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Exposure to receptor-activator of NF κ B ligand renders pre-osteoclasts resistant to IFN- γ by inducing terminal differentiationWillis Huang¹, Regis J O'Keefe² and Edward M Schwarz²¹The Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA²The Center for Musculoskeletal Research, University of Rochester Medical Center, Rochester, NY, USACorresponding author: Edward M Schwarz (e-mail: edward_schwarz@urmc.rochester.edu)

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Arthritis Res Ther 2003, **5**:R49-R59 (DOI 10.1186/ar612)© 2003 Huang *et al.*, licensee BioMed Central Ltd (Print ISSN 1478-6354; Online ISSN 1478-6362). This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any non-commercial purpose, provided this notice is preserved along with the article's original URL.**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 NF κ B 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 maximally anti-osteoclastogenic doses of IFN- γ . These cells were also resistant to IFN- γ -induced nitric oxide

production, morphological change, and surface upregulation of CD11b and receptor-activator of NF κ B, 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 NF κ B ligand (RANKL), a tumor necrosis factor superfamily ligand expressed by stromal cells, osteoblasts, and activated T cells, binds to its cognate receptor-activator of NF κ B (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

BSA = bovine serum albumin; DMEM = Dulbecco's modified Eagle's medium; FACS = fluorescence-activated cell sorting; Fc = crystallizable fragment; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; MHC = major histocompatibility complex; NF = nuclear factor; NO = nitric oxide; RANK = receptor-activator of NF κ B; RANKL = receptor-activator of NF κ B ligand; Th = T helper cells; TRAP = tartrate-resistant acid phosphatase.

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 tuberculoid 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 presence 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 osteopetrosis 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.

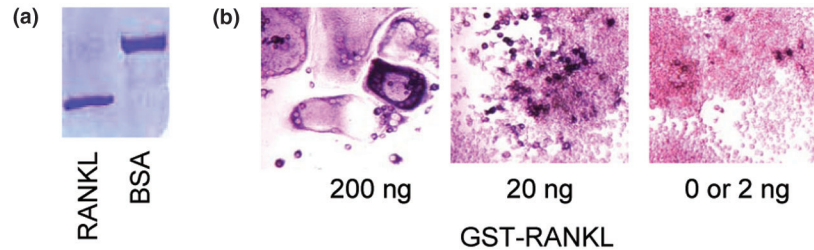
Cell culture and animals

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 96-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 \times 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-

Figure 1

Receptor-activator of NF κ B ligand (RANKL) induces osteoclastogenesis in RAW264 cells. **(a)** Bacterially produced GST-RANKL protein was purified and visualized with SDS-PAGE/Coomassie Blue against 99% pure BSA standards. **(b)** RAW cells were cultured with the indicated doses of GST-RANKL for 4 days and then stained for tartrate-resistant acid phosphatase.

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 be 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.

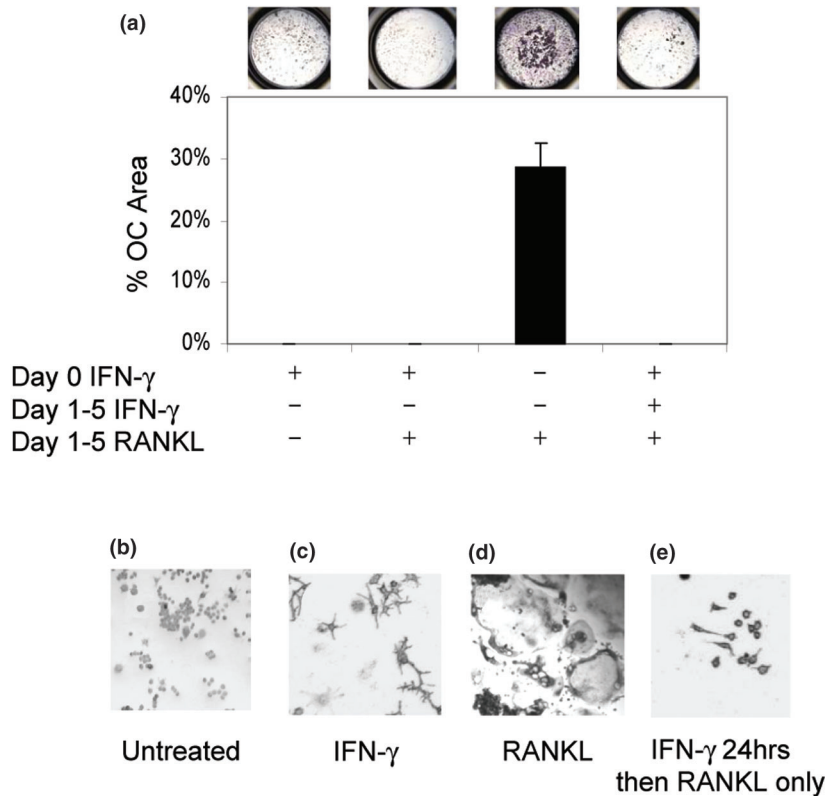
Western blotting

Cells were lysed with hypotonic lysis buffer with protease inhibitors (Roche/Boehringer Mannheim, Indianapolis, USA). Thirty micrograms of cytoplasmic lysates or 20 μ g 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 Dickinson, Bedford, MA, USA) and were analyzed by Cellquest software (version 3.1, Beckton Dickinson). 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.

Figure 2



IFN-γ dominantly inhibits receptor-activator of NFκB ligand (RANKL)-mediated osteoclastogenesis. (a) RAW cells received the indicated cytokine treatments of IFN-γ (10 ng/ml) and/or RANKL (200 ng/ml). Cells were stained for the osteoclast marker tartrate-resistant acid phosphatase (TRAP) on day 5 and osteoclast numbers were counted; a 1x micrograph of a representative well is shown above its corresponding column. TRAP positivity and osteoclast formation are seen only in the absence of IFN-γ. (b)–(e) Micrographs (10x) of the same groups show that IFN-γ stimulates a stellate morphology and inhibits osteoclastogenesis. All panels represent continuous cytokine treatment, except for the IFN-γ washout group (e). OC, osteoclast.

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 irre-

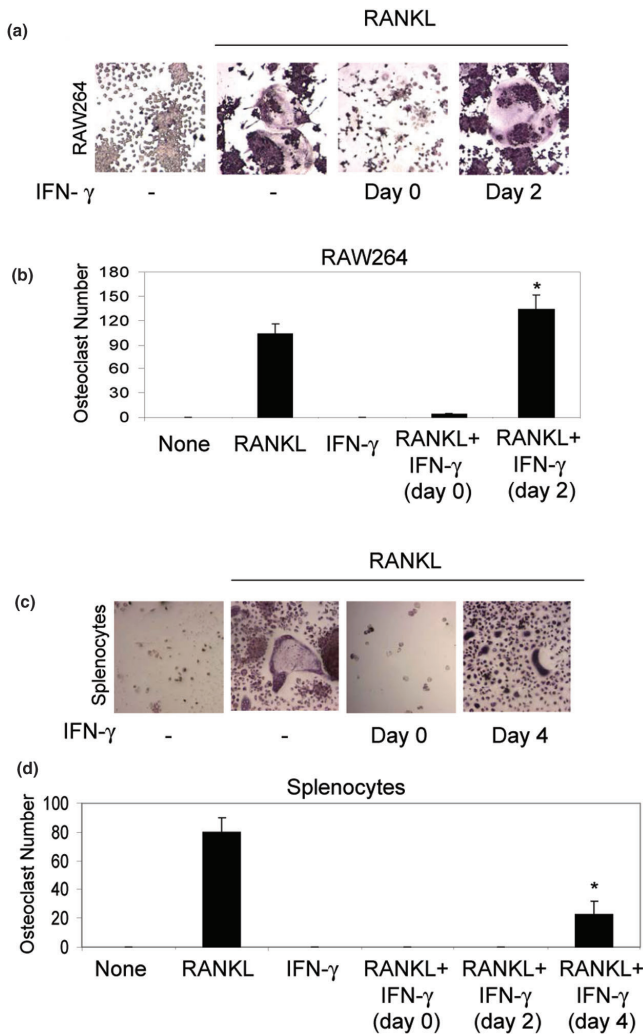
versible effects of IFN-γ on osteoclast inhibition indicate a dominant effect of this cytokine over RANKL via induction of monocyte differentiation toward the activated macrophage fate, as opposed to the osteoclast fate, when concomitantly administered.

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

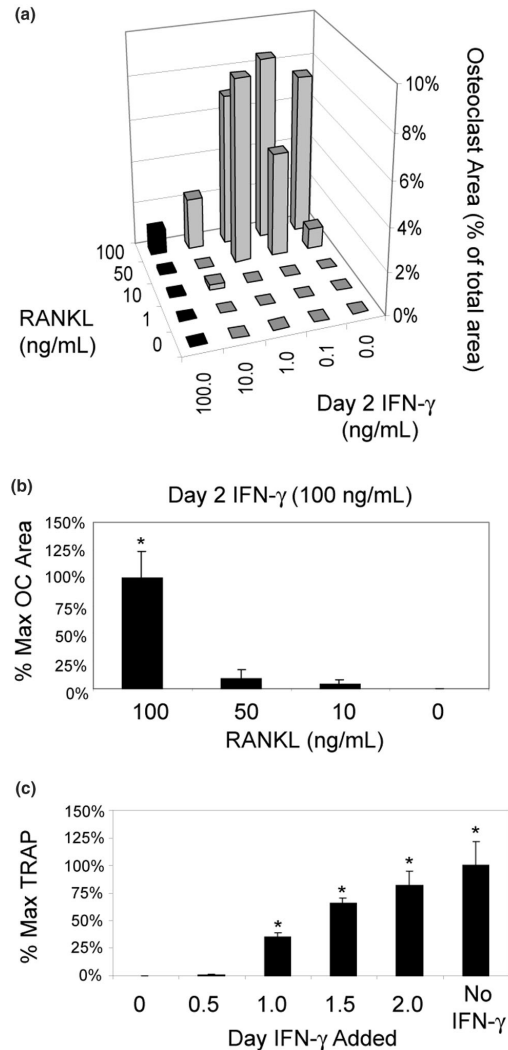
Figure 3



Pretreatment with receptor-activator of NF κ B ligand (RANKL) permits osteoclastogenesis in the presence of maximally inhibitory IFN- γ in a dose-dependent and time-dependent manner. RAW cells were grown in the presence of continuous RANKL (100 ng/ml) for 4 days, in the absence or presence of IFN- γ as indicated on either day 0 or 2 of culture. **(a)** Representative photographs from each group are shown at 10 \times magnification, and **(b)** the average number of osteoclasts per well \pm SEM is presented. **(c)**, **(d)** The experiments were repeated with primary osteoclast precursors in splenocyte cultures. For both RAW cells and primary osteoclast precursors, RANKL pretreatment allowed for osteoclastogenesis despite the presence of a maximally inhibitory dose of IFN- γ (10 ng/ml). * $P < 0.05$ compared with either untreated controls or cells treated with IFN- γ for the whole culture period.

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.

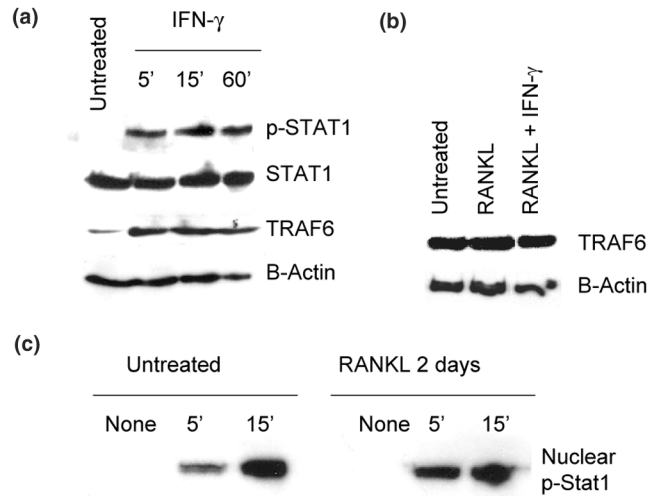
Figure 4



Osteoclast formation in the presence of IFN- γ depends upon receptor-activator of NF κ B 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 , were then fixed and tartrate-resistant acid phosphatase (TRAP) stained after day 4. **(b)** The mean \pm 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 \geq 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

Figure 5



TRAF6 levels and STAT1 signaling are intact in late pre-osteoclasts. **(a)** Untreated RAW cells were stimulated with IFN- γ for 5, 15 and 60 min, and then protein extracts were analyzed by immunoblotting as described in Materials and methods. Multiple signals were achieved by stripping and reprobing the same blot. TRAF6 expression increased with IFN- γ signaling. **(b)** RAW cells were cultured for 4 days in the presence of the indicated cytokines. Osteoclasts were observed in the receptor-activator of NF κ B ligand (RANKL) group, while activated macrophages were observed in the RANKL + IFN- γ group similar to that described in the previous experiments. TRAF6 expression was remarkably consistent in these cells. **(c)** Nuclear extracts were prepared from untreated RAW cells and late pre-osteoclasts (treated with RANKL for 2 days) and immunoblotted with antiphospho-Stat1 antibodies to assess nuclear translocation in extracts obtained at the indicated time points following IFN- γ treatment. Late pre-osteoclasts translocated phospho (p)-Stat1 as well as untreated RAW cells.

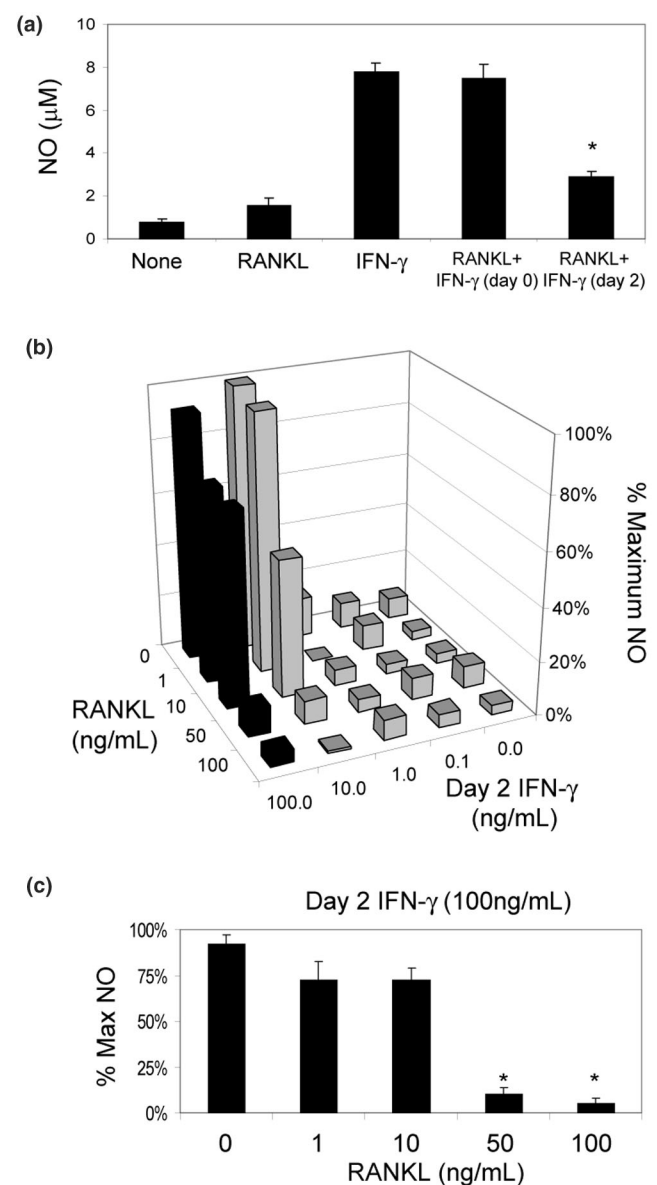
established that RANK signaling is mediated primarily through TRAF6 [35] and that IFN- γ signaling is mediated primarily through STAT1 [36,37], we examined their expression by western blot. We failed to observe significant degradation of TRAF6 after stimulation with IFN- γ or RANKL in short-term or long-term cultures (Fig. 5a,b).

Interestingly, IFN- γ -induced STAT1 phosphorylation and nuclear translocation was retained in 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

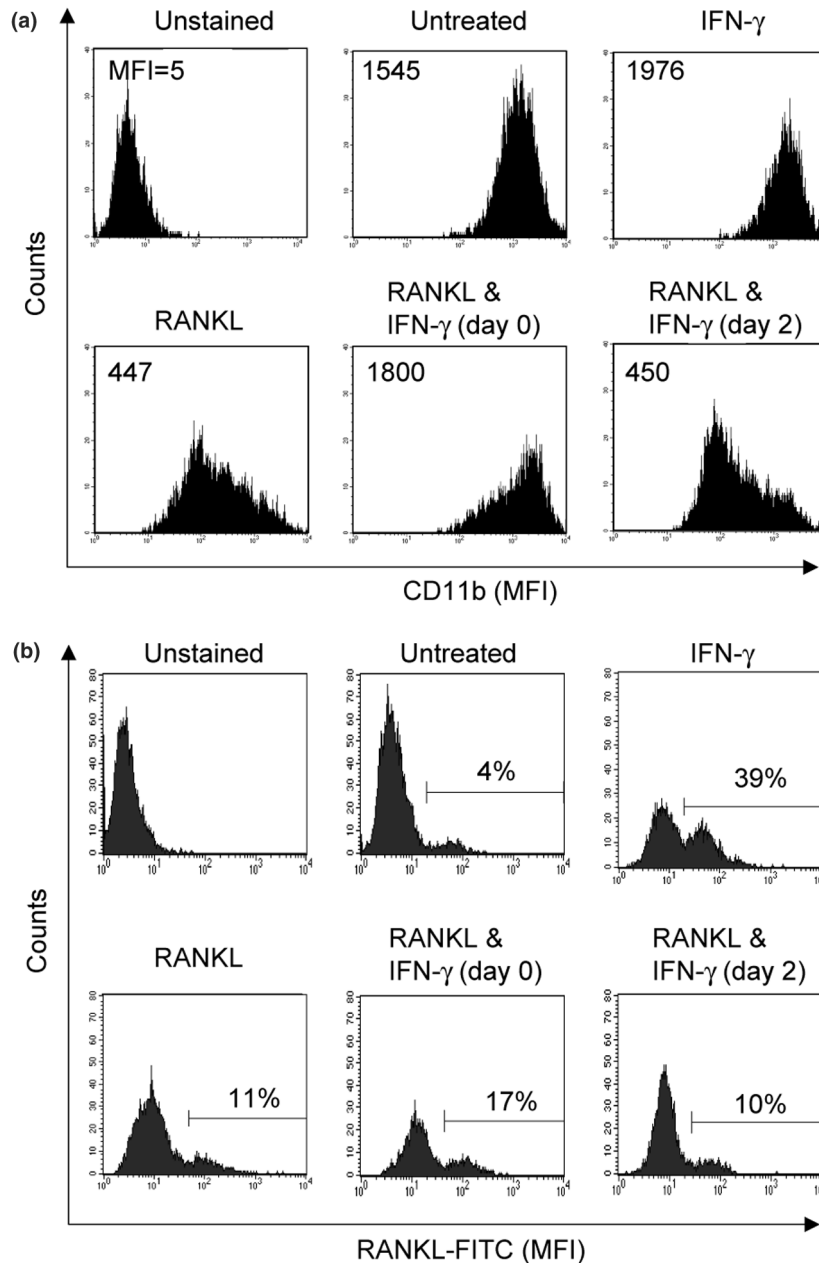
Figure 6



Receptor-activator of NF κ B ligand (RANKL) pretreatment impairs IFN- γ -induced nitric oxide (NO) production in a dose-dependent manner. **(a)** RAW cells were treated as in Figure 2a, and supernatants assayed for NO production on day 4. NO production was significantly inhibited by pretreatment with RANKL (* $P < 0.01$ versus the IFN- γ only group). **(b)** RAW cells were grown in the presence of various doses of RANKL, then supplemented with various doses of IFN- γ and maximal GST-RANKL (200 ng/ml) on day 2. NO was assayed on day 4. Black bars indicate cells treated on day 2 with 100 ng/ml IFN- γ . **(c)** This group (indicated by black bars) with statistics. Inhibition of NO production by RANKL pretreatment is dose dependent, with more inhibition with higher doses of RANKL (* $P < 0.05$ versus 0 ng/ml RANKL).

cells. IFN- γ -induced NO production in macrophages was blunted in osteoclasts, probably due to specialization for

Figure 7



Late pre-osteoclasts are resistant to IFN- γ -induced surface expression of CD11b and receptor-activator of NF κ B (RANK). **(a)** Cells were treated with the indicated cytokines for 4 days, then analyzed for CD11b expression by FACS. Late pre-osteoclasts (RAW cells pretreated with 200 ng/ml receptor-activator of NF κ B ligand [RANKL] for 2 days prior to IFN- γ treatment) expressed CD11b at levels nearly identical to RANKL-only treated cells, suggesting that late pre-osteoclasts are resistant to IFN- γ -induced CD11b upregulation. **(b)** Identically treated cells were analyzed for RANK expression by FACS. The proportion of RANK^{hi} cells was nearly identical in RANKL-only treated cells and late pre-osteoclasts, suggesting that late pre-osteoclasts are resistant to IFN- γ -induced upregulation of RANK. MFI, mean fluorescence intensity.

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 circulating, activated T cells [20,40]. In the bone microenvironment, however, more abundant RANKL produced by osteoblasts/stromal cells may influence certain monocytes to commit to the osteoclast lineage [41,42].

The present results predict that IFN- γ will not activate these cells, as they have received an early RANKL signal. The strength of the early RANKL signal may be altered in the setting of inflammatory bone diseases such as rheumatoid arthritis, in which high levels 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 NF κ B 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.

Acknowledgements

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References

- Teitelbaum SL: **Bone resorption by osteoclasts.** *Science* 2000, **289**:1504-1508.
- Childs LM, Goater JJ, O'Keefe RJ, Schwarz EM: **Efficacy of etanercept for wear debris-induced osteolysis.** *J Bone Miner Res* 2001, **16**:338-347.
- Arthritis Foundation [http://www.arthritis.org/Answers/Disease-Center/ra.asp].
- Suda T, Kobayashi K, Jimi E, Udagawa N, Takahashi N: **The molecular basis of osteoclast differentiation and activation.** *Novartis Found Symp* 2001, **232**:235-247.
- Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL: **The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption.** *J Bone Miner Res* 2000, **15**:2-12.
- Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM: **OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis.** *Nature* 1999, **397**:315-323.
- Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: **Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.** *Cell* 1998, **93**:165-176.
- Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T: **Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL.** *Proc Natl Acad Sci USA* 1998, **95**:3597-3602.
- Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL: **TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand.** *J Clin Invest* 2000, **106**:1481-1488.
- Mundy GR: *Cytokines and Bone Remodeling.* San Diego, CA: Academic Press; 1996.
- Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, Suda T: **Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells.** *Exp Cell Res* 1999, **247**:84-93.
- Horwood NJ, Elliott J, Martin TJ, Gillespie MT: **IL-12 alone and in synergy with IL-18 inhibits osteoclast formation in vitro.** *J Immunol* 2001, **166**:4915-4921.
- Hong MH, Williams H, Jin CH, Pike JW: **The inhibitory effect of interleukin-10 on mouse osteoclast formation involves novel tyrosine-phosphorylated proteins.** *J Bone Miner Res* 2000, **15**: 911-918.
- Fox SW, Chambers TJ: **Interferon-gamma directly inhibits TRANCE-induced osteoclastogenesis.** *Biochem Biophys Res Commun* 2000, **276**:868-872.
- Takahashi N, Mundy GR, Roodman GD: **Recombinant human interferon-gamma inhibits formation of human osteoclast-like cells.** *J Immunol* 1986, **137**:3544-3549.
- OMIM, interferon-gamma listing. NCBI. 14 May 2002 [electronic citation].
- Tau G, Rothman P: **Biologic functions of the IFN-gamma receptors.** *Allergy* 1999, **54**:1233-1251.
- Farrar MA, Schreiber RD: **The molecular cell biology of interferon-gamma and its receptor.** *Annu Rev Immunol* 1993, **11**: 571-611.
- Takahashi N, Udagawa N, Suda T: **A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function.** *Biochem Biophys Res Commun* 1999, **256**:449-455.

20. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM: **Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand.** *Nature* 1999, **402**:304-309.
21. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P: **Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice.** *J Immunol* 1997, **158**:5507-5513.
22. Manoury-Schwartz B, Chiochia G, Bessis N, Abehsira-Amar O, Batteux F, Muller S, Huang S, Boissier MC, Fournier C: **High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors.** *J Immunol* 1997, **158**:5501-5506.
23. Arnoldi J, Gerdes J, Flad HD: **Immunohistologic assessment of cytokine production of infiltrating cells in various forms of leprosy.** *Am J Pathol* 1990, **137**:749-753.
24. Desai SD, Birdi TJ, Antia NH: **Correlation between macrophage activation and bactericidal function and *Mycobacterium leprae* antigen presentation in macrophages of leprosy patients and normal individuals.** *Infect Immun* 1989, **57**:1311-1317.
25. Firestein GS, Alvaro-Gracia JM, Maki R, Alvaro-Garcia JM: **Quantitative analysis of cytokine gene expression in rheumatoid arthritis.** *J Immunol* 1990, **144**:3347-3353.
26. Ritchlin C, Haas-Smith SA, Hicks D, Cappuccio J, Osterland CK, Looney RJ: **Patterns of cytokine production in psoriatic synovium.** *J Rheumatol* 1998, **25**:1544-1552.
27. Park SH, Min DJ, Cho ML, Kim WU, Youn J, Park W, Cho CS, Kim HY: **Shift toward T helper 1 cytokines by type II collagen-reactive T cells in patients with rheumatoid arthritis.** *Arthritis Rheum* 2001, **44**:561-569.
28. Cannon GW, Pincus SH, Emkey RD, Denes A, Cohen SA, Wolfe F, Saway PA, Jaffer AM, Weaver AL, Cogen L, et al.: **Double-blind trial of recombinant gamma-interferon versus placebo in the treatment of rheumatoid arthritis.** *Arthritis Rheum* 1989, **32**:964-973.
29. Veys EM, Mielants H, Verbruggen G, Grosclaude JP, Meyer W, Galazka A, Schindler J: **Interferon gamma in rheumatoid arthritis – a double blind study comparing human recombinant interferon gamma with placebo.** *J Rheumatol* 1988, **15**:570-574.
30. Veys EM, Menkes CJ, Emery P: **A randomized, double-blind study comparing twenty-four-week treatment with recombinant interferon-gamma versus placebo in the treatment of rheumatoid arthritis.** *Arthritis Rheum* 1997, **40**:62-68.
31. Key LL, Jr., Rodriguiz RM, Willi SM, Wright NM, Hatcher HC, Eyre DR, Cure JK, Griffin PP, Ries WL: **Long-term treatment of osteopetrosis with recombinant human interferon gamma.** *N Engl J Med* 1995, **332**:1594-1599.
32. Lam J, Nelson CA, Ross FP, Teitelbaum SL, Fremont DH: **Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity.** *J Clin Invest* 2001, **108**:971-979.
33. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L: **A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function.** *Nature* 1997, **390**:175-179.
34. Hsu H, Lacey DL, Dunstan CR, Solovyyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ: **Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand.** *Proc Natl Acad Sci USA* 1999, **96**:3540-3545.
35. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW: **TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling.** *Genes Dev* 1999, **13**:1015-1024.
36. Bach EA, Aguet M, Schreiber RD: **The IFN gamma receptor: a paradigm for cytokine receptor signaling.** *Annu Rev Immunol* 1997, **15**:563-591.
37. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD: **Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway.** *Cell* 1996, **84**:431-442.
38. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T: **T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma.** *Nature* 2000, **408**:600-605.
39. Canete JD, Martinez SE, Farres J, Sanmarti R, Blay M, Gomez A, Salvador G, Munoz-Gomez J: **Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies.** *Ann Rheum Dis* 2000, **59**:263-268.
40. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT: **Activated T lymphocytes support osteoclast formation in vitro.** *Biochem Biophys Res Commun* 1999, **265**:144-150.
41. Quinn JM, Horwood NJ, Elliott J, Gillespie MT, Martin TJ: **Fibroblastic stromal cells express receptor activator of NF-kappa B ligand and support osteoclast differentiation.** *J Bone Miner Res* 2000, **15**:1459-1466.
42. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E, Higashio K, Gillespie MT, Martin TJ, Suda T: **Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand.** *Bone* 1999, **25**:517-523.
43. Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM: **Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint.** *Rheumatology (Oxford)* 2001, **40**:623-630.
44. Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, Takahashi K, Furuya T, Ishiyama S, Kim KJ, Saito S, Nishikawa T, Takahashi N, Togari A, Tomatsu T, Suda T, Kamatani N: **Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients.** *Arthritis Rheum* 2001, **44**:1003-1012.
45. Weitzmann MN, Cenci S, Rifas L, Haug J, Dipersio J, Pacifici R: **T cell activation induces human osteoclast formation via receptor activator of nuclear factor kappaB ligand-dependent and -independent mechanisms.** *J Bone Miner Res* 2001, **16**:328-337.
46. Kobayashi N, Kadono Y, Naito A, Matsumoto K, Yamamoto T, Tanaka S, Inoue J: **Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis.** *EMBO J* 2001, **20**:1271-1280.
47. Yang S, Madyastha P, Ries W, Key LL: **Characterization of interferon gamma receptors on osteoclasts: effect of interferon gamma on osteoclastic superoxide generation.** *J Cell Biochem* 2002, **84**:645-654.
48. Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C, Susa M: **Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFkappa B.** *J Biol Chem* 2002.
49. Abu-Amer Y: **IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-kappaB.** *J Clin Invest* 2001, **107**:1375-1385.
50. Wei S, Wang MW, Teitelbaum SL, Ross FP: **Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF-kappaB and MAP kinase signaling.** *J Biol Chem* 2001, **276**:2121-2126.
51. Haanen JB, de Waal MR, Res PC, Kraakman EM, Ottenhoff TH, de Vries RR, Spits H: **Selection of a human T helper type 1-like T cell subset by mycobacteria.** *J Exp Med* 1991, **174**:583-592.
52. Boissier MC, Chiochia G, Bessis N, Hajnal J, Garotta G, Nicoletti F, Fournier C: **Biphasic effect of interferon-gamma in murine collagen-induced arthritis.** *Eur J Immunol* 1995, **25**:1184-1190.
53. Machold KP, Neumann K, Smolen JS: **Recombinant human interferon gamma in the treatment of rheumatoid arthritis: double blind placebo controlled study.** *Ann Rheum Dis* 1992, **51**:1039-1043.

54. Ortmann RA, Shevach EM: **Susceptibility to collagen-induced arthritis: cytokine-mediated regulation.** *Clin Immunol* 2001, **98**: 109-118.
55. Cottard V, Mulleman D, Bouille P, Mezzina M, Boissier MC, Bessis N: **Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis.** *Gene Ther* 2000, **7**:1930-1939.
56. Lubberts E, Joosten LA, Chabaud M, van Den BL, Oppers B, Coenen-De Roo CJ, Richards CD, Miossec P, van Den Berg WB: **IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion.** *J Clin Invest* 2000, **105**:1697-1710.
57. Watanabe S, Imagawa T, Boivin GP, Gao G, Wilson JM, Hirsch R: **Adeno-associated virus mediates long-term gene transfer and delivery of chondroprotective IL-4 to murine synovium.** *Mol Ther* 2000, **2**:147-152.

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