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Does TNF Promote or Restrain Osteoclastogenesis and Inflammatory Bone Resorption?

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Abstract

Chronic inflammation is one of the most evident and common pathological conditions leading to deregulated osteoclastogenesis and bone remodeling. Tumor necrosis factor (TNF) as a pleiotropic cytokine plays a key role, not only in inflammation, but also in bone erosion in diseases associated with bone loss. TNF can stimulate the proliferation of osteoclast precursors and, in most conditions, act together with other cytokines and growth factors such as receptor activator of nuclear factor (NF)-[kappa]B ligand (RANKL), interleukin-6, and transforming growth factor beta to synergistically promote osteoclast formation and bone resorption *in vivo*. A longstanding enigma in the field is why TNF alone is not able to induce osteoclast differentiation as effectively as the same superfamily member RANKL, a physiological master osteoclastogenic cytokine. Recent studies have highlighted several lines of evidence showing the intrinsic mechanisms through RBP-J, NF-[kappa]B p100/TNF receptor-associated factor 3, or interferon regulatory factor-8 that restrain TNF-induced osteoclast differentiation and bone resorption. These feedback inhibitory mechanisms driven by TNF shed light into the current paradigm of osteoclastogenesis and would provide novel therapeutic implications on controlling inflammatory bone resorption.

Keywords

TNF; osteoclasts; bone resorption; inflammation

I. INTRODUCTION

Osteoimmunology is an emerging interdisciplinary field linking immunology and bone biology. Recent studies from the osteoimmunology field highlight the important roles of the immune system in the regulation of bone homeostasis in both physiological and pathological conditions.^{1–5} The adult skeleton undergoes constant and dynamic remodeling in order to maintain a healthy bone mass that is appropriately regulated by osteoclast-mediated bone resorption and associated osteoblast/osteocyte-mediated bone formation.^{6–8} Osteoclasts, derived from monocyte/macrophage precursors, are the exclusive cell type responsible for bone resorption. Osteoclast differentiation is effectively induced by the major osteoclastogenic cytokine receptor activator of nuclear factor (NF)-[kappa]B ligand (RANKL), which acts in concert with macrophage colony-stimulating factor (M-CSF) and

immunoreceptor tyrosine-based activation motif (ITAM)-mediated co-stimulatory signals. Binding of RANKL to its receptor RANK activates a broad range of signaling cascades, including canonical and non-canonical NF-[kappa]B pathways, mitogen-activated kinase pathways leading to the activation of AP-1 and CREB transcription factors, and calcium signaling, to induce the expression of key transcription factors Blimp1 and NFATc1 for osteoclast differentiation.^{9–13} Chronic inflammation such as that occurs in rheumatoid arthritis, periodontitis, and periprosthetic osteolysis is one of the most common pathological conditions associated with excessive bone resorption. In these diseases, abnormally enhanced generation and/or function of osteoclasts results in inflammatory bone erosion.^{14–17}

Tumor necrosis factor (TNF) is a pleiotropic cytokine important for immunity and inflammation. In addition to driving chronic inflammation, TNF plays a key role in inflammatory bone destruction observed in inflammatory arthritis, periodontitis, and periprosthetic osteolysis. The importance of TNF in the pathogenesis of rheumatoid arthritis and the resounding success of TNF blockade therapy in the treatment of rheumatoid arthritis and its associated bone erosion provide clinical evidence that TNF promotes pathologic bone resorption. Interestingly, however, compared with RANKL, TNF alone does not effectively induce osteoclast precursors to differentiate to osteoclasts directly, which has been demonstrated by both genetic evidence and osteoclastogenesis in human CD14-positive cells.^{18–20} TNF acts directly on osteoclast precursors, mostly in synergy with RANKL, to promote pathologic osteoclastogenesis and bone resorption.^{16–17,21–26} TNF may function *in vivo* to increase osteoclast precursors.^{27–30} In certain circumstances, transforming growth factor-beta may prime osteoclast precursors, which potentiates TNF to induce osteoclast differentiation.²⁴ Other than the direct effects on osteoclast lineage, TNF executes their indirect osteoclastogenic effect through augmentation of RANK expression on osteoclast precursors, M-CSF, and RANKL expression in stromal cells such as osteoblasts and synovial fibroblasts^{16,31–32} (Fig. 1).

TNF and RANKL belong to the same TNF super family, but their osteoclastogenic capacity differs drastically. The intrinsic mechanisms that restrain TNF-induced osteoclastogenesis are much less understood than those that promote osteoclastogenesis in response to RANKL. In this review, we focus on and highlight recent discoveries (Fig. 1) by our group and others of several key feedback mechanisms that control the direct osteoclastogenic properties of TNF.^{33–36}

II. RBP-J

RBP-J was originally identified as a master transcription factor in the canonical Notch signaling pathway,³⁷ in which the Notch intracellular domains translocate to the nucleus after activation by Notch ligands, bind to RBP-J, and induce RBP-J-mediated transcriptional activation of Notch target genes such as Hes1 and Hey1. Accumulating evidence recently showed that RBP-J also functions as a critical transcriptional regulator in many other signaling pathways, such as the TNF,³⁴ Toll-like receptor (TLR),^{38–39} Wnt- β -catenin,⁴⁰ NF-[kappa]B,^{41–42} TAK1,⁴³ and ITAM signaling pathways,³⁵ and is also targeted by viral proteins^{42,44} and cellular proteins of unknown function.^{45–46} Therefore, RBP-J is a central

transcription factor that receives inputs from various signaling pathways. Most cell types express a basic level of RBP-J that acts as either a transcriptional repressor or activator depending on the partner proteins with which it interacts.³⁷ RBP-J regulates cell proliferation, differentiation, cell fate decisions, and diverse cellular functions, including neurogenesis, stem cell maintenance, and lymphocyte development.^{37,47} In myeloid lineage cells, RBP-J has been implicated in inflammatory macrophage activation and function,^{38–39,48} dendritic cell (DC) differentiation, and maintenance of CD8⁺ DC populations.^{49–50} Although many of these functions are related to its role in Notch signaling, RBP-J function is context dependent and, under inflammatory conditions, RBP-J plays a key role in expression of immune response genes unrelated to canonical Notch signaling.³⁹

We found that RBP-J expression level was suppressed in the synovial fluid macrophages isolated from rheumatoid arthritis (RA) patients, supporting a pathological relevance of RBP-J to RA. Indeed, RBP-J allelic variants have been linked with RA.^{51–52} The synovial fluid macrophages can function as osteoclast precursors. However, the role of RBP-J signaling in pathological osteoclastogenesis and bone resorption is not well understood. Notch-RBP-J signaling in osteoclasts plays a minor role in homeostatic bone resorption based on genetic evidence that mice with a myeloid-specific deletion of RBP-J (RBP-J^{f/f} LysM-Cre), deletion of Notch 1/2/3, or constitutively active NICD1 expression in the myeloid compartment (NICD1^M) do not exhibit significant bone defects under physiological conditions.^{34,53} Consistent with these phenotypes, RBP-J modestly suppresses RANKL-induced osteoclastogenesis *in vitro*.^{34,54} In striking contrast, myeloid-specific deletion of RBP-J driven by LysMCre dramatically increases TNF-induced osteoclast differentiation to a level comparable to that induced by RANKL in control cells.³⁴ Furthermore, this enhanced osteoclastogenesis leads to severe bone destruction in a TNF-induced inflammatory bone resorption model. TNF is able to induce osteoclast differentiation and inflammatory bone resorption in RBP-J-deficient cells and mice even in the absence of RANK signaling. Therefore, RBP-J deletion reveals the full osteoclastogenic potential of TNF. Complementary evidence shows that forced activation of RBP-J suppresses inflammatory arthritic bone resorption. Mechanistically, RBP-J suppresses NFATc1 transcription by attenuating c-Fos activation and Blimp1 induction, thereby preventing the downregulation of the transcriptional repressor IFN regulatory factor-8 (IRF-8) that blocks osteoclast differentiation.³⁴ These studies shed insight into a poorly characterized field, in which RBP-J functions as a critical transcriptional repressor to suppress inflammatory and TNF-mediated osteoclastogenesis and bone resorption.

Our results revealed a predominant role of RBP-J in suppressing TNF-induced osteoclastogenesis compared with that induced by RANKL. Subsequent studies found an important clue for the relatively modest regulation of RANKL-induced osteoclastogenesis by RBP-J, which is likely attributed to the endogenous presence of ITAM-mediated calcium signaling.³⁵ The ITAM-mediated signaling pathways play important roles in various cellular activities, including immune response and cancer activation. The main ITAM-containing adaptors expressed by myeloid osteoclast precursors are DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcR γ). These adaptors associate with and mediate signaling by various receptors, including DAP12-associated triggering receptor expressed in myeloid cells 2 (TREM2) and signal-regulatory protein β 1 (SIRP β 1),

FcR γ -associated osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor-A (PIR-A) and FcRs. Osteoclasts require ITAM-mediated co-stimulation of RANK signaling for their appropriate differentiation during bone homeostasis.⁵⁵ RBP-J deficiency almost completely reverses the defects of osteoclast differentiation program and significantly rescues the osteopetrotic bone phenotype of *Dap12* knockout (KO) or *Dap12/Fcrg* double-KO mice by bypassing the requirement for costimulation of osteoclastogenesis during bone homeostasis.³⁵ In inflammatory conditions, RBP-J restrains ITAM signaling and limits cross-talk between RANK or TNFR- and ITAM-associated receptors by suppressing PLC γ 2 expression and keeping PLC γ 2-calcium signaling in a repressed basal state. RBP-J deficiency enables TNF to induce osteoclast formation and bone resorption in *Dap12* KO mice.³⁵ These data show that RBP-J deficiency allows osteoclast differentiation to occur independently of ITAM-mediated costimulation during homeostatic bone remodeling and inflammatory bone destruction. Therefore, RBP-J imposes the requirement for ITAM-mediated co-stimulation. Our data suggests a model in which the balance between ITAM-mediated induction and RBP-J-mediated suppression determines the level of basal PLC γ 2/calcium signaling, which further determines whether stimulation of osteoclast precursors with factors such as RANKL or TNF induces sufficient calcium signaling to cross the threshold required to effectively induce osteoclast differentiation.

These studies provided an important inhibitory mechanism mediated by RBP-J that can at least partially to explain the longstanding enigma in the field of why TNF alone is not able to induce osteoclast differentiation as effectively as the same superfamily member RANKL. The balance between the activating and inhibitory pathways fine tunes osteoclastogenesis and determines the strength of activating signals required for osteoclastogenesis in physiological and pathological settings. The expression and function of RBP-J can be altered in response to environmental cues in pathological states. For example, RBP-J expression level was significantly decreased in synovial fluid macrophages derived from RA patients.³⁵ The lower RBP-J level could contribute to the excessive osteoclastic bone resorption in RA.

A question plaguing the field is whether upstream Notch signaling plays a similar role as RBP-J in controlling TNF-mediated osteoclastogenesis. Interestingly, deficiency of Notch1/2/3 or ADAM metallopeptidase domain 10 (ADAM10) in osteoclast precursors does not promote TNF-induced osteoclastogenesis (author's unpublished data), suggesting that the canonical Notch-mediated osteoclastogenesis does not recapitulate RBP-J-suppressed osteoclastogenesis in response to TNF signaling. The pan inhibition of canonical Notch signaling may compromise the function of each specific Notch receptor. Although we cannot exclude the possibility that specific individual Notch receptors may function upstream of RBP-J to inhibit TNF-induced osteoclastogenesis, RBP-J may also act in a Notch-independent manner in this scenario. Furthermore, we did not find that TNF was able to induce osteoclast differentiation in the Hes1- and/or Hey1-deficient osteoclast precursors (author's unpublished data). This suggests that the targets of RBP-J other than the canonical Notch targets presumably control the negative regulation of TNF-induced osteoclastogenesis. Indeed, modern genome-wide analysis using RBP-J or NICD chromatin immunoprecipitation sequencing identifies a distinct set of sites where RBP-J recruits neither NICD nor Histone acetyltransferase p300 and binds DNA statically irrespective of Notch activity.^{56–58} These findings significantly modify the current paradigm of canonical

Notch/RBP-J signaling pathway and provide genomic evidence of a Notch-independent RBP-J signaling pathway. Appropriate manipulation of RBP-J activity in inflammatory arthritis mouse models has a significant impact on bone, but no discernable effects on TNF-mediated inflammation,^{34,59} suggesting a possibility of selective control of RBP-J activity in inflammatory bone destruction without affecting inflammation induced by TNF. Identification of RBP-J targets induced by TNF would provide a selective therapeutic strategy to prevent TNF-mediated bone resorption associated with inflammatory diseases without a significant impact on physiological bone remodeling.

III. NF-[KAPPA]B P100 AND TRAF3

The NF-[kappa]B family is comprised of RelA (p65), RelB, c-Rel, NF-[kappa]B1 (p50 and its precursor p105), and NF-[kappa]B2 (p52 and its precursor p100). NF-[kappa]B activation is induced by two major signaling pathways; the classical pathway mainly involves IKK β -induced I[kappa]B α degradation and subsequent RelA/p50 activation and the alternative pathway involves NIK-induced p100 processing to p52 and RelB/p52 activation. RelB is not only required for RANKL-induced osteoclast maturation, but is also important for TNF-induced bone resorption.⁶⁰ Activation of IKK β is sufficient for RANK-independent osteoclast differentiation and osteolysis.⁶¹ There are cross-talks between these two pathways and NF-[kappa]B activation from these two pathways plays important positive roles in inducing osteoclastogenesis.^{9,60,62–63} Conversely, clear evidence shows that NF-[kappa]B p100 functions as a negative regulator of osteoclastogenesis by binding to NF-[kappa]B 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*.^{60,62–65} TNF does not seem to activate the alternative NF-[kappa]B pathway as efficiently as RANKL because it induces an accumulation of p100 in osteoclast precursors via induction of TRAF3, thus limiting TNF-induced osteoclastogenesis.⁶⁴ TNF-Tg mice lacking NF-[kappa]B p100 exhibit more severe joint erosion than do their TNF-Tg littermates.⁶⁴ These data suggest that inhibition of NF-[kappa]B p100 processing may represent a novel therapeutic strategy for inflammatory bone loss such as that occurs in RA, periodontitis, or periprosthetic osteolysis.

IV. IRF-8

In addition to activating positive signaling pathways, RANKL needs to overcome the “brakes” imposed on osteoclast differentiation by transcriptional repressors that include inhibitors of differentiation/DNA binding (Ids),^{49,66} Eos,²⁷ v-maf musculoaponeurotic fibrosarcoma oncogene family protein B (MafB),⁶⁷ IRF-8,³⁶ and B-cell lymphoma 6 (Bcl6).⁶⁸ Among these transcriptional repressors, IRF-8 deficiency dramatically promotes TNF-induced osteoclastogenesis and results in increased NFATc1 expression, indicating that IRF-8 has a suppressive role in TNF-induced osteoclastogenesis. IRF-8 deficiency also significantly attenuates TLR-induced inhibition of osteoclastogenesis, suggesting that 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 their 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 RA. Identification of signaling pathways, additional factors, and mechanisms that regulate IRF-8 expression and function represents a promising approach to control inflammatory bone loss. The mechanisms by which the expression of these repressors is downregulated are largely unknown. Recently, inspiring studies highlighted novel epigenetic regulatory mechanisms that control IRF-8 downregulation,^{69–70} which moved the field one step ahead. Epigenetic regulation is universal in cellular processes that require transcription dynamics and genetic stability. Epigenetic modifications contribute to regulation of gene expression by transcriptional activation or repression. There are two main types of epigenetic modulation: DNA methylations such as via *de novo* DNA methyltransferase (DNMTs) DNMT3a and DNMT3b and histone post-translational modifications (histone tail modifications) such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation of histones. Epigenetic repressors generally include DNMTs, histone deacetylases (HDACs) and polycomb group proteins (PcGs). Epigenetic repression of IRF-8 by Dnmt3a-mediated DNA hypermethylation leads to decreased IRF-8 expression and enhanced osteoclastogenesis and bone resorption.⁶⁹ Our group found that EZH2 directly silences IRF-8 expression.⁷⁰ EZH2 is the catalytic subunit of polycomb repressive complex 2 (PRC2) that catalyzes the addition of methyl groups to lysine 27 of histone 3 (H3K27me). EZH2 binds to the IRF-8 promoter and induces the transcriptional repressor marker H3K27me3 at the IRF-8 locus in response to RANKL.⁷⁰ There are functional links among PRC2, HDACs, and DNMTs.⁷¹ It is thus likely that the regulation of IRF-8 expression can be controlled by both DNA methylation and histone modification in a synergistic manner. It would be of great interest to investigate whether these regulatory mechanisms also apply to TNF-induced osteoclastogenesis and if inflammatory conditions would alter the action between these mechanisms.

V. CONCLUSIONS

Osteoclastogenesis *in vivo* is mediated by various factors including cytokines, growth factors, signaling molecules, and transcription factors that directly target osteoclast precursors and affect their differentiation and/or indirectly mediate osteoclastogenesis by targeting other cell populations such as osteoblastic/stromal cells, synovial cells, and T-cells. In the latter case, for example, TNF stimulates RANKL production from these cells. Both direct and indirect effects are integrated together to regulate osteoclastogenesis. The extent of inflammatory bone resorption is often determined by the balance between opposing factors. This includes not only balance between positive osteoclastogenic factors and negative regulators, but also different effects of individual factors on different cell types. For example, TNF does not induce effective osteoclast differentiation directly, but at the same time it promotes osteoclastogenesis indirectly by activating RANKL expression on stromal, osteoblastic, and T-cells. A recent finding showed that Lgr4 is a new receptor for RANKL and the signal through Lgr4 suppresses osteoclastogenesis.⁷² It would be of interest to investigate whether TNF stimulates Lgr4 expression and TNF interacts with Lgr4-mediated signaling pathways to restrain osteoclast differentiation. In acute infection or chronic inflammatory diseases such as RA, TNF is often predominantly expressed. Osteoclast precursors in the inflammatory microenvironment are refractory to inhibitors of osteoclastogenesis such as IL-27,⁷³ leading to excessive bone resorption. Therefore, the

identification of additional mechanisms and factors that increase the potency of intrinsic repressors or restore cellular responses to suppressive factors may represent effective therapies for pathologic bone loss.

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ABBREVIATIONS:

DAP12	TYRO protein tyrosine kinase-binding protein
FasL	Fas ligand
FcRg	Fc receptor g
GM-CSF	granulocyte M-CSF
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
M-CSF	macrophage colony-stimulating factor
MITF	microphthalmia-associated transcription factor
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NF-[kappa]B	nuclear factor [kappa]B
OPG	osteoprotegerin
OSCAR	osteoclast-associated receptor
PI3K	phosphatidylinositol-3-kinase
RANK	receptor activator of NF-[kappa]B
RANKL	RANK ligand
RBP-J	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
TREM-2	triggering receptor expressed by myeloid cells-2

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FIG. 1:

Model showing a dual role of TNF in osteoclastogenesis. TNF can stimulate proliferation of osteoclast precursors, increase RANK expression, and in most conditions act together with other cytokines and growth factors such as RANKL, IL-6, and TGF- β to synergistically promote osteoclast formation and bone resorption *in vivo*. TNF also indirectly promotes osteoclastogenesis by targeting other cell populations such as osteoblastic/stromal cells, synovial cells, and T-cells by stimulating M-CSF or RANKL production from these cells. Recent studies have highlighted several lines of evidence showing that TNF restrains osteoclastogenesis through intrinsic feedback mechanisms such as via RBP-J, NF-[kappa]B p100/TRAF3, or IRF-8.