Research article

Exposure to receptor-activator of NFκB ligand renders pre-osteoclasts resistant to IFN-γ by inducing terminal differentiation

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Abstract

While it has been established that IFN-γ is a strong activator of macrophages and a potent inhibitor of osteoclastogenesis in vitro, it is also known that this cytokine is produced in particular settings of inflammatory bone loss, such as infection and psoriatic arthritis. Because of the different kinetics between rapid IFN-γ macrophage activation (< 24 hours) and the slower receptor-activator of NFκB ligand (RANKL) osteoclast differentiation (7 days), we postulated that IFN-γ would have different effects on early-stage and late-stage osteoclast precursors. In RAW264.7 cells and primary splenocyte cultures, pretreatment with RANKL rendered these cells resistant to IFN-γ-induced nitric oxide production, morphological change, and surface upregulation of CD11b and receptor-activator of NFκB, suggesting that early exposure of osteoclast precursors to RANKL induces a broad resistance to the cellular effects of IFN-γ. Changes in STAT1 activation did not correlate with this resistance, as IFN-γ activated STAT1 equally in both early-stage and late-stage pre-osteoclasts. Furthermore, we failed to observe changes in TRAF6 expression following IFN-γ treatment in pre-osteoclasts. Together these data support a model of inflammatory bone loss in which early exposure to RANKL can prime osteoclast precursors to form in the presence of high levels of IFN-γ using mechanisms independent of the signal molecules STAT1 and TRAF6.

Keywords: cellular differentiation, cytokines, monocytes/macrophages

Introduction

Osteoclasts are large, multinucleated bone-resorbing cells derived from the monocyte–macrophage lineage [1]. Their bone-resorbing capacity is unique; no other cell shares this capability. Osteoclasts are critical for both the continuous remodeling of normal bone tissue as well as the repair of fractured bones [2]. During normal bone remodeling, osteoclast-mediated bone resorption is balanced by osteoblast-mediated bone formation, resulting in the maintenance of skeletal bone mass. As a consequence, osteoclast dysregulation leads to osteoporosis (decreased bone mass caused by excess osteoclast activity) or to osteopetrosis (increased bone mass caused by insufficient osteoclast activity). Considering the tremendous morbidity and cost of metabolic bone diseases [3], improving our molecular understanding of osteoclast development and function is critical towards the design of therapies to combat these prevalent diseases.

The molecular signals required for osteoclastogenesis have recently been elucidated [1,4,5]. Receptor-activator of NFκB ligand (RANKL), a tumor necrosis factor superfamily ligand expressed by stromal cells, osteoblasts, and activated T cells, binds to its cognate receptor-activator of NFκB (RANK) receptor on macrophages/monocytes, inducing a signal that gradually transforms the macrophages into osteoclasts over a period of several days [6,7]. While this interaction occurs largely in the bone microenvironment, it has been shown that monocytes from the spleen, peripheral blood, and synovium are...
all capable of RANKL-dependent osteoclast formation [8]. Convincing evidence has been generated indicating that the RANK–RANKL interaction is absolutely required for osteoclastogenesis [7]; in the absence of these molecules, osteoclastogenesis cannot occur [9].

While the RANK–RANKL signal is absolutely required for osteoclastogenesis, the efficiency of this process is influenced by cytokines. The proinflammatory cytokines tumor necrosis factor alpha, IL-1, and IL-6 augment osteoclastogenesis [9–11], while IL-10, IL-12, IL-18, and IFN-γ antagonize osteoclastogenesis in vitro [12–15]. It is probable that cytokines play a vital role in the delicate balance of bone remodeling, and that therapies based upon their natural biological function can be designed.

IFN-γ is a cytokine secreted primarily by activated T cells and NK cells whose role in bone biology is only beginning to be clarified. It was originally characterized as a powerful macrophage activator that upregulated nitric oxide (NO) production and MHC expression in macrophages [16]. It has since been shown to stimulate antiviral and antibacterial activities, to differentiate Th0 cells toward Th1 fates, and to activate endothelial cells for leukocyte adhesion (reviewed in [17,18]).

With respect to osteoclast formation, IFN-γ is known to potently inhibit RANKL-mediated osteoclastogenesis in both spleen-derived macrophage cultures and bone marrow coculture systems [19–21]. It has also been demonstrated that mice defective in IFN-γ signaling have a more rapid onset of arthritis and bone resorption compared with wild-type mice, suggesting a protective role of IFN-γ in early arthritis [21,22].

There are situations, however, in which the anti-osteoclastogenic effects of IFN-γ are not so clear. While IFN-γ is not highly expressed in joints of patients with rheumatoid arthritis, diseases such as erosive tuberculous leprosy [23,24] and psoriatic arthritis [25] are associated with high Th1 cytokines such as IFN-γ [26]. In these conditions, tissue destruction has been shown to correlate with Th1-mediated immune responses and the production of IFN-γ, indicating that osteoclastogenesis may occur in the absence of elevated IFN-γ [27].

Furthermore, several clinical studies have failed to demonstrate efficacy of IFN-γ administration as an anti-osteoclastogenic agent to prevent bone loss [28–30]. There seem to be situations in which IFN-γ does not act as an anti-osteoclastogenic agent. In particular, IFN-γ has been shown to be efficacious in the treatment of osteoporosis in humans [31].

In the present study, we demonstrate that early exposure to RANKL renders osteoclast precursors resistant to the effects of IFN-γ, including inhibition of osteoclastogenesis. These effects were irreversible and not caused by inhibition of proximal signaling at the level of STAT1 or TRAF6, suggesting that resistance to IFN-γ is caused by a complex differentiation program specifying the osteoclast fate. These data may help explain the contradictory findings regarding the effects of IFN-γ as an inhibitor of osteoclastogenesis, and also suggest a model of erosive disease in the presence of IFN-γ whereby osteoclast precursors are exposed to RANKL before they enter the IFN-γ-rich environment.

Materials and methods
Cytokines and growth factors
Murine IFN-γ, macrophage colony-stimulating factor, tumor necrosis factor alpha, IL-1, IL-10 and IL-6 were obtained from R&D Systems (Minneapolis, USA). GST-mRANKL was generated as described later. Human RANKL was a gift from Immunex (Seattle, USA) and was used to verify the efficiency of GST-RANKL protein as verified by splenic osteoclast induction.

Cell culture and animals
The RAW264.7 mouse macrophage cell line was obtained from ATCC (Manassas, USA) and grown in a humidified 5% CO2 environment at 37°C. Cultures were maintained with DMEM (R&D Systems) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. RAW cell osteoclasts were generated by plating exactly 5000 RAW cells per well in a 24-well dish with 200 ng/ml GST-RANKL or additional cytokines and culturing for 4–5 days. In some cultures, administration of IFN-γ was delayed to allow for an early effect of RANKL. Osteoclasts were large, multinucleated cells, and expressed high levels of tartrate-resistant acid phosphatase (TRAP). For western blots, RAW cells were grown in six-well dishes at 100,000 cells/well with the same cytokine treatments.

CBA/BL6 mice (Jackson Labs, Bar Harbor, USA) were sacrificed at 6–8 weeks and spleens aseptically removed. Splenocytes were mechanically dissociated by disruption through steel mesh, and red blood cells were lysed using a hypotonic ammonium chloride solution (82.9 g ammonium chloride, 10 g potassium bicarbonate, 0.37 g EDTA, 1 l sterile water for a 10× solution). The remaining white cells were plated in 96-well plates at 200,000 cells/well with 50 ng/ml murine macrophage colony-stimulating factor (R&D Systems) to preferentially maintain monocyte proliferation. Large, multinucleated, TRAP+ osteoclasts were generated by further addition of 200 ng/ml GST-RANKL and culturing for 4–6 days. IFN-γ (10 ng/ml) was added on various days in some cultures.

TRAP staining and quantitation
Cultures were stained with the osteoclast-specific marker TRAP using a kit from Sigma (St Louis, USA). In osteo-
clast precursors, RANKL induced increasing TRAP expression until virtually 100% of mononuclear cells were highly TRAP-positive. Fusion and multinucleation subsequently occurred in proportion to the magnitude of the RANKL stimulus.

Osteoclasts were counted using three equivalent methods, optimized for the extent of osteoclastogenesis. For extremely robust osteoclast cultures, manual tracing and digital quantitation of a photographed osteoclast area was the best measure due to extensive fusion and multinucleation between cells (Fig. 1). Less robust cultures with 80–150 osteoclasts per well were more accurately quantitated by counting individual osteoclasts (Fig. 2). For cultures utilizing lower levels of RANKL and low osteoclast numbers, densitometric quantitation of TRAP was a more sensitive measure of osteoclastogenesis (Fig. 2d).

Purification of GST-RANKL
GST-RANKL was purified as described previously [32]. Briefly, a fragment of murine RANKL cDNA was cloned inframe into the pGEX-4T vector (Amersham Pharmacia, Piscataway, USA) and expressed in BL21 bacteria (Amersham Pharmacia) induced with 0.1 mM IPTG (Gibco) for 5 hours at 30°C. Bacteria were lysed, and soluble proteins were recovered using glutathione-agarose beads (Amersham Pharmacia). Protein purity was assessed by SDS-PAGE with Coomassie Blue by comparison with 99% pure BSA standards (Sigma) and shown to of equivalent purity (Fig. 1a). The bioactivity of GST-RANKL was verified by RAW cell osteoclastogenesis and TRAP expression before experimental use. A dose of 200 ng/ml GST-RANKL had equivalent bioactivity to 100 ng/ml eukaryotically expressed human RANKL protein (donated by Immunex).

NO measurement
NO production was measured by the Greiss reaction (Promega, Madison, USA), which spectrophotometrically detects nitrite, a stable breakdown product of NO whose accumulation reflects NO production. RAW cells were plated in 96-well plates, and were stimulated with GST-RANKL (200 ng/ml) and/or IFN-γ (10 ng/ml). Cell supernatants were harvested and reacted with 1% sulfanilamide and 0.1% naphthyl-ethylenediamine. A standard curve was constructed using dilutions of sodium nitrite, and the absorbance was measured at 550 nm. Similar data were obtained from protein-normalized data to control for variation in cell number or proliferation.

Western blotting
Cells were lysed with hypotonic lysis buffer with protease inhibitors (Roche/Boehringer Mannheim, Indianapolis, USA). Thirty micrograms of cytoplasmic lysates or 20 µg nuclear lysates were loaded onto 12% SDS-PAGE gels and immunoblotted using chemiluminescent antibodies. Short exposures of blots were also performed to verify that signals shown did not result from blot overexposure. All antibodies were from Santa Cruz (Santa Cruz, USA) except anti-actin, which was purchased from Sigma.

Flow cytometry
After red blood cell lysis, a single cell suspension was incubated with antimurine CD16/32 (Pharmingen, San Diego, USA) to block Fc receptor-mediated antibody binding. Cells were then labeled with phycoerythrin-conjugated anti-CD11b antibodies (Pharmingen) or fluorescein-conjugated RANKL (a gift from M Tondravi, American Red Cross, Rockville, MD, USA) as described previously [33]. Data were acquired using a FACScalibur instrument (Beckton Dickenson, Bedford, MA, USA) and were analyzed by Cellquest software (version 3.1, Beckton Dickenson). CD11b expression followed a normal-type distribution in all groups indicative of one population, and the mean fluorescent intensity was thus the unit of measure. RANK staining indicated two populations, and the relative size of the high RANK expressing (RANKhi) population was thus expressed on a percentage basis.
Results

IFN-γ potently and irreversibly inhibits RANKL-induced osteoclastogenesis

To evaluate the effects of IFN-γ on osteoclastogenesis, we utilized the mouse macrophage cell line RAW264.7 (RAW cells) or splenic macrophages from CBA/BL6 mice as osteoclast precursors. These cells have been demonstrated to recapitulate critical aspects of osteoclast formation and activity, including bone resorption, expression of calcitonin receptor, multinuclear fusion, and TRAP expression [6,34].

A dose of 200 ng/ml RANKL generated numerous large, multinucleated osteoclasts. Lower doses induced the osteoclast-specific marker TRAP in mononuclear cells but generated substantially fewer osteoclasts (Fig. 1a,b). A 24-hour exposure to IFN-γ was sufficient to potently and irreversibly inhibit osteoclastogenesis in the presence of maximal doses of RANKL (Fig. 2). The exposure also induced a stellate cellular morphology consistent with that of an activated macrophage (Fig. 2). The rapid and irreversible effects of IFN-γ on osteoclast inhibition indicate a dominant effect of this cytokine over RANKL via induction of monocyte differentiation toward the activated macrophage fate, as opposed to the osteoclast fate, when concomitantly administered.

RANKL-pretreated RAW cells are resistant to the anti-osteoclastogenic effect of IFN-γ

The different kinetics between IFN-γ-mediated macrophage activation (<24 hours) and RANKL-mediated osteoclastogenesis (4–5 days) prompted us to investigate the effects of IFN-γ on RANKL-pretreated cells (Fig. 3). Pretreatment of RAW cells with RANKL for 48 hours rendered them resistant to IFN-γ; these mononuclear cells formed multinuclear osteoclasts despite the presence of maximally inhibitory doses of IFN-γ (10 ng/ml).

To characterize this RANKL-mediated resistance to IFN-γ, we performed a series of dose–response and time course experiments. Pretreatment with lower doses of RANKL was unable to overcome IFN-γ inhibition, indicating that
resistance to IFN-γ required high levels of RANKL pretreatment (Fig. 4a,b). The duration of RANKL pretreatment was also important; the longer the pretreatment phase, the greater the resistance to IFN-γ (Fig. 4c). Together these data show that pretreatment with RANKL increases resistance to IFN-γ-mediated osteoclast inhibition in a dose-dependent and time-dependent manner.

Osteoclast formation in the presence of IFN-γ depends upon receptor-activator of NFκB ligand (RANKL) pretreatment in a dose-dependent and time-dependent manner. (a) RAW cells were pretreated with the indicated doses of RANKL for 2 days. On day 3, cells were switched to media with RANKL (200 ng/ml GST-RANKL) and various doses of IFN-γ, then fixed and tartrate-resistant acid phosphatase (TRAP) stained after day 4. (b) The mean ± SEM of three independent experiments containing the highest dose of IFN-γ in (a) with statistics. *P = 0.05 compared with RANKL pretreatment at 0 ng/ml. (c) RAW cells treated continuously with a suboptimal dose of GST-RANKL (50 ng/ml), to slow osteoclastogenesis, were given IFN-γ (10 ng/ml) at the indicated time points. All cells were fixed and stained for TRAP on day 6. TRAP staining was quantitated densitometrically as described in Materials and methods. RANKL-treated RAW cells (no IFN-γ) stained on day 2 were only 10–20% TRAP-positive. All cultures with RANKL pretreatment ≥ 24 hours underwent osteoclastogenesis. *P = 0.05 compared with the IFN-γ day 0 group.

The mechanism by which IFN-γ and RANKL reciprocally inhibit each other in pre-osteoclasts is independent of TRAF-6 and STAT1 signal transduction. Since it is well
established that RANK signaling is mediated primarily through TRAF6 [35] and that IFN-γ signaling is mediated primarily through STAT1 [36,37], we examined their expression by western blot. We failed to observe significant degradation of TRAF6 after stimulation with IFN-γ or RANKL in short-term or long-term cultures (Fig. 5a,b). Interestingly, IFN-γ-induced STAT1 phosphorylation and nuclear translocation was retained in late pre-osteoclasts (Fig. 5c). This result was confirmed by electrophoretic mobility shift assays and the absence of detectable amounts of the STAT1 antagonists SOCS1 and SOCS3 (data not shown). These results show that late pre-osteoclasts are capable of signaling via JAK-STAT1, and suggest that signaling events distal to TRAF6 and STAT1 modulate the resistance of these cells to IFN-γ.

**RANKL pretreatment inhibits IFN-γ-induced macrophage activation**

We next investigated whether other IFN-γ effects besides osteoclast inhibition were blunted in RANKL-pretreated cells. IFN-γ-induced NO production in macrophages was blunted in osteoclasts, probably due to specialization for...
bone resorption in the latter differentiated cell (Fig. 6). Pretreatment with RANKL inhibited IFN-γ-induced NO production in a dose-dependent and time-dependent manner (Fig. 6b,c).

To expand these findings, we investigated the regulation of the cell surface markers CD11b and RANK in response to induction by RANKL and IFN-γ (Fig. 7). Both markers were strongly upregulated by IFN-γ. Consistent with our
prior results, concomitant treatment of IFN-γ and RANKL showed dominance of the IFN-γ upregulation, while pre-treatment with RANKL for 2 days showed impaired upregulation. These data support the hypothesis that sensitivity to IFN-γ decreases with increasing osteoclast differentiation and that the mechanism by which RANKL and IFN-γ reciprocally inhibit each other in pre-osteoclasts is mediating the irreversible commitment into the osteoclast or activated macrophage lineages.

**Discussion**

The role of cellular immunity (Th1) in inflammatory bone loss such as that seen in osteomyelitis and erosive arthritis remains unclear. The resolution of this issue is further clouded by the apparent contradictory findings that IFN-γ is an extremely potent anti-osteoclastogenic factor [38] but it can be found at high levels in sites of osteolysis [39]. To reconcile this controversy we performed a series of experiments aimed at understanding how IFN-γ could induce either bactericidal effects via macrophage activation or osteoclastic bone resorption, by influencing the same progenitor cell in the same bone environment.

We demonstrate that pre-osteoclasts stimulated with RANKL for 2 days are rendered resistant to the IFN-γ-induced inhibition of osteoclastogenesis, NO production, and upregulation of CD11b and RANK surface expression. Surprisingly, this resistance is not mediated by inhibition of the JAK-STAT1 pathway, as STAT1 phosphorylation, nuclear translocation, and DNA-binding capabilities are all preserved in the late pre-osteoclast. Together this evidence suggests that RANKL modifies IFN-γ effects downstream of STAT1.

The present results predict that timing of IFN-γ exposure will be an important determinant of its biological function during *in vivo* osteoclastogenesis. In the case of circulating or peripheral macrophages that have not encountered osteoclastogenic quantities of RANKL, IFN-γ rapidly induces macrophage activation and subsequent NO production, recruiting these cells for immune responses. The dominance of IFN-γ over RANKL, which we observed *in vitro* (1 ng IFN-γ counteracts 200 ng RANKL when simultaneously administered), probably allows this macrophage activation to occur even in the presence of RANKL expressed on either synovial cells or infiltrating T cells [43,44] may induce osteoclast formation despite the presence of exogenous or endogenous IFN-γ.

The potency of IFN-γ as a macrophage activator and osteoclast inhibitor suggests a prominent role for T cells and/or NK cells in the regulation of bone resorption, as these cells constitute the major source of IFN-γ [18]. A link between activated T cells and osteoclast inhibition by IFN-γ has been highlighted in a report by Takayanagi *et al*., providing the first *in vivo* evidence that the immune system may influence osteoclastogenesis [38]. Interestingly, other groups have demonstrated that activated T cells induce osteoclastogenesis via upregulation of surface RANKL, presenting a dilemma regarding the role of activated T cells in osteoclastogenesis [20,40,45].

It is possible that RANKL and IFN-γ expression by activated T cells may be a major mechanism by which T cells control the fate of osteoclasts, and that relative expression of both molecules will dictate whether osteoclasts are induced or inhibited. The present results suggest that the influence of activated T cells on osteoclasts will probably change depending on when and where the T cells encounter the osteoclasts. Bone-resident macrophages pre-exposed to RANKL in the stromal environment may be resistant to the immunoregulatory effects of IFN-γ and may thus be better suited for bone-remodeling tasks. In contrast, peripheral blood macrophages not exposed to RANKL may be more responsive to the macrophage-activating immunoregulatory effects of IFN-γ, and may thus be better suited for antimicrobial activities.

The molecular mechanisms underlying the contrasting effects of IFN-γ and RANKL remain elusive. Based on the phenotype of knockout mice, it has become clear that TRAF6 is the critical adapter molecule required for RANK signaling during osteoclastogenesis [35]. The importance of TRAF6 has been further explored in deletion studies, which correlated its various domains with its osteoclastogenic potential [46]. A link between IFN-γ and RANK signaling via TRAF6 has also been demonstrated in bone marrow cultures, in which IFN-γ was shown to accelerate the degradation of TRAF6 [38]. In our studies, however, we failed to observe this TRAF6 degradation, as its expression remained constant in both short-term and long-term cultures under conditions where IFN-γ completely inhibited osteoclastogenesis. While this discrepancy could be explained by the possibility that RAW cells represent a stage of pre-osteoclast differentiation that is not sensitive to IFN-γ-mediated TRAF6 degradation, the present studies clearly indicate that IFN-γ can inhibit osteoclastogenesis via a completely different mechanism.

The retention of Jak-STAT1 signaling in RANKL-stimulated pre-osteoclasts is intriguing, given their demonstrated
insensitivity to IFN-γ. Since it is known that osteoclasts express IFN-γ receptors [47,48], it begs the question as to why this pathway remains operant. One possibility is that IFN-γ-induced STAT1 activation in osteoclasts leads to the expression of a unique set of genes that is distinct from that in macrophages. Future studies designed to understand IFN-γ signaling in mature osteoclasts are needed to resolve this issue.

It is noteworthy to point out that the dominant/irreversible inhibitory effects of IFN-γ on osteoclastogenesis are fundamentally different to the transient/reversible inhibitory effects of IL-4 on this process, which are mediated by STAT6 inhibition of NFκB activation [49,50]. In the present article, we demonstrate that RANKL-induced osteoclastogenesis cannot be recovered following exposure to IFN-γ. In contrast, Wei et al. have demonstrated that pre-osteoclasts do not lose their potential to differentiate into mature osteoclasts following a similar exposure to IL-4 [50]. It thus appears that IFN-γ anti-osteoclast activity is mediated by inducing terminal differentiation away from the osteoclast lineage, while IL-4 directly interferes with RANKL signaling during osteoclastogenesis. Collectively, these findings are consistent with the observations that Th1 cells are associated with erosive disease [39,50,51], that IFN-γ does not have antiresorptive activity [52–54], and that IL-4 inhibits bone resorption [55–57].

The present results underscore the importance of developing a complete understanding of osteoclastogenesis in vivo with regard to location, time, and the signal transduction pathways involved. It is probable that signals such as IFN-γ will be interpreted differently by precursors at various stages of development, with consequent effects on disease. Future studies in this area are needed to better understand how T cells producing both IFN-γ and RANKL mediate immunity and bone resorption, and to better elucidate their role in the pathogenesis of diseases such as osteomyelitis and erosive arthritis.

Conclusion

We have demonstrated, using in vitro methods, that osteoclast precursors exposed to RANKL for 1–2 days can be rendered resistant to maximal osteoclast-inhibitory doses of IFN-γ. These IFN-γ-resistant pre-osteoclasts produced low levels of NO upon IFN-γ stimulation and were resistant to IFN-γ-induced, Mac-1-induced and RANKL-induced surface expression, suggesting a broad resistance to the cellular effects of IFN-γ. The Jak-Stat1 pathway was intact in these cells, indicating that downstream transcriptional events are involved in the inhibition. These results imply a model for arthritic joints in which macrophage precursors entering an inflamed joint are exposed to RANKL and are subsequently rendered resistant to the anti-osteoclastogenic effects of IFN-γ expressed by activated T cells in the synovium.
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