cells does not significantly contribute to the physiological regulation of osteoclastogenesis, although the specific function of the RANKL on T cells in the pathological bone destruction that occurs in autoimmune diseases such as RA remains to be elucidated.

The intracellular signaling mechanism of RANKL

RANK is a transmembrane molecule expressed on osteoclast progenitor cells and mature osteoclasts [13]. Binding of RANK to RANKL results in the commitment of monocyte/macrophage precursor cells to the osteoclast lineage and the activation of mature osteoclasts [1]. RANK lacks intrinsic enzymatic activity in its intracellular domain, and transduces signals by recruiting adaptor molecules such as the TNF receptor-associated factor (TRAF) family of proteins [1]. Genetic approaches followed by intensive molecular analyses have identified TRAF6 as the main adaptor molecule that links RANK to both the differentiation and function of osteoclasts (Figure 2) [41,42]. By an as yet unknown mechanism, RANKL binding to RANK induces the trimerization of RANK and TRAF6, which leads to the activation of NF-κB and MAPKs [1,21]. It has not yet been determined how RANK alone, among the TRAF6-binding receptors, is able to stimulate osteoclastogenesis potently under physiological conditions. Additional RANK-specific adaptor molecules might exist which link RANK signaling to other pathways [1,21]. For example, the molecular scaffold Grb2-associated binding protein 2 (Gab2) and Fhl2 have been shown to be associated with RANK and to have an important role in its signal transduction [43,44].

The essential role of NF-κB in osteoclastogenesis has been demonstrated genetically [45,46]. NF-κB p50 and p52 double-deficient mice develop severe osteopetrosis because of a defect in osteoclastogenesis. The activator protein 1 (AP-1) transcription factor complex is also essential for osteoclastogenesis [47]. RANK activates AP-1 through the induction of c-Fos, which was recently shown to be dependent on the activation of calcium/calmodulin-dependent protein kinase type IV (CaMKIV) and cAMP-response element-binding protein (CREB) (Figure 2) [48].

NFATc1: the key transcription factor in osteoclast development

RANKL both specifically and potently induces nuclear factor of activated T cells cytoplasmic 1 (NFATc1), the

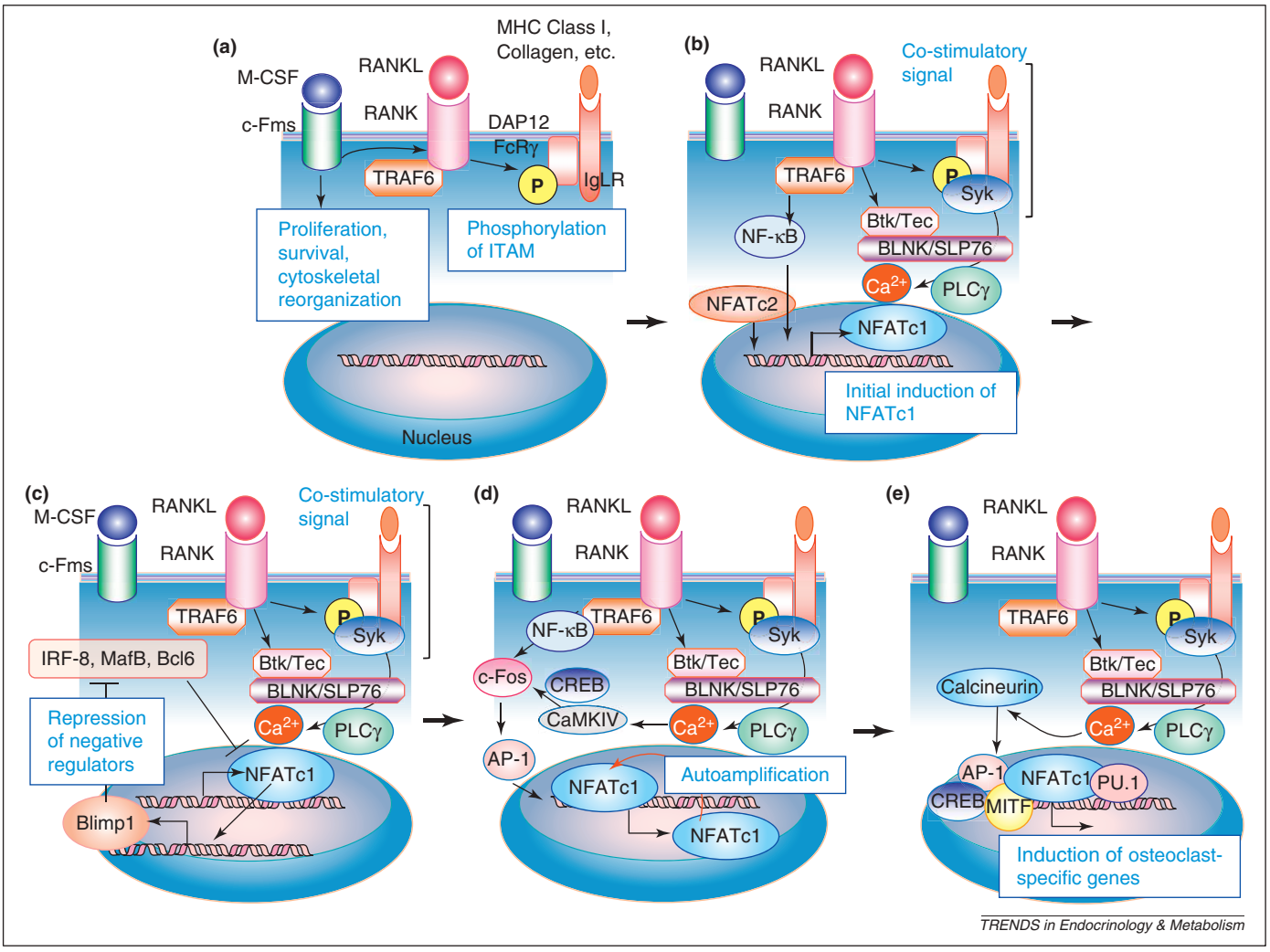


Figure 2. Signal transduction in osteoclast differentiation. Osteoclastogenesis is cooperatively induced by M-CSF, RANKL and its co-stimulatory factor, immunoglobulin-like receptor. **(a)** Precursor cell stage: the binding of M-CSF to its receptor, c-Fms, activates the proliferation, survival and cytoskeletal reorganization of osteoclast precursor cells of the monocyte/macrophage lineage and induces RANK expression. The co-stimulatory receptors appear to be stimulated in the early stages. Proximal RANK signals: RANKL binding to RANK results in the recruitment of TRAF6 and, at the same time, the phosphorylation of the ITAM in DAP12 and FcRγ, which are adaptor proteins associating with distinct immunoglobulin-like receptors. **(b)** Initial induction of NFATc1: NFATc1, a master transcription factor for osteoclastogenesis, is initially induced by the TRAF6-activated NF-κB and NFATc2 that are present in the cell before RANKL stimulation. RANK and ITAM signals cooperate to phosphorylate PLCγ and activate calcium signaling, which is crucial for the activation of NFATc1. The tyrosine kinases Btk and Tec are activated by RANK and are important for the phosphorylation of PLCγ, thus linking the two pathways. **(c)** Disinhibition of NFATc1: NFATc1 activity is negatively regulated by other transcription factors, such as IRF-8, MafB and Bcl6. The expression of such negative regulators was observed to be repressed in osteoclastogenesis. Blimp1, which is induced by RANKL through NFATc1 during osteoclastogenesis, functions as a transcriptional repressor of anti-osteoclastogenic genes. **(d)** Autoamplification of NFATc1: calcium signal-mediated persistent activation of NFATc1, as well as cooperation with AP-1, are prerequisites for the robust induction of NFATc1. AP-1 activation is mediated by the induction and activation of c-Fos by CaMKIV-stimulated CREB and c-Fms. The NFATc1 promoter is epigenetically activated through histone acetylation and NFATc1 binds to an NFAT-binding site on its own promoter. **(e)** Induction of osteoclast-specific genes: NFATc1 works together with other transcription factors, such as AP-1, PU.1, CREB and MITF, to induce various osteoclast-specific genes. Abbreviations: AP-1, activator protein 1; Bcl6, B cell lymphoma 6; Blimp1, B lymphocyte-induced maturation protein-1; Btk, Bruton's tyrosine kinase; CREB, cAMP-responsive element-binding protein; DAP12, DNAX-activating protein; FCRγ, Fc receptor common γ subunit; Gab2, Grb2-associated binding protein 2; IRF-8, Interferon regulatory factor-8; ITAM, immunoreceptor tyrosine-based activation motif; JNK, JUN N-terminal kinase; MafB, v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein B; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; NF-κB, nuclear factor-κB; OSCAR, osteoclast-associated receptor; PIR-A, paired immunoglobulin-like receptor A; PIR-B, paired immunoglobulin-like receptor B; PLCγ, phospholipase Cγ; RANKL, receptor activator of nuclear factor-κB ligand; SIRPβ1, signal-regulatory protein β1; Syk, spleen tyrosine kinase; TRAF6, tumor necrosis factor receptor-associated factor 6; TREM-2, triggering receptor expressed in myeloid cells 2.

master regulator of osteoclast differentiation, and this induction is dependent on both the TRAF6–NF-κB and c-Fos pathways [49]. The NFAT family of transcription factors was originally discovered in T cells, hence the name, but they are in fact involved in the regulation of various biological systems [50]. The activation of NFAT is mediated by a specific phosphatase, calcineurin, which is activated by calcium–calmodulin signaling. The essential and sufficient role of the *Nfatc1* gene in osteoclastogenesis has been demonstrated both *in vitro* and *in vivo* [49,51,52]. The *Nfatc1* promoter contains NFAT binding sites and NFATc1 specifically autoregulates its own promoter during osteoclastogenesis, resulting in robust induction of NFATc1 [51]. AP-1 containing c-Fos, together with continuously activated calcium signaling, is crucial for this autoamplification [49]. NFATc1 regulates several osteoclast-specific genes in cooperation with other transcription factors, such as AP-1, PU.1 and MITF [1]. Osteoclasts mature into multinuclear giant cells by the fusion of mononuclear osteoclasts. The expression of fusion-mediating molecules such as the d2 isoform of the vacuolar ATPase V0 domain and the dendritic cell-specific transmembrane protein are

directly regulated by NFATc1[21]. A previous study reported that CREB, activated by CaMKIV, also cooperates with NFATc1 in the activation of osteoclast-specific genes (Figure 2) [48].

NFATc1 activity is negatively regulated by specific transcription factors such as interferon regulatory factor-8 (IRF-8), B cell lymphoma 6 (Bcl6), and v-Maf musculoaponeurotic fibrosarcoma oncogene family member protein B (MafB) [53–55]. The expression of such negative regulators was observed to be repressed in the course of osteoclastogenesis. This repression is consistent with the notion that high NFATc1 activity is a prerequisite for efficient osteoclastogenesis, but the actual mechanism by which the expression of these anti-osteoclastogenic regulators is repressed during RANKL-induced osteoclastogenesis remains obscure. Recent studies indicate that B lymphocyte-induced maturation protein-1 (Blimp1), which is induced by RANKL via NFATc1 during osteoclastogenesis, functions as a transcriptional repressor of anti-osteoclastogenic genes such as *Irf8*, *Bcl6* and *Mafb* [54,56]. Therefore, NFATc1 choreographs the determination of cell fate in the osteoclast lineage by inducing the repression of negative regulators as well as through its effect on positive regulators (Figure 2).

Calcium signaling and immunoreceptors in osteoclastogenesis

Phospholipase Cγ (PLCγ), which mediates Ca^{2+} release from intracellular stores, is crucially important for the activation of the key transcription factor NFATc1 via calcineurin (Figure 2) [49]. However, despite the evident importance of the calcium–NFAT pathway, it had long been unclear until recently how RANKL specifically activates calcium signals. RANK belongs to the TNF receptor family, which has yet to be directly connected to calcium signaling. The activation of PLCγ by RANK requires the protein tyrosine kinase Syk, along with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules, such as DNAX-activating protein 12 (DAP12) and the Fc receptor common γ chain (FcRγ) [57]. In the osteoclast lineage, the immunoglobulin-like receptors (IgLR) associated with DAP12 include the triggering receptor expressed in myeloid cells 2 (TREM-2) and signal-regulatory protein β1 (SIRPβ1); those associated with FcRγ include osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor A. 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 while osteoclasts are undergoing differentiation [58]. Because ITAM signals are essential for osteoclastogenesis, but by themselves cannot induce osteoclastogenesis, these signals should properly be considered as co-stimulatory signals 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 [59]. Thus, RANKL and M-CSF signals appear to converge on the ITAM signaling pathway.

It is also conceivable that RANK activates an as yet unknown pathway that specifically synergizes with or upregulates ITAM signaling. Recently, it was shown that Tec family tyrosine kinases such as Btk and Tec are activated by RANK, and are involved in the phosphorylation of PLCγ, which leads to the release of calcium from endoplasmic reticulum through the generation of IP3 [60]. An osteopetrotic phenotype in Tec and Btk double-deficient mice revealed these two kinases play an essential role in the regulation of osteoclastogenesis [60]. Tec and Btk were previously reported to play a key role in proximal BCR signaling, but this study established their crucial role in linking the RANK and ITAM signals (Figure 2). 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.

Bone destruction with arthritis as a RANKL-driven disease

The original identification of osteoclast-like giant cells at the interface between the synovium and bone in rheumatoid joints dates back to the early 1980s [61]. These multinucleated giant cells were subsequently further characterized as being positive for TRAP and calcitonin receptor, which are characteristic features of authentic osteoclasts. TRAP-positive multinucleated cells are frequently observed in the synovium, which is not in contact with bone [62]. These pathological findings led investigators to hypothesize that osteoclasts play an important role in bone resorption in arthritis and that osteoclasts are formed in the synovium [62,63]. Can osteoclasts be generated from synovial cells alone? This question was answered affirmatively by generating osteoclasts in synovial cell culture without the addition of any other cells, thus demonstrating that rheumatoid synovial cells contain both osteoclast precursor and osteoclastogenesis-supporting cells [63]. Further studies indicated that synovial fibroblasts express membrane-bound factor(s) that stimulate osteoclastogenesis and induce the differentiation of synovial macrophages into osteoclasts, but it was not until RANKL was cloned that the membrane-bound factor on synovial cells was brought to light [64].

Importantly, inflammatory cytokines such as IL-1, IL-6 and TNF-α, which are abundant in the synovial fluid and synovium of RA patients, have a potent capacity to induce RANKL on synovial fibroblasts and thus accelerate RANKL signaling, thereby directly contributing to the bone destruction process [1]. Several groups have demonstrated a high level of RANKL expression in the synovium of RA patients [64,65]. RANKL was found to be expressed by synovial and T cells, both of which are found in inflamed synovium [64–66], but at that point it was unclear which cell types were the major RANKL-expressing cells. Since then, a series of reports have established that the pathological bone damage associated with inflammation is caused by an abnormal expression of RANKL. In addition, osteoclast-deficient mice and ARO patients are protected from bone erosion in arthritis [1,67]. In the absence of osteoclasts, bone destruction did not occur, despite a similar level of inflammation, indicating that RANKL and osteoclasts are indispensable for the inflammation-associated bone loss [68]. Blocking RANKL significantly prevents bone destruction in adjuvant arthritis [66]. Consistent

with this, 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, as well as in the treatment of an animal model of arthritis [1].

Concluding remarks

The skeletal and immune systems share a remarkable array of molecules and regulatory mechanisms, and osteoimmunology has attracted considerable attention in recent years. Osteoclast biology has been the driving force in this trend. Most of the molecules involved in osteoclastogenic signaling were originally identified and extensively studied in the field of immunology, but these molecules have turned out to be auspicious therapeutic targets for bone diseases as well. This approach will be facilitated by the increasing availability of genetically modified mice. Such animal models will surely lead to a deeper understanding of the molecular basis for the cell lineage specifications, and perhaps most importantly, cell type-specific treatments, despite the similarity and indeed evident overlap of the skeletal and immune systems. The identification of osteocytes as the major source of RANKL in bone remodeling has shed light on the regulatory network of bone homeostasis and may provide a basis for future therapeutic strategies.

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