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REVIEW

Negative regulation of osteoclastogenesis and bone resorption by cytokines and transcriptional repressors

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Abstract

Bone remodeling in physiological and pathological conditions represents a balance between bone resorption mediated by osteoclasts and bone formation by osteoblasts. Bone resorption is tightly and dynamically regulated by multiple mediators, including cytokines that act directly on osteoclasts and their precursors, or indirectly by modulating osteoblast lineage cells that in turn regulate osteoclast differentiation. The critical role of cytokines in inducing and promoting osteoclast differentiation, function and survival is covered by the accompanying review by Zwerina and colleagues. Recently, it has become clear that negative regulation of osteoclastogenesis and bone resorption by inflammatory factors and cytokines, downstream signaling pathways, and a newly described network of transcriptional repressors plays a key role in bone homeostasis by fine tuning bone remodeling and restraining excessive bone resorption in inflammatory settings. In this review we discuss negative regulators of osteoclastogenesis and mechanisms by which these factors suppress bone resorption.

Introduction

Osteoclasts, the exclusive bone resorptive cells, play an important role not only in physiological bone development and remodeling, but also function actively as a central pathogenic factor ('culprit') leading to musculo-skeletal tissue damage and accelerating pathogenesis of

diseases characterized by inflammatory osteolysis, including rheumatoid arthritis, psoriatic arthritis, periodontitis and peri-prosthetic loosening. In these diseases, abnormally enhanced osteoclast formation and activity cause bone loss that can result in pain, deformity, osteopenia, osteoporosis and even fracture.
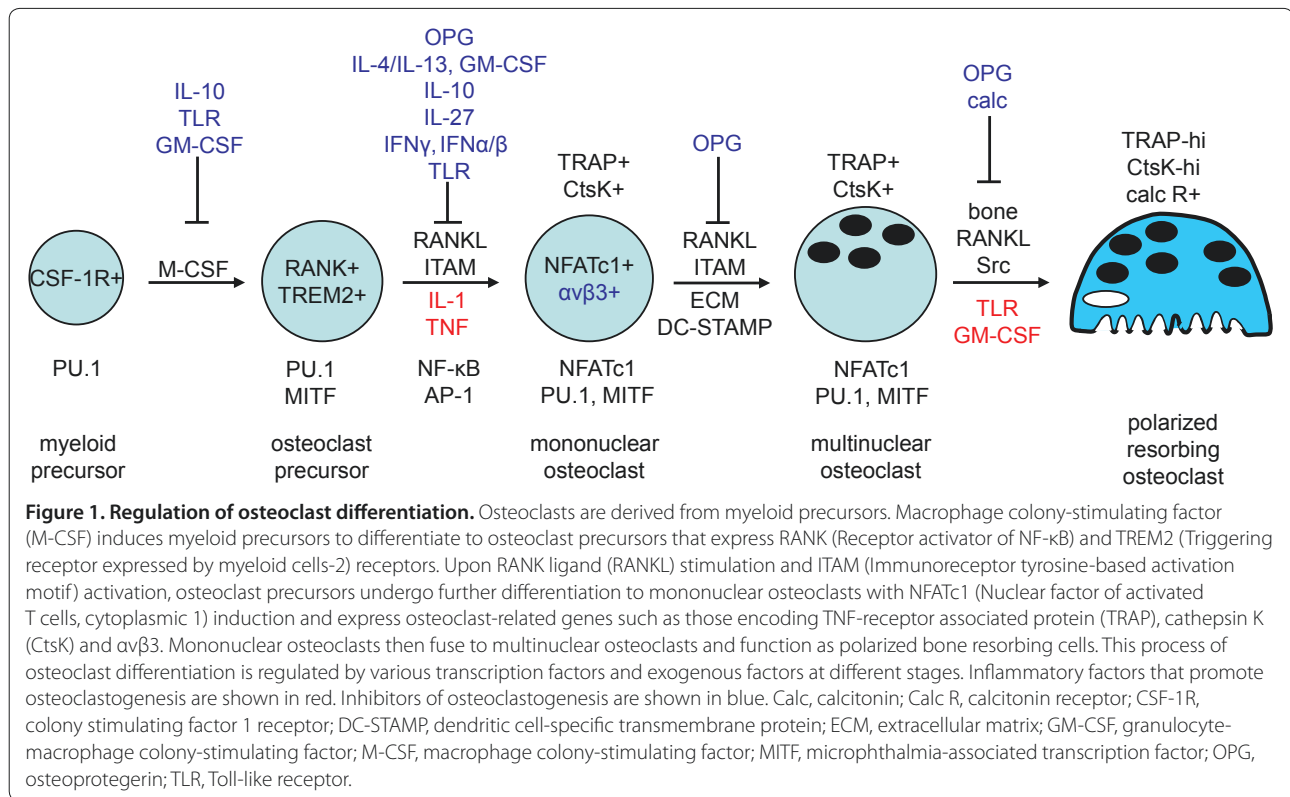
It has been extensively documented that a variety of inflammatory molecules, such as TNF-α, IL-1β, IL-17, and Toll-like receptor (TLR) ligands, promote osteoclastogenesis synergistically with RANKL (Receptor activator of NF-κB ligand) to induce pathological bone resorption in inflammatory settings. In addition, there is a great deal of recent evidence that various inflammatory factors produced by activated immune cells act as anti-osteoclastogenic factors by different mechanisms (Figure 1). Suppression of osteoclastogenesis by inflammatory factors and cytokines functions as a feedback inhibition system that limits bone resorption and tissue damage associated with infection or inflammation. These inflammatory factors can suppress osteoclastogenesis directly by inhibiting differentiation of osteoclast precursors or indirectly by regulating differentiation and expression of RANKL and osteoprotegerin (OPG) by mesenchymal cells, such as osteoblastic/stromal cells and synovial fibroblasts, or by T cells.

Osteoclast differentiation is physiologically triggered by RANKL in the presence of macrophage colony-stimulating factor (M-CSF) and unknown co-stimulatory factors. Upon RANK stimulation, a broad range of signaling cascades are activated, such as canonical and non-canonical NF-κB pathways, protein tyrosine kinases, such as BtK/Tec, calcium signaling, and mitogen-activated protein kinase (MAPK) pathways, including p38 and Erk. These signaling cascades, which are reviewed in depth in the accompanying review by Zwerina and colleagues [1], lead to induction of the transcription factor NFATc1 (Nuclear factor of activated T cells, cytoplasmic 1), which serves as a 'master regulator' of osteoclastogenesis, together with other transcription factors, such as NF-κB

Figure 1. Regulation of osteoclast differentiation. Osteoclasts are derived from myeloid precursors. Macrophage colony-stimulating factor (M-CSF) induces myeloid precursors to differentiate to osteoclast precursors that express RANK (Receptor activator of NF-κB) and TREM2 (Triggering receptor expressed by myeloid cells-2) receptors. Upon RANK ligand (RANKL) stimulation and ITAM (Immunoreceptor tyrosine-based activation motif) activation, osteoclast precursors undergo further differentiation to mononuclear osteoclasts with NFATc1 (Nuclear factor of activated T cells, cytoplasmic 1) induction and express osteoclast-related genes such as those encoding TNF-receptor associated protein (TRAP), cathepsin K (CtsK) and αvβ3. Mononuclear osteoclasts then fuse to multinuclear osteoclasts and function as polarized bone resorbing cells. This process of osteoclast differentiation is regulated by various transcription factors and exogenous factors at different stages. Inflammatory factors that promote osteoclastogenesis are shown in red. Inhibitors of osteoclastogenesis are shown in blue. Calc, calcitonin; Calc R, calcitonin receptor; CSF-1R, colony stimulating factor 1 receptor; DC-STAMP, dendritic cell-specific transmembrane protein; ECM, extracellular matrix; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; OPG, osteoprotegerin; TLR, Toll-like receptor.

and c-fos, to drive osteoclastogenesis [2] (Figure 2). More recently, transcriptional repressors that suppress RANKL-induced gene expression and differentiation have been described (Figure 2). These repressors can work as homeostatic factors in regulating osteoclastogenesis in physiological bone development and remodeling, and also as feedback inhibitors that limit bone resorption associated with inflammation. The extent of bone destruction in inflammatory diseases is determined by the balance between osteoclastogenic and anti-osteoclastogenic factors.
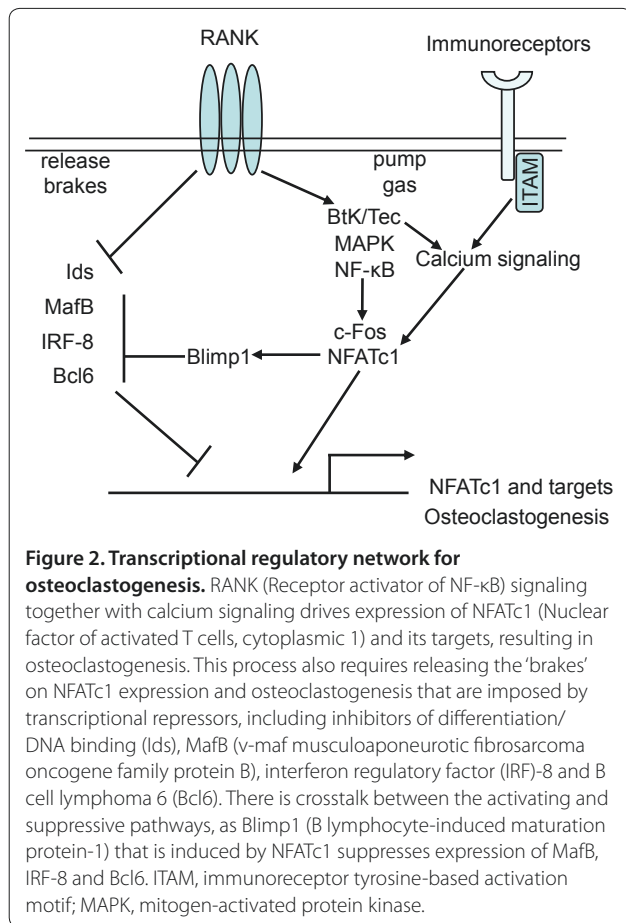
Cytokines
IL-4/IL-13 and granulocyte-macrophage colony-stimulating factor
IL-4 and IL-13 have pleiotropic immune functions and are produced by Th2 lymphocytes, although IL-13 can also be produced by stromal cells. Since IL-4 and IL-13 utilize closely related receptor complexes, they have many overlapping features, including downstream signaling and some biological functions. IL-4, more effectively than IL-13, directly prevents osteoclast precursors from differentiating into osteoclasts in a signal transducer and activator of transcription (STAT)6-dependent manner [3,4]. IL-4 suppresses RANK expression, NF-κB, MAPK and calcium signaling, and expression of NFATc1 and c-Fos during osteoclastogenesis [3-5]. In addition, IL-4

inhibits bone resorption and actin ring formation in human mature osteoclasts by suppressing NF-κB and calcium signaling. On the other hand, IL-4 and IL-13 indirectly suppress osteoclastogenesis by inhibiting RANKL but enhancing OPG expression in osteoblastic cells [3,4]. Although IL-4 suppresses spontaneous or parathyroid hormone-related protein (1-34)-stimulated osteoclast formation in mice, IL-4 transgenic mice exhibit an osteoporotic phenotype that is attributed to a more dominant suppressive effect of IL-4 on osteoblast formation *in vivo* relative to its role in suppressing osteoclastogenesis. Thus, it is important to note that the net effect of IL-4 on bone turnover *in vivo* represents an integrated outcome of its influence on various cell populations.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibits osteoclastogenesis by diverting osteoclast precursors to a macrophage lineage [6]. The osteoclast suppressive mechanism was recently suggested to involve proteolytic cleavage of cell surface M-CSF receptor after treatment with GM-CSF and IL-4 [7]. The combination of GM-CSF and IL-4 enhances expression and activity of TACE (TNF-α converting enzyme)/ ADAM17 (a disintegrin and metalloproteinase 17) in human monocytes. This results in cleavage of cell surface M-CSF receptor, leading to disruption of M-CSF signaling and thereby suppressing osteoclastogenesis and diverting the cells toward the dendritic cell lineage [7].

Figure 2. Transcriptional regulatory network for osteoclastogenesis. RANK (Receptor activator of NF-κB) signaling together with calcium signaling drives expression of NFATc1 (Nuclear factor of activated T cells, cytoplasmic 1) and its targets, resulting in osteoclastogenesis. This process also requires releasing the 'brakes' on NFATc1 expression and osteoclastogenesis that are imposed by transcriptional repressors, including inhibitors of differentiation/DNA binding (Ids), MafB (v-maf musculoaponeurotic fibrosarcoma oncogene family protein B), interferon regulatory factor (IRF)-8 and B cell lymphoma 6 (Bcl6). There is crosstalk between the activating and suppressive pathways, as Blimp1 (B lymphocyte-induced maturation protein-1) that is induced by NFATc1 suppresses expression of MafB, IRF-8 and Bcl6. ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase.

IL-10

IL-10, produced by T and B lymphocytes and myeloid lineage cells, is predominantly an immunosuppressive and anti-inflammatory cytokine that is best known as a potent deactivator of dendritic cells and macrophages. It plays a critical role in limiting tissue injury during infections and in preventing autoimmunity by limiting the duration and intensity of immune and inflammatory reactions. A large body of work has established an important role for IL-10 in suppressing osteoclastogenesis *in vitro* and *in vivo* [8-12]. For example, IL-10 is expressed in periodontitis, and IL-10 polymorphisms have been linked to periodontitis in multiple studies. In periodontitis, IL-10 is a key negative regulator of bone resorption [8,9]. IL-10 directly inhibits osteoclast precursors by suppressing RANKL-induced NFATc1, c-Fos and c-Jun expression [10,11]. Inhibition of RANKL expression and an increase in OPG expression due to IL-10 were found in dental follicle cells that support osteoclastogenesis, suggesting that IL-10 can also indirectly inhibit osteoclastogenesis via modulation of RANKL and OPG expression. A key biological activity of IL-10 is to attenuate inflammation by suppressing TNF-α

and IL-1 production and by antagonizing TNF-α and IL-1 function; thereby, IL-10 may suppress TNF-α- and IL-1-stimulated bone resorption. Recently, our lab, using human osteoclast precursors, showed that IL-10 inhibits calcium signaling by suppressing transcription of TREM-2, an important co-stimulatory receptor for osteoclastogenesis. Downregulation of TREM-2 (Triggering receptor expressed by myeloid cells-2) expression leads to diminished calcium/calmodulin-dependent protein kinase (CaMK)-MEK-ERK activation induced by RANKL [12].

IL-27

IL-27 is produced by antigen-presenting cells and belongs to the IL-12 family of cytokines. IL-27 has pleiotropic immune functions with either activating or suppressive roles in various infectious and inflammatory models. The IL-27 receptor is an IL-27Ra (WSX-1)/gp130 heterodimer. IL-27 mildly suppresses osteoclast differentiation in murine systems, potentially due to the low levels of WSX-1 expression on murine osteoclast precursors, limiting the response of these cells to IL-27 [13-15]. Aggravated arthritic bone erosions and enhanced osteoclastogenesis were observed in *Escherichia coli* cell wall lysate-induced arthritis models in WSX-1 knockout mice compared to wild-type mice [14]. It should, however, be noted that the enhanced inflammation and excessive Th17 cells in WSX-1 knockout arthritis models could also explain the increase in osteoclastogenesis [14]. On the other hand, our lab and other groups [13,14] reported that IL-27 potently inhibits RANKL-induced human osteoclastogenesis and osteoclastic resorptive activity *in vitro* by downregulation of RANK and TREM-2 expression, inhibition of RANKL-activated ERK, p38 and NF-κB signaling, and by suppression of AP-1 (c-Fos and c-Jun) and NFATc1 expression in human osteoclast precursors. IL-27-induced STAT1 activation also partially contributes to its inhibitory function [14]. While expression of IL-27 is observed in human rheumatoid arthritis, synovial fluid macrophages harvested from active rheumatoid arthritis patients are refractory to IL-27 [13]. This suggests that IL-27 has the capacity to protect bone tissue from resorption, but this homeostatic role of IL-27 might be compromised in an active inflammatory microenvironment, such as occurs in RA.

Interferons

IFN-γ, the sole type II IFN, is a product of innate immune cells and Th1 cells. In bone marrow-derived macrophage culture systems, IFN-γ strongly inhibits osteoclastogenesis [16] by suppressing RANK signaling via rapid TNF receptor-associated factor (TRAF)6 degradation in murine osteoclast precursors [16]. IFN-γ also inhibits human osteoclastogenesis, but TRAF6 expression is not

significantly affected [17], suggesting that IFN-γ acts through distinct mechanisms in humans versus mice. Our lab recently found that IFN-γ, alone or in synergy with TLR stimulation, suppresses expression of the M-CSF receptor c-Fms, c-Fms's target *RANK*, and co-stimulatory receptor TREM2 in human osteoclast precursors [17]. In both collagen-induced arthritis and lipopolysaccharide-induced inflammatory bone resorption mouse models, loss of IFN-γ receptor leads to enhanced osteoclast formation and bone destruction [16,18]. IFN-γ also inhibits osteoclast formation to prevent tumor-associated bone loss [19]. These data support an inhibitory role of IFN-γ in osteoclastogenesis *in vivo*. However, administration of recombinant IFN-γ to rodents or osteopetrotic patients stimulates osteoclast formation and bone erosion [20,21]. These contradictory observations of the *in vivo* role of IFN-γ may result from differences in the disease models and, more importantly, the impact of IFN-γ on various cell types. For example, recent data suggest that IFN-γ can not only directly inhibit differentiation of osteoclast precursors, but can also indirectly promote osteoclastogenesis by stimulating T-cell activation and secretion of the osteoclastogenic factors RANKL and TNF-α [22].

Type I IFNs, IFN-α and IFN-β, have also been implicated in suppression of bone resorption. During osteoclastogenesis, RANKL induces IFN-β expression in osteoclast precursors, and IFN-β, in turn, functions as a negative-feedback regulator to suppress osteoclast differentiation by decreasing c-Fos expression [23]. Mice deficient in the type I IFN receptor component IFNAR1 spontaneously develop severe osteopenia with enhanced osteoclastogenesis due to interference of this feedback loop [23]. STAT3 and SOCS (Suppressor of cytokine signaling) proteins downstream of Jak1 are also likely involved in the IFN-β-induced inhibition of osteoclastogenesis, and the ubiquitin-mediated degradation of Jak1 after RANKL stimulation may limit the suppressive effect of IFN-β on osteoclastogenesis [24-26]. IFN-α also blunts *in vitro* osteoclastogenesis, but exogenous IFN-α has no obvious effect on bone turnover *in vivo*. Interestingly, type I IFNs appear to protect from erosive arthritic lesions in the setting of an IFN-driven mouse model of systemic lupus erythematosus, potentially explaining the lack of erosive arthritis in human systemic lupus erythematosus [27].

Additional inhibitory cytokines: TRAIL, IL-12, IL-18, IL-6
TRAIL (TNF-related apoptosis inducing ligand), a TNF family member, impedes osteoclast differentiation [28] and induces apoptosis of osteoclasts [29]. IL-12 plays an inhibitory role in osteoclastogenesis, but it is still controversial whether IL-12 directly inhibits osteoclast precursors or targets other cell types such as stromal/

osteoblastic cells or T cells to indirectly suppress osteoclastogenesis [30]. Apoptosis induced by interactions between IL-12-induced FasL and TNF-α-induced Fas contributes to the inhibitory mechanisms of IL-12 in TNF-α-induced osteoclastogenesis [31]. IL-18 inhibits osteoclastogenesis by a variety of mechanisms, including stimulation of GM-CSF [32] and induction of IFN-γ and OPG. IL-18 alone or synergistically with IL-12 inhibits TNF-α-induced osteoclastogenesis through Fas-FasL-induced apoptosis. IL-18 is induced in rheumatoid arthritis, but contrarily it indirectly stimulates osteoclastogenesis via its induction of RANKL on synovial T cells. IL-6 has been regarded as a stimulator of osteoclastogenesis and bone resorption by stimulating osteoblastic/stromal cell-mediated osteoclast differentiation, but recent studies described an opposite effect of IL-6 that directly targets osteoclast precursors to suppress their differentiation [33,34].

Toll-like receptors and interplay with interferons
TLRs are the best characterized 'pattern recognition receptors' that recognize conserved microbial molecules and mediate immune and inflammatory cellular responses to infection and microbial products and in some cases responses to endogenous factors generated during cell death, inflammation, and tissue damage. Activation of various TLRs directly inhibits the early stages of RANKL-induced osteoclastogenesis [35,36]. The underlying molecular mechanisms include TLR-induced production of IFN-β that suppresses RANKL-induced c-Fos, and inhibition of NFATc1 by decreased JNK activation in response to TLR ligands [37]. However, in a human osteoclast culture system, TLRs can inhibit human osteoclastogenesis independently of type I IFNs [17]. TLR ligands can suppress human osteoclastogenesis by inhibiting expression of c-Fms, RANK and TREM2, thereby rendering osteoclast precursors refractory to M-CSF and RANKL stimulation [17]. Inhibition of RANK expression by TLRs was also observed in murine osteoclast precursors but to a lesser extent [17], suggesting that TLR-induced inhibition of osteoclastogenesis can be mediated by distinct IFN-dependent and IFN-independent mechanisms that can act in parallel. Moreover, TLRs cooperate with IFN-γ to inhibit osteoclastogenesis by synergistically suppressing expression of RANK and c-Fms [17]. These data revealed a complex interplay between TLRs and IFN-γ in the inhibition of osteoclastogenesis, and new mechanisms by which TLRs and IFN-γ prevent osteoclast precursors from differentiating to osteoclasts, while directing them toward becoming inflammatory macrophages. Interferon regulatory factor (IRF)-8, induced by IFN-γ, is a critical negative regulator for osteoclastogenesis in humans and mice, and its downregulation by RANKL is essential for osteoclastogenesis

[38]. We found that RANKL-induced downregulation of IRF-8 is abrogated by TLR activation (Zhao B *et al.*, unpublished data). The inhibitory effect of TLRs on osteoclastogenesis is compromised by IRF-8 deficiency [38], suggesting that regulation of IRF-8 is involved in the mechanisms by which TLRs and IFN-γ inhibit osteoclastogenesis.

TLRs are activated during acute infection, during chronic microbial colonization and invasion as occur in periodontitis, and during chronic sterile inflammation as occurs in rheumatoid arthritis, most likely by tissue degradation products. TLRs are highly expressed on hematopoietic cells and are also expressed on various other cell types, including epithelial cells, fibroblasts, and osteoblasts. Therefore, it is not surprising that, in contrast to their direct inhibitory effect on osteoclast precursors, TLRs can stimulate inflammatory osteolysis *in vivo* by affecting various cell populations and by distinct mechanisms. TLRs have been implicated in the induction of RANKL and TNF-α expression on osteoblastic/stromal cells and thus are involved in stimulating osteoblast/stromal cell-mediated osteoclastogenesis and bone resorption [39]. In addition, TLRs are among the most potent inducers of inflammatory cytokines such as TNF-α and IL-1, which then act to increase RANKL expression on stromal cells and also synergize with RANK signals to drive osteoclastogenesis. Furthermore, TLR activation accelerates differentiation of committed osteoclasts, and promotes mature osteoclast survival [39-41]. Thus, the net effect of TLRs on osteoclastogenesis *in vivo* is mediated by various cell types and is determined by the potency of pro-osteoclastogenic versus anti-osteoclastogenic mechanisms.

Cytotoxic T-lymphocyte antigen 4 and regulatory T cells

Recent exciting work has identified a role for regulatory T cells (Tregs) in restraining osteoclastogenesis and limiting bone resorption [42,43]. Tregs suppress osteoclast precursors directly by a mechanism predominantly dependent on cytotoxic T-lymphocyte antigen 4 (CTLA-4). CTLA-4 is expressed on the surface of activated T cells and Tregs and transmits an inhibitory signal to T cells after binding to its cognate ligands, CD80 and CD86 (also known as B7.1 and B7.2), on antigen-presenting cells. Recent work showed that CTLA-4, which is constitutively expressed by Tregs, directly inhibits osteoclast formation by binding to CD80 and CD86 expressed by osteoclast precursors. This suggests that CTLA-4-mediated ligation of its counter-receptors CD80 and CD86 delivers a negative signal to osteoclast precursors, and provides a potential new explanation for the anti-erosive effect of abatacept, a CTLA-4 immunoglobulin fusion protein used for the treatment of rheumatoid arthritis [42,43].

Inhibitory signaling molecules
NF-κB p100

The NF-κB family comprises RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). NF-κB activation is elicited by two major signaling pathways; the classical pathway mainly involves IκB kinase-β-induced IκBα degradation and subsequent RelA/p50 activation, and the alternative pathway involves NF-kappa-B-inducing kinase-induced p100 processing to p52 and RelB/p52 activation. There is crosstalk between these two pathways, and NF-κB activation from these two pathways plays important positive roles in inducing osteoclastogenesis [2]. On the other hand, recent emerging evidence shows that NF-κB p100 functions as a negative regulator of osteoclastogenesis by binding to NF-κB complexes and preventing their nuclear translocation. Cytosolic accumulation of p100 impairs osteoclastogenesis, whereas p100 deficiency leads to enhanced osteoclastogenesis that contributes to an osteopenic phenotype *in vivo* [44,45]. TNF-α, unlike RANKL, does not seem to activate the alternative NF-κB pathway efficiently, as it induces an accumulation of p100 in osteoclast precursors via induction of TRAF3, thus limiting TNF-α-induced osteoclastogenesis [44]. TNF-Tg mice lacking NF-κB p100 exhibit more severe joint erosion than that of TNF-Tg littermates [44]. Although there is some controversy whether TNF-α positively regulates osteoclastogenesis [44,46], these data suggest that blockade of NF-κB p100 processing might represent a novel therapeutic strategy for inflammatory bone loss as occurs in RA.

Cytosolic phosphatase: SHIP1

SHIP1 (Src homology 2-containing inositol-5-phosphatase 1) is preferentially expressed in hematopoietic cells, including T and B lymphocytes, mast cells and macrophages. M-CSF induces tyrosine phosphorylation of SHIP1 and the association of SHIP1 with c-*fms* via the adaptor protein Shc, whereby SHIP1 specifically dephosphorylates phosphatidylinositol 3,4,5-triphosphate and thus inactivates phosphatidylinositide-3-kinase/Akt-mediated signaling. Genetic evidence from SHIP1-deficient mice showed that SHIP1 negatively regulates osteoclast formation and function. Compared to wild-type mice, SHIP1-deficient mice exhibit increased proliferation of osteoclast precursors with hypersensitivity to M-CSF and RANKL, and increased osteoclasts with prolonged survival and enhanced bone resorptive activity, thus leading to an osteoporotic phenotype [47]. SHIP1 suppresses osteoclastogenesis and bone erosions in K/BxN mouse serum-induced inflammatory arthritis models [48]. The underlying mechanisms of the suppressive effect of SHIP1 on osteoclastogenesis involve negative regulation of M-CSF-dependent Akt activity and

consequent negative regulation of D-type cyclins, up-regulation of cyclin-dependent kinase inhibitor p27, and negative regulation of retinoblastoma and cell prolifera-tion [48]. A recent study revealed a novel mechanism in which SHIP1 interacts with DAP12 (DNAX-activating protein of 12 kDa) via its SH2 domain, thereby directly blocking the binding and activation of phosphatidyl-inositide-3-kinase, and thus limiting TREM2- and DAP12-mediated co-stimulatory signaling for osteoclasto-genesis [49]. It is also interesting to note the morpho-logical and functional similarities between SHIP1 knockout osteoclasts and osteoclasts in patients with Paget's disease, and similar high IL-6 expression [47]. However, the possibility of SHIP1 involvement in Paget's disease requires genetic analysis and additional support-ing evidence.

Notch signaling pathway
The Notch signaling pathway regulates cell proliferation, differentiation and survival. In mammalian cells, there are four Notch receptors (Notch 1 to 4) and five notch ligands (Jagged1, Jagged2, Delta-like (DLL)1, DLL3, and DLL4). Ligation of Notch receptors by their ligands leads to proteolytic cleavage of Notch by ADAM family proteases that releases the extracellular domain followed by intramembranous cleavage by γ-secretase that releases the Notch intracellular domain. The Notch intracellular domain translocates to the nucleus, binds to the DNA-binding protein RBP-J (recombinant recognition sequence binding protein at the Jκ site; also named CSL or CBF1), and activates Notch target genes such as *Hes* and *Hey*. Induction of Notch ligand Jagged1 and expres-sion of Notch receptors 1, 2, and 3 were observed during RANKL-induced osteoclastogenesis [50-52]. Some investigators found that activation of the Notch signaling pathway inhibits RANKL-induced osteoclast differen-tiation [50,51], whereas others described the opposite [52]. The genetic evidence obtained by using bone marrow-derived macrophages from Notch 1/2/3 knock-out mice or Notch 1 or Notch 3 knockout mice, however, solidify the finding that Notch negatively regulates osteo-clastogenesis [51]. The osteoclast inhibitory mechanisms include the suppression of osteoclast precursor proliferation by Notch, likely through inhibition of the expression of the M-CSF receptor c-Fms [51]. On the other hand, Notch also indirectly blunts osteoclasto-genesis by affecting osteoblastic/stromal cells to decrease the OPG/RANKL ratio [51] or M-CSF gene expression [50]. However, it should be noted that the inhibitory effect of Notch on RANKL-induced osteoclastogenesis is modest since the mice with Notch 1/2/3-specific deficiency in the osteoclast lineage do not exhibit significant defects in physiological bone development [51]. In addition, Notch signaling plays an important role

in proliferation, differentiation and expression of RANKL and OPG by osteoblast lineage cells [53-55], and thus indirectly regulates osteoclastogenesis *in vivo*. The role of the Notch pathway in inflammatory bone resorption has not been investigated, and future studies in this area may reveal new opportunities for therapeutic intervention.

Transcriptional repressors: Ids, Eos, MafB, C/EBPβ, IRF-8, BcL6
Balanced osteoclast differentiation is precisely controlled and maintained by complex mechanisms at various levels. In the past two decades, extensive studies have focused on the activation of signaling cascades that lead to activation of transcription factors such as NF-κB, AP-1 and NFATc1 that promote osteoclast differentiation (Figure 2, right). More recently, accumulating evidence has revealed that transcriptional repressors expressed constitutively in osteoclast precursors function to oppose the action of RANK and to restrain osteoclastogenesis (Figure 2, left). Thus, in addition to activating positive signaling pathways, RANK needs to overcome the 'brakes' imposed on osteoclast differentiation by trans-criptional repressors that include inhibitors of differen-tiation/DNA binding (Ids) [56,57], Eos [58], MafB (v-maf musculoaponeurotic fibrosarcoma oncogene family protein B) that is in turn induced by C/EBPβ (CCAAT-enhancer-binding protein β) [59], IRF-8 [38] and B cell lymphoma (Bcl)6 [60]. RANK signaling appears to overcome transcriptional repression of genes important for osteoclast differentiation and functions, at least in part, by downregulating expression of these transcrip-tional repressors. The need for removal of transcriptional repressors for osteoclast differentiation to occur high-lights their critical roles in negative regulation of osteoclastogenesis.

The expression levels of the currently identified nega-tive transcription factors Id, Eos, MafB, IRF-8 and Bcl6 are downregulated by RANKL during osteoclastogenesis with different kinetics. Ids, IRF-8 and MafB are decreased at the early stage of osteoclasogenesis, within 24 hours after RANKL stimulation, whereas Eos and Bcl6 expression appear to decrease at later time points. Forced expression of Id, MafB, IRF-8 or Bcl6 strongly inhibits RANKL-induced osteoclastogenesis *in vitro*. Eos targets Microphthalmia-associated transcription factor (MITF)/PU.1 target genes for repression, whereas inhibition of NFATc1 induction by the other repressors represents a common mechanism of suppression of osteoclast differentiation. Id proteins associate directly with MITF to downregulate expression of osteoclast-associated receptor (OSCAR) as well as NFATc1, without affecting the expression of TREM2, DAP12 or Fc receptor γ. MafB proteins interfere with the DNA-binding ability of c-Fos, MITF, and NFATc1, thereby inhibiting the transactivation

of NFATc1 and OSCAR. IRF-8 binds to NFATc1 and suppresses its DNA binding ability and transcriptional activity, thereby inhibiting NFATc1 autoamplification and expression of NFATc1 target osteoclast marker genes. Bcl6 directly binds to the promoters of NFATc1, dendritic cell-specific transmembrane protein (DC-STAMP) and cathepsin K, which are NFATc1 targets, to suppress osteoclastogenesis.

Deficiency of IRF-8 [38], Id1 [57] or Bcl6 [60] in mice leads to enhanced osteoclast formation and different extents of osteoporosis, indicating IRF-8, Id1 and Bcl6 play an inhibitory role in *in vivo* osteoclastogenesis and physiological bone metabolism. The role of MafB in physiological bone metabolism *in vivo* has not been reported. Expression of MafB, IRF-8 and Bcl6 is relatively selective for hematopoietic cells, whereas expression of Ids is observed in diverse cell types, including osteoblasts. Thus, the role of Ids seems to be more complex *in vivo*. Hypoxia-induced Id2 expression is found in rheumatoid arthritis synovial fibroblasts, and promotes synovial fibroblast-dependent osteoclastogenesis [61]. Another study showed that overexpression of Id1 in prostate cancer cells has an important role in promoting prostate cancer-mediated osteoclast differentiation, probably via certain secreted factors [62]. Therefore, the role of Id proteins during *in vivo* osteoclastogenesis in physiological and pathological conditions might be regulated by different cells and dependent on a particular environment.

The role of IRF-8 in inflammatory bone resorption was studied *in vitro* and *in vivo* [38]. Inflammatory bone erosion stimulated by RANK signaling is enhanced by inflammatory cytokines such as TNF-α that activate osteoclastogenesis directly or indirectly via activation of stromal cells and osteoblasts. IRF-8 deficiency dramatically promotes TNF-α-induced osteoclastogenesis *in vitro*, and results in increased NFATc1 expression, indicating that IRF-8 has a suppressive role in TNF-α-induced osteoclastogenesis. IRF-8 deficiency significantly attenuates TLR-induced inhibition of osteoclastogenesis, suggesting IRF-8 plays an important part in the inhibitory mechanisms of TLRs. In a lipopolysaccharide-induced inflammatory bone resorption model, IRF-8-deficient mice exhibit enhanced osteoclast formation and more dramatic bone destruction than wild-type littermates. These data indicate that this homeostatic role of IRF-8 may be important to limit bone resorption during acute infections and also in chronic inflammatory conditions such as rheumatoid arthritis. IRF-8 expression is also downregulated during RANKL-induced human osteoclastogenesis and silencing of *IRF8* mRNA in human osteoclast precursors with small interfering RNAs leads to enhanced osteoclast differentiation, indicating the function of IRF-8 in osteoclastogenesis is well conserved in humans and mice.

The mechanisms by which the expression of these repressors is downregulated are largely unknown. Recently, the transcriptional repressor Blimp1 (B lymphocyte-induced maturation protein-1), which is induced by NFATc1 in response to RANKL stimulation, was shown to suppress the expression of IRF-8, MafB [63] and Bcl6 [60] (Figure 2). Blimp1 deficiency attenuates downregulation of IRF-8, MafB and Bcl6 expression after RANKL stimulation, and thus Blimp1 promotes osteoclast differentiation by suppressing expression of its repressors. Conversely, Bcl6 can regulate Blimp1 expression and IRF-8 can regulate Bcl6 expression. These findings suggest a complex network of transcriptional repressors that control osteoclast differentiation, and it will be important to identify RANKL-induced signaling pathways and upstream molecules that control this transcriptional network. It will be also interesting to clarify whether these transcriptional repressors mediate the effects of inhibitory cytokines and inflammatory factors on osteoclasts. For example, factors that induce or maintain IRF-8 expression in the presence of RANKL would act to restrain osteoclast differentiation. IRF-8 expression is induced by IFN-γ, and augmented IRF-8 expression may contribute to the inhibitory effects of IFN-γ on osteoclastogenesis, and also to the well documented suppressive effects of TLRs on osteoclast precursor cells. Identification of signaling pathways, additional factors, and mechanisms that regulate IRF-8 expression and function represents a promising approach to control inflammatory bone loss.

Conclusion

Osteoclastogenesis *in vivo* is mediated by various factors, including cytokines, signaling molecules and transcription factors that directly affect osteoclast precursors and/or indirectly mediate osteoclastogenesis by targeting other cell populations, such as osteoblastic/stromal cells, synovial cells and T cells. In the latter case, the balance of RANKL versus OPG is often regulated to modulate osteoclastogenesis. Both direct and indirect effects need to be studied to fully understand the regulation of osteoclastogenesis. In addition, many inflammatory factors also influence osteoblast differentiation/function and osteoblastic bone formation, for example, the induction of Wnt pathway inhibitors Dickkopf (DKK) proteins and Frizzled-related proteins in inflammatory arthritis [64,65]. Regulation of osteoblast differentiation will impact on RANKL/OPG expression [66,67] and anabolic function and thus play an important part in physiological and pathological bone turnover *in vivo;* discussion of osteoblast differentiation is beyond the scope of this review.

It is interesting that the effects of most direct inhibitors are highly dependent on the timing of exposure and

inhibit most strongly when present prior to or shortly after RANKL administration (Figure 1). Strikingly, exposure of pre-osteoclasts to TLR ligands and GM-CSF several days after the RANK-mediated osteoclast differentiation program has been initiated actually results in increased osteoclastogenesis and bone resorption, possibly by mechanisms related to increased cell survival. Another attractive explanation for this timing phenomenon could be related to the downregulation of transcriptional repressors such as IRF-8 at the early stage of osteoclastogensis, thereby diminishing the suppressive function of inflammatory factors that utilize these repressors to suppress osteoclastogenesis.

One key principle that we have tried to develop is that the extent of inflammatory bone resorption is often determined by the balance between opposing factors. This includes not only the balance between positive osteoclastogenic factors and negative regulators, but also opposing effects of individual factors on different cell types. A striking example of opposing effects is offered by TLR ligands that promote osteoclastogenesis by activating RANKL expression on stromal cells, yet at the same time restrain the amount of bone resorption by directly inhibiting early osteoclast precursors. In acute infection or chronic inflammatory diseases such as rheumatoid arthritis, osteoclastogenic factors, including RANKL, TNF-α and IL-1, are often predominant and/or osteoclast precursors in the inflammatory microenvironment are refractory to inhibitors of osteoclastogenesis, such as IL-27, leading to excessive and pathologic bone resorption. Thus, identification of additional mechanisms and factors that increase the potency of repressors or restore cellular responses to suppressive factors may represent effective therapies for bone loss.

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Abbreviations

Bcl, B cell lymphoma; CTLA4, cytotoxic T-lymphocyte antigen 4; DAP12, DNAX-activating protein of 12 kDa; Id, inhibitors of differentiation/DNA binding; IL, interluekin; IRF, interferon regulatory factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; NF, nuclear factor; NFATc1, Nuclear factor of activated T-cells; OPG, osteoprotegerin; OSCAR, osteoclast-associated receptor; RANK, receptor activator of NF-κB; RANKL, RANK ligand; SHIP1, Src homology 2-containing inositol-5-phosphatase 1; STAT , signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; Treg, regulatory T cell; TREM-2, triggering receptor expressed by myeloid cells-2.

Competing interests

The authors declare that they have no competing interests.

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