Molecular Mechanisms of the Biphasic Effects of Interferon- γ on Osteoclastogenesis

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Although interferon- γ (IFN- γ) potently inhibits osteoclastogenesis, the suppressive effect is significantly reduced when osteoclast precursors are pre-exposed to the receptor activator of NF- κ B (RANK) ligand (RANKL). However, the molecular mechanism underlying the biphasic effects of IFN- γ on osteoclastogenesis remains elusive. Here, we recapitulate the biphasic functions of IFN- γ in osteoclastogenesis in both tissue culture dishes and on bone slices. We further demonstrate that IFN- γ markedly suppresses the RANKL-induced expression of nuclear factor of activated T-cells c1 (NFATc1) in normal, but not RANKL-pretreated bone marrow macrophages (BMMs). Similarly, IFN- γ impairs the activation of the nuclear factor- κ B (NF- κ B) and c-Jun N-terminal kinase (JNK) pathways in normal, but not RANKL-pretreated, BMMs. These findings indicate that IFN- γ inhibits osteoclastogenesis partially by suppressing the expression of NFATc1 and the activation of the NF- κ B and JNK pathways. Moreover, IFN- γ inhibits the RANKL-induced expression of osteoclast genes, but RANKL pretreatment reprograms osteoclast genes into a state in which they can no longer be suppressed by IFN- γ , indicating that IFN- γ inhibits osteoclastogenesis by blocking the expression of osteoclast genes. Finally, the IVVY^{535–538} motif in the cytoplasmic domain of RANK is responsible for rendering BMMs refractory to the inhibitory effect of IFN- γ . Taken together, these findings provide important mechanistic insights into the biphasic effects of IFN- γ on osteoclastogenesis.

Introduction

INTERFERON- γ (IFN- γ), A CYTOKINE produced primarily by activated T cells and natural killer cells, plays a critical role in activating macrophages, promoting type I T helper (Th1) cell differentiation, and stimulating antiviral and antibacterial activities (Schroder and others 2004). Moreover, it has been well established that IFN- γ is also crucially involved in osteoimmunology by regulating the differentiation of osteoclasts (Lorenzo and others 2008), which are the sole bone resorbing cells that not only play an important role in skeletal development and adult bone remodeling but are also implicated in various bone disorders (Teitelbaum 2000).

Osteoclasts are multinucleated giant cells which differentiate from mononuclear cells of the monocyte-macrophage lineage upon stimulation by the macrophage/monocyte-colony stimulating factor (M-CSF) and the receptor activator of NF- κ B (RANK) ligand (RANKL) (Boyle and others 2003). RANKL (also known as osteoprotegerin ligand, osteoclast differentiation factor, and TNF-related activation-induced cytokine), a member of the tumor necrosis factor (TNF) superfamily, was identified independently by 2 bone groups (Lacey and others 1998; Yasuda and others 1998) and 2 im-

munology groups (Anderson and others 1997; Wong and others 1997) in the late 1990s. RANKL plays a pivotal role in bone metabolism by mediating osteoclast differentiation, function, and survival (Lacey and others 1998) and in the immune functions by regulating dendritic cell survival and activation (Wong and others 1997; Josien and others 1999, 2000), T-cell activation (Bachmann and others 1999; Kong and others 1999), B-cell differentiation (Dougall and others 1999; Kong and others 1999), and lymph node development (Dougall and others 1999; Kong and others 1999; Kim and others 2000). Moreover, RANKL is also involved in modulating other physiological processes such as mammary gland development (Fata and others 2000) and thermoregulation in females/fever response in inflammation (Hanada and others 2009).

In bone, RANKL is primarily expressed in osteoblasts and stromal cells (Yasuda and others 1998) and regulates osteoclast differentiation, function, and survival by activating its receptor RANK, a member of the TNF receptor (TNFR) superfamily, in osteoclast precursors and mature osteoclasts. RANK employs 3 TNFR-associated factor (TRAF)-binding sites in its cytoplasmic tail (PFQEP^{369–373}, PVQEET^{559–564}, and PVEQG^{604–609}) (Armstrong and others 2002; Liu and others 2004) to recruit various TRAFs to activate the nuclear

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factor- κ B (NF- κ B) and 3 mitogen-activated protein kinase pathways (JNK, c-Jun N-terminal kinase; extracellular signal-regulated kinase; and p38) in osteoclast precursors (Boyle and others 2003; Liu and others 2004; Feng 2005). In particular, PFQEP^{369–373} has been previously shown to interact with TRAF6 (Ye and others 2002), which plays a crucial role in osteoclast formation and/or function (Lomaga and others 1999; Naito and others 1999). In addition, RANKL induces the expression of nuclear factor of activated T-cells c1 (NFATc1), which has been recognized as a master regulator of osteoclastogenesis (Takayanagi and others 2002; Takayanagi 2007; Aliprantis and others 2008). Importantly, RANK also possesses a TRAF-independent RANK cytoplasmic motif (IVVY^{535–538}) that plays a vital role in osteoclastogenesis by committing bone marrow macrophages (BMMs) to the osteoclast lineage *in vitro* (Xu and others 2006) and stimulates the osteoclast formation and function *in vivo* (Kim and others 2009).

Although numerous previous studies demonstrated that IFN- γ is a potent negative regulator of osteoclast differentiation (Takahashi and others 1986; Lacey and others 1995; Fox and Chambers 2000; Kamolmatyakul and others 2001), several groups have shown that IFN- γ exerts a stimulating effect on osteoclastogenesis *in vitro* and *in vivo* (Vignery and others 1990; Madyastha and others 2000), raising concerns about the precise role played by IFN- γ in osteoclast biology. Emerging evidence indicates that IFN- γ may play a biphasic role in osteoclastogenesis (Huang and others 2003; Gao and others 2007). Nonetheless, the molecular mechanism by which IFN- γ regulates osteoclastogenesis has not been fully elucidated. In this study, we further investigated the role of IFN- γ in osteoclastogenesis by independently carrying out detailed *in vitro* studies using mouse BMMs. Our data support the notion that IFN- γ has biphasic functions in osteoclastogenesis. Moreover, we also investigated the molecular mechanism by which IFN- γ exerts the biphasic effects on osteoclastogenesis. We show that IFN- γ exerts the inhibitory effects by suppressing the expression of the NFATc1, impairing the activation of the NF- κ B and JNK pathways, and reprogramming of the activation state of osteoclast genes.

Materials and Methods

Chemicals and biological reagents

Chemicals were from Sigma (St. Louis, MO) unless indicated otherwise. Synthetic oligonucleotides were purchased from Sigma-Genosys (Woodlands, TX). Recombinant Mouse IFN- γ was purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant GST-RANKL was prepared as previously described (McHugh and others 2000). Mouse M-CSF was prepared as culture supernatants from a M-CSF-producing cell line, CMG14-12, kindly provided by Dr. Sunao Takeshita (Takeshita and others 2000). M-CSF concentration was determined by performing MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assays with M-CSF dependent cell line M-NFS-60 from ATCC (Manassas, VA) using recombinant mouse M-CSF from R&D Systems, Inc. (Minneapolis, MN) as standards. The following antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA): antibodies against nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkB α) (Cat. No. 9242), phospho-IkB α (2859s), JNK (9252), and phospho-JNK (9251s). Anti-NFATc1 (sc-7294) and anti-

TRAF6 (sc-8409) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human Fas activating antibody (Fas-AB) was purchased from Millipore (Temecula, CA). Anti-human Fas antibody conjugated with phycoerythrin (sc-21730PE) for flow cytometry was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

In vitro osteoclastogenesis assays using primary mouse BMMs

C57BL/6 mice were obtained from Harlan Industries (Indianapolis, IN) and the experiments involving mice were performed in accordance with the regulations of the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee. BMMs were isolated from long bones of young (4–6-week-old) mice and cultured in α -minimal essential medium (α -MEM) containing 10% heat-inactivated fetal bovine serum (FBS) in the presence of M-CSF (220 ng/mL) as previously described (Feng and others 2001). To prepare osteoclasts from BMMs, 5×10^4 cells per well were cultured in 24-well tissue culture plates in the presence of 44 ng/mL M-CSF and different concentrations of purified GST-RANKL and/or IFN- γ as indicated in individual experiments. The osteoclastogenesis cultures were stained for tartrate resistant acid phosphatase (TRAP) activity using Leukocyte Acid Phosphatase Kit (387-A) from Sigma (St. Louis, MO).

In vitro bone resorption assay

BMMs were plated on bovine cortical bone slices in 24-well plates (5×10^4 /well), then treated with M-CSF (44 ng/mL) with or without RANKL (100 ng/mL) or IFN- γ (1.2 ng/mL) as indicated in individual experiments for 8 days. Bone slices were harvested and cells removed from the bone slices with 0.25 M ammonium hydroxide and mechanical agitation. Bone resorption pits were imaged using a Leica DMIRBE inverted UV SP1 confocal microscope system with Leica confocal software at the Imaging Facility of UAB. Four areas in bone slices from each bone resorption assay were chosen randomly, and the percentage of resorbed area was determined using ImageJ analysis software obtained from National Institutes of Health (NIH; <http://rsb.info.nih.gov/ij/>).

Western blot analysis

BMMs were cultured in α -MEM (serum-free) with M-CSF for 16 h before treatment with RANKL and/or IFN- γ for various times as indicated in individual experiments, then washed twice with ice-cold PBS and lysed in lysis buffer (Cat. No. 9803) from Cell Signaling Technology, Inc. (Beverly, MA), 1 $\times$ protease inhibitor mixture 1 (P-2850) and 1 $\times$ protease inhibitor mixture 2 (P-5726) from Sigma were added. Cell lysates were boiled in the presence of sodium dodecyl sulfate (SDS) sample buffer [0.5 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 10% glycerol, and 0.05% (w/v) bromophenol blue] for 5 min and loaded for electrophoresis on 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Cat. No. 162-0147) from Bio-Rad (Hercules, CA) using a semidry blotter. Membranes were blocked in blocking solution (5% nonfat dry milk in TBS buffer (Tris buffered saline) containing 0.1% Tween 20) for 1 h and then washed 3 times with TBS-T (TBS buffer containing 0.1% Tween 20). Membranes were incubated with primary antibodies in TBS-T containing 5%

bovine serum albumin (A-7030) from Sigma at 4°C overnight, membranes were then washed 3 times with TBS-T and incubated with secondary antibody in TBS-T containing 5% nonfat dry milk at room temperature for 1 h. Membranes were washed extensively and an enhanced chemiluminescence detection assay was performed using the SuperSignal West Dura kit from Pierce (Rockford, IL). The density of bands was determined using ImageJ analysis software obtained from NIH.

Semi-quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from BMMs using Trizol reagent (Cat. No. 15596-018) from Invitrogen (Carlsbad, CA). 1 µg total RNA was reversed transcribed to cDNA with oligo (dT) using the SuperScript® III First-Strand Synthesis System (Cat. No. 18080-051) from Invitrogen (Carlsbad, CA). After completion of reverse transcription, 40 µL H₂O was added to the reaction. Using 5 µL for PCR amplification of the matrix metalloproteinase 9 (*MMP9*), cathepsin K (*Ctsk*), *TRAP*, carbonic anhydrase II (*Car2*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNAs using the following condition: preheating at 95°C for 2 min, (denaturing 95°C for 30 s, annealing 58°C for 30 s, and extension 72°C for 30 s) × 20 cycles for *MMP9*, *Ctsk*, *TRAP* and *GAPDH* transcripts or × 25 cycle for *Car2*, followed by final extension 72°C for 5 min. PCR was performed with GoTaq Flexi DNA polymerase (Cat. No. M8291) from Promega (Madison, WI) in a 50 µL reaction volume. PCR primers are for *GAPDH* 5'-ACATCATCCCTGCATCCACTG-3' and 5'-TCATTGAGAGCAATGCCAGC-3'; for *MMP9* 5'-CTTCTCTCTGGACGTCAAATG-3' and 5'-CATTTTGAAACTCACACGCC-3'; for *TRAP*, 5'-GCCAAGATGGATTCATGGGTGG-3' and 5'-CAGAGACATGATGAAGTCAGCG-3'; for *Ctsk* 5'-GATGCTTACCATATGTGGGC-3' and 5'-CATATCCTTTGTTTCCCCAGC-3'; for *Car2* 5'-AGAGAACTGGCACAAGGACTT-3' and 5'-CCTCCTTTCAGCACTGCATTGT-3'. Twenty microliters of PCR mixture was loaded on 2% agarose gel for electrophoretic analysis.

Preparation of retrovirus and infection of BMMs

293GPG cells were cultured in DMEM with 10% heat-inactivated FBS supplemented with tetracycline, puromycin, G418, and penicillin/streptomycin. The chimeric receptor constructs, pMX-puro-hFas-RANK [wild type (WT)] and pMX-puro-hFas-PM3 [mutant (Mu)], were generated in previous studies (Xu and others 2004). The retroviral vectors were transiently transfected into 293GPG cells using Lipofectamine Plus Reagent (Invitrogen; Cat. No. 18324-012). After transfection, virus supernatant was collected at 48, 72, and 96 h. BMMs were then infected with virus in the presence of M-CSF (220 ng/mL) and 8 µg/mL polybrene for 24 h. For selection and expansion of transduced cells, cells were further cultured in the presence of M-CSF (220 ng/mL) and 2 µg/mL puromycin for 2 days. Selected cells were subsequently used for various studies. For assays involving the activation of the chimeric receptor, human Fas-AB was added at concentrations as indicated in individual assays.

Flow cytometric analysis

Retrovirally infected BMMs (1×10^6) were suspended in 200 µL α -MEM containing 10% heat-inactivated FBS in the

presence of M-CSF (44 ng/mL). Cells were then blocked with 1 µg of 2.4G2 antibody for 30 min at 4°C. Under dim light, 10 µL human Fas antibody conjugated with phycoerythrin was added to the cell suspension, and cells were incubated for 30 min at 4°C before centrifuging at 2000 rpm for 5 min. Cells were then washed 3 times with 1 mL α -MEM and then suspended in 1 mL α -MEM for cytometric analysis. Flow cytometric analysis was performed using a BD LSR II Flow Cytometer from the Center for AIDS Research at UAB.

Statistical analysis

Bone resorption data are expressed as mean ± standard deviation. Statistical significance was determined using Student's *t*-test and *P* values < 0.05 were considered significant.

Results

IFN- γ exerts biphasic effects on osteoclast formation

The role of IFN- γ in osteoclastogenesis remains controversial (Takahashi and others 1986; Vignery and others 1990; Lacey and others 1995; Fox and Chambers 2000; Madyastha and others 2000; Kamolmatyakul and others 2001). To delineate the precise role of IFN- γ in osteoclastogenesis, we independently and thoroughly carried out numerous assays to examine the effect of IFN- γ on *in vitro* osteoclastogenesis using primary BMMs (namely, authentic primary osteoclast precursors) from 4- to 6-week-old male C57BL/6 mice. First, BMMs were treated with M-CSF, RANKL and different doses of IFN- γ (0–2.4 ng/mL) for 4 days (Fig. 1A). However, IFN- γ inhibited osteoclastogenesis in a dose-dependent manner and completely blocked osteoclastogenesis at 1.2 ng/mL (Fig. 1A), replicating the conclusion from the previous studies (Takahashi and others 1986; Lacey and others 1995; Fox and Chambers 2000; Kamolmatyakul and others 2001). Next, we determined the effect of IFN- γ on osteoclastogenesis from BMMs that were pretreated by RANKL. BMMs were treated with M-CSF and RANKL for 1 day and then different doses of IFN- γ were added to the cultures to complete the remaining process. As shown in Fig. 1B, a few small osteoclasts formed in the cultures treated with 1.2 ng/mL or 2.4 ng/mL IFN- γ , indicating that the inhibitory effect of IFN- γ on RANKL-mediated osteoclastogenesis is reduced when osteoclast precursors are pretreated by RANKL for 1 day. It was previously shown that pretreatment of BMMs for a longer period can further reduce the inhibitory effect of IFN- γ on RANKL-mediated osteoclastogenesis (Huang and others 2003). To independently validate this finding, we pretreated BMMs with RANKL for 2 days before addition of IFN- γ (Fig. 1C). Our results demonstrate that numerous large osteoclasts formed in the cultures treated with 1.2 ng/mL and 2.4 ng/mL IFN- γ when BMMs were pretreated with RANKL for 2 days (Fig. 1C), recapitulating the previous observation (Huang and others 2003). Conversely, pretreatment of BMMs with IFN- γ for 1 day weakly impairs the ability of BMMs to differentiate into osteoclasts in response to subsequent RANKL stimulation in a dose-dependent manner (Fig. 1D). Moreover, we also repeated the assay shown in Fig. 1A by pretreating BMMs with IFN- γ for 1 day before addition of RANKL (Fig. 1E). The results indicate that pretreatment of BMMs with IFN- γ for 1 day before addition of RANKL more significantly suppresses osteoclastogenesis

FIG. 1. IFN- γ exerts biphasic effects on osteoclastogenesis. **(A)** BMMs were treated with 44 ng/mL M-CSF and 100 ng/mL RANKL for 4 days in the presence of increasing concentrations of IFN- γ (0, 0.15, 0.3, 0.6, 1.2, or 2.4 ng/mL). **(B)** BMMs were pretreated with 44 ng/mL M-CSF and 100 ng/mL RANKL for 1 day. Different doses of IFN- γ (0, 0.15, 0.3, 0.6, 1.2, or 2.4 ng/mL) were then added to the cultures for the remaining 3 days. **(C)** The assays in **B** were repeated by treating BMMs with M-CSF (44 ng/mL) and RANKL (100 ng/mL) for 2 days and different doses of IFN- γ were added to the cultures for the remaining 2 days. **(D)** BMMs were pretreated with 44 ng/mL M-CSF and different doses of IFN- γ (0, 0.15, 0.3, 0.6, 1.2, or 2.4 ng/mL) for 1 day. The cultures were continued with 44 ng/mL M-CSF and 100 ng/mL RANKL for 4 days. **(E)** BMMs were pretreated with 44 ng/mL M-CSF and different doses of IFN- γ (0, 0.15, 0.3, 0.6, 1.2, or 2.4 ng/mL) for 1 day. About 100 ng/mL RANKL was then added to the cultures for remaining 4 days. The cultures in **A–E** were stained for TRAP activity. Each condition in the assays had 3 replicates (wells). The assays were independently repeated at least 2 times. A representative area of the cultures from each condition is shown. IFN- γ , interferon- γ ; BMMs, bone marrow macrophages; M-CSF, monocyte-colony stimulating factor; RANK, receptor activator of nuclear factor- κ B; RANKL, RANK ligand; TRAP, tartrate resistant acid phosphatase.

since IFN- γ at dose of 0.15 ng/mL blocked osteoclastogenesis (Fig. 1E), compared with the inhibitory effect of IFN- γ at this dose in the assay in Fig. 1A. Taken together, these data indicate that IFN- γ can exert biphasic effects on osteoclastogenesis. When IFN- γ is exposed to BMMs before or simultaneously with RANKL, it potentially inhibits osteoclastogenesis. In contrast, if IFN- γ is added to BMMs after 1-day RANKL pretreatment, the inhibitory effect of IFN- γ on osteoclastogenesis is impaired.

To obtain more physiologically relevant data, we further asked whether we can recapitulate the biphasic effects of IFN- γ on osteoclastogenesis on bone slices. Toward this end,

we performed bone resorption assays by plating and culturing BMMs on bone slices (Fig. 2). As a negative control, BMMs grown on bone slices in the presence of M-CSF alone resulted in no bone resorption pits (Fig. 2A). M-CSF and RANKL were sufficient to promote the formation of functional osteoclasts, indicated by the presence of numerous bone resorption pits. In contrast, IFN- γ significantly inhibited the formation of functional osteoclasts, reflected by reduced bone resorption activity (Fig. 2A, B). However, if the cells were first pretreated with M-CSF and RANKL for 2 days and then IFN- γ was added to the culture for the remaining 6 culturing days, the ability of IFN- γ to block the formation of

FIG. 2. Effects of IFN- γ on osteoclastogenesis on bone slices. **(A)** BMMs on bone slices were treated with 44 ng/mL M-CSF (M) alone, 44 ng/mL M and 100 ng/mL RANKL (R), or 44 ng/mL M and 100 ng/mL R plus 1.2 ng/mL IFN- γ (I) for 8 days. Also, BMMs on bone slices were primed with 44 ng/mL M and 100 ng/mL R for 2 days and then treated with 44 ng/mL M, 100 ng/mL R, and 1.2 ng/mL I for 6 days. Bone resorption pits on bone slices were analyzed by confocal microscopy. The assays were independently repeated 2 times and a representative view from each assay was shown. **(B)** Quantification of the bone resorption assays. Four areas in bone slices from each bone resorption assay shown in A were chosen randomly, and the percentage of resorbed area was determined as described in the Materials and Methods section. Data are presented as mean $\pm$ standard deviation.

functional osteoclasts on bone slices was significantly impaired (Fig. 2A, B), indicating that pretreatment of BMMs with RANKL can also reduce the inhibitory effect of IFN- γ on osteoclastogenesis on bone slices. This finding further substantiates that IFN- γ plays biphasic roles in osteoclastogenesis.

IFN- γ inhibits osteoclastogenesis by impairing the activation of the JNK and NF- κ B pathways and suppressing the expression of NFATc1

It was initially shown that IFN- γ inhibits osteoclast formation by inducing degradation of TRAF6 (Takayanagi and others 2000). However, two other groups later failed to replicate this finding (Huang and others 2003; Ji and others 2009). To address this contention, we independently examined the capacity of IFN- γ to promote the degradation of TRAF6. As shown in Fig. 3A, 1-day IFN- γ stimulation exerts no effect on stability of TRAF6 in BMMs. To further address the issue, we repeated the assay with longer IFN- γ treatments and our data demonstrate that the stimulation with IFN- γ as long as 3 days is unable to induce degradation of TRAF6 (Fig. 3B). These findings support the notion that IFN- γ inhibits osteoclast formation by mechanism(s) other than degradation of TRAF6 (Huang and others 2003; Ji and others 2009).

To elucidate the precise mechanism by which IFN- γ suppresses osteoclastogenesis, we examined the effect of IFN- γ on the expression of NFATc1, which is a master regulator of

osteoclastogenesis (Takayanagi and others 2002; Takayanagi 2007; Aliprantis and others 2008). To this end, we treated BMMs with RANKL for 24 h in the absence or presence of IFN- γ and the expression of NFATc1 was assessed by western blot analysis. As shown in Fig. 3A, while RANKL dramatically up-regulated NFATc1 expression (lane 2), the addition of IFN- γ almost completely abrogated the RANKL-induced NFATc1 expression (lane 3). Given that we and others have demonstrated that 2-day RANKL pretreatment significantly reduces the inhibitory effect of IFN- γ on osteoclastogenesis (Fig. 1C) (Huang and others 2003), we further examined whether RANKL pretreatment abrogates the IFN- γ -mediated inhibitory effect on the expression of NFATc1. The data demonstrate that while NFATc1 expression induced by 2-day RANKL pretreatment does not significantly decrease during the following 1-day M-CSF-only stimulation period (lanes 1 and 2, Fig. 3C), RANKL treatment further enhances NFATc1 expression after 2-day RANKL pretreatment (lanes 2 and 3, Fig. 3C). Moreover, IFN- γ slightly inhibits RANKL-induced additional NFATc1 expression after 2-day RANKL pretreatment (lanes 3 and 4, Fig. 3C). Taken together, these findings indicate that IFN- γ inhibits osteoclastogenesis in part by suppressing the RANKL-induced activation of NFATc1.

Previously it was shown that IFN- γ inhibits osteoclastogenesis by suppressing the RANKL-induced activation of the NF- κ B and JNK signaling pathways in osteoclast precursors (Takayanagi and others 2000). To further delineate the

FIG. 3. IFN- γ exerts no effect on TRAF6 stability but regulates differently the expression of NFATc1 in normal BMMs and RANKL-pretreated BMMs. (A) BMMs were treated with 44 ng/mL M-CSF (M) and 100 ng/mL RANKL (R) for 1 day in the absence or presence of IFN- γ (I). TRAF6 and NFATc1 expression was assessed by western blot analysis. Blots were stripped and then reprobed with β -actin antibody as loading control. (B) BMMs were treated with 44 ng/mL M and 100 ng/mL R for 1, 2, or 3 days in the absence or presence of IFN- γ (I). TRAF6 expression was determined as in A. (C) BMMs were treated with 44 ng/mL M and 100 ng/mL R for 2 days. Cells were then either lysed immediately after the 2-day RANKL pretreatment or continued with 44 ng/mL M alone, 44 ng/mL M and 100 ng/mL R, or 44 ng/mL M and 100 ng/mL R plus 1.2 ng/mL I for 1 day before lysis for western blot analysis.

NFATc1 expression was determined as in A. The assays were independently repeated at least 2 times. Representative data are shown. TRAF, tumor necrosis factor receptor-associated factor; NFATc1, nuclear factor of activated T-cells c1.

molecular mechanism by which IFN- γ suppresses osteoclastogenesis, we also examined the effect of IFN- γ on RANKL-induced activation of the NF- κ B and JNK pathways in BMMs. As shown in Fig. 4, BMMs were treated with RANKL for 5 or 10 min without or with IFN- γ and the activation of the NF- κ B and JNK pathways was assessed. Our results show that IFN- γ impaired the activation of the NF- κ B and JNK signaling pathways (compare lanes 2 vs. 4, and lanes 3 vs. 5, Fig. 4). Moreover, we also examined whether RANKL pretreatment abrogates the IFN- γ -mediated inhibitory effect on the activation of the NF- κ B and JNK signaling pathways. Our data demonstrate that that IFN- γ can no longer exert the inhibitory effect on the activation of the NF- κ B and JNK signaling pathways once BMMs are pretreated with RANKL for 2 days (compare lanes 7 vs. 9, and lanes 8 vs. 10, Fig. 4). These

findings indicate that IFN- γ inhibits osteoclastogenesis also by interfering with the activation of the JNK and NF- κ B pathways.

IFN- γ inhibits osteoclastogenesis by preventing the activation of the expression of osteoclast genes

RANKL promotes osteoclast differentiation by altering the expression of a large number of genes (Cappellen and others 2002; Ishida and others 2002; Rho and others 2002). Among them are those encoding MMP9, Car2, Ctsk, and TRAP. To further decipher the molecular mechanism by which IFN- γ inhibits osteoclastogenesis, we examined the effect of IFN- γ on the expression of the four osteoclast genes (Fig. 5). BMMs were treated with RANKL for 1, 2, or 4 days in the absence or

FIG. 4. IFN- γ inhibits RANKL-mediated activation of the NF- κ B and JNK pathways in normal BMMs but not in RANKL-pretreated BMMs. Normal BMMs or BMMs pretreated with RANKL for 2 days were cultured with serum-free media for 16 h before treatment with RANKL for 0, 5, or 10 min in the absence or presence of IFN- γ . Activation of the NF- κ B and JNK pathways was assessed as phosphorylation of I κ B and JNK using western analysis with antibodies against phospho-I κ B (p-I κ B) and phospho-JNK (p-JNK). The same volume of lysates was run and then probed with antibodies against I κ B and JNK as loading control. The assays were independently repeated 3 times. A representative set of data is shown. Band densities were determined as described in the Materials and Methods section and presented as p-I κ B/I κ B (total) and p-JNK/JNK (total) ratios below the images. NF- κ B, nuclear factor- κ B; JNK, c-Jun N-terminal kinase.

FIG. 5. IFN- γ inhibits RANKL-mediated expression of osteoclast genes in normal BMMs but not in RANKL-pretreated BMMs. **(A)** BMMs were treated with M-CSF (M, 44 ng/mL) alone, M (44 ng/mL) and RANKL (R, 100 ng/mL), or M (44 ng/mL) and R (100 ng/mL) plus IFN- γ (I, 1.2 ng/mL) for 1, 2, or 4 days. Gene expression was determined by semi-quantitative RT-PCR. **(B)** BMMs were treated with M (44 ng/mL) and R (100 ng/mL) for 1 day (*left panel*) or 2 days (*right panel*) and then cultured with M (44 ng/mL) alone, M (44 ng/mL) and R (100 ng/mL), or M (44 ng/mL) and R (100 ng/mL) plus I (1.2 ng/mL) for 3 days (*left panel*) or 2 days (*right panel*). Gene expression was determined by semi-quantitative RT-PCR. The assays were independently repeated 2 times.

presence of IFN- γ . Although RANKL significantly increased the expression of these genes with time, the presence of IFN- γ in the cultures completely blocked the activation of the expression of these genes (Fig. 5A), indicating that IFN- γ inhibits osteoclastogenesis by preventing the activation of the expression of osteoclast genes.

We then examined whether RANKL pretreatment can eliminate the inhibitory effect of IFN- γ on the expression of the 4 osteoclast genes (Fig. 5B). BMMs were pretreated with RANKL for 1 or 2 days and then the cultures were continued with M-CSF alone, M-CSF and RANKL, or M-CSF and RANKL plus IFN- γ . Although IFN- γ still inhibited the expression of the *MMP9*, *TRAP*, *Car2*, and *Ctsk* genes in BMMs pretreated with RANKL for 1 day, it failed to inhibit the expression of these genes in BMMs pretreated with RANKL for 2 days. The findings indicate that while IFN- γ inhibits osteoclastogenesis by preventing the activation of the expression of osteoclast genes, the RANKL pretreatment renders BMMs refractory to the inhibitory effect of IFN- γ by altering osteoclast genes into a state in which IFN- γ is no longer able to suppress the activation of the expression of osteoclast genes.

The RANK IVVY⁵³⁵⁻⁵³⁸ motif plays a critical role in rendering osteoclast precursors refractory to the inhibition of IFN- γ

A TRAF-independent RANK cytoplasmic motif (IVVY⁵³⁵⁻⁵³⁸) has been shown to play a crucial role in osteoclastogenesis by priming BMMs to the osteoclast lineage *in vitro* (Xu and others 2006). Thus, we investigated whether this IVVY motif also plays a critical role in rendering osteoclast precursors refractory to the inhibition of IFN- γ . To do so, we took ad-

vantage of 2 chimeric receptors that were previously constructed and validated (Xu and others 2004, 2006). Briefly, the chimeras were prepared by linking the external domain of human Fas to the transmembrane and cytoplasmic domains of normal mouse RANK (WT) or RANK bearing inactivating mutations in the IVVY motif (Mu; Fig. 6A) (Xu and others 2004). These chimeras can be activated by a human-specific Fas-AB to exclusively activate RANK without affecting endogenous mouse Fas (Xu and others 2004). Flow cytometric analysis showed that WT and Mu were expressed at comparable levels on the surface of infected cells (Fig. 6B). WT-expressing or Mu-expressing cells were treated with M-CSF and Fas-AB for 2 days before the cultures were switched to M-CSF alone, M-CSF and RANKL, or M-CSF and RANKL plus IFN- γ for 4 days (Fig. 6C). WT-expressing cells formed a few small osteoclasts in the presence of M-CSF alone after 2-day Fas-AB treatment. This is likely to result from the overexpression of the WT chimeric receptor in a few cells in the infected BMM population and it is well known that the overexpression of TNF family members can lead to self-activation (Inoue and others 2000). As expected, Fas-pretreated BMMs formed large multinucleated osteoclasts in response to subsequent M-CSF and RANKL stimulation for 4 days. Moreover, addition of IFN- γ to the culture still resulted in significant osteoclast formation from WT BMMs. Thus, this finding has recapitulated our previous observations that the chimera containing the external domain of human Fas to the transmembrane and cytoplasmic domains of normal mouse RANK (WT) can be activated by Fas-AB to transmit RANK signaling pathways (Xu and others 2004). Mu-expressing cells formed no osteoclasts in the presence of M-CSF alone after 2-day Fas-AB treatment, but the cells formed large multinucleated osteoclasts in

FIG. 6. The RANK IVVY535-538 motif plays a critical role in rendering BMMs refractory to the inhibition of IFN- γ . **(A)** Schematic diagram of wild-type (WT) chimeric receptor with normal RANK cytoplasmic domain and mutant (Mu) chimeric receptor with mutated RANK IVAF535-538 motif (VY is replaced by AF). **(B)** Flow cytometric analysis of BMMs infected with virus encoding WT and Mu. **(C)** BMMs expressing WT or Mu were treated with M-CSF (M, 44 ng/mL) and Fas activating antibody (F, 100 ng/mL) for 2 days and the cultures were then continued with M (44 ng/mL), M (44 ng/mL) and R (100 ng/mL), or M (44 ng/mL) and R (100 ng/mL) plus I (1.2 ng/mL) for 4 days. The cultures were stained for TRAP activity. The assays were independently repeated twice. A representative view of the cultures from each condition is shown.

response to subsequent M-CSF and RANKL stimulation for 4 days (Fig. 6C). Importantly, IFN- γ was able to inhibit formation of osteoclasts from Mu-expressing BMMs after 2-day Fas-AB treatment. These results indicate that the RANK IVVY⁵³⁵⁻⁵³⁸ motif plays a critical role in rendering osteoclast precursors refractory to the inhibition of IFN- γ .

Discussion

An inhibitory effect of IFN- γ on bone resorption was initially discovered almost a quarter of century ago in an *in vitro* neonatal mouse bone resorption assay (Gowen and Mundy 1986). Numerous subsequent studies further demonstrated that IFN- γ exerts the suppressive effect on bone resorption primarily by blocking osteoclastogenesis (Takahashi and others 1986; Lacey and others 1995; Fox and Chambers 2000; Kamolmatyakul and others 2001). However, the physiological significance of the inhibitory role of this Th1 cytokine in osteoclastogenesis had remained elusive until the unraveling the RANKL/RANK system. In the late 1990s, RANKL was almost simultaneously discovered by bone biologists (Lacey and others 1998; Yasuda and others 1998) and immunologists independently (Anderson and others 1997; Wong and

others 1997). Although bone researchers demonstrated that RANKL is a potent stimulator of bone resorption via promoting osteoclast formation, function, and survival (Lacey and others 1998; Yasuda and others 1998), immunologists showed that RANKL is also an important regulator of immune functions such as dendritic cell survival and activation (Wong and others 1997; Josien and others 1999, 2000), T-cell activation (Bachmann and others 1999; Kong and others 1999), and B-cell differentiation (Dougall and others 1999; Kong and others 1999). In particular, it was shown that RANKL expression is up-regulated in activated T cells to promote dendritic cell survival and activation (Wong and others 1997; Josien and others 1999, 2000). This raised a question: how is extensive bone resorption prevented in the constant immune response involving the activation of T cells? Not long after the unraveling of the RANKL/RANK system, it was revealed that IFN- γ , produced by activated T cells, plays an important role in preventing abnormal bone resorption during normal T-cell-mediated immune response by counteracting the stimulatory effect of T-cell-produced RANKL on osteoclastogenesis (Takayanagi and others 2000).

Activated T cells are present in a number of chronic inflammatory conditions such as rheumatoid arthritis (Cope and

others 2007) and periodontitis (Taubman and Kawai 2001), which are characterized by extensive bone loss. So, this leads to another intriguing issue: why is IFN- γ , produced by activated T cells, unable to prevent excessive bone resorption by blocking the RANKL-induced osteoclastogenesis in these pathological conditions? Intriguingly, a study showed that IFN- γ failed to inhibit osteoclastogenesis from a macrophage cell line or spleen cells which were pretreated by RANKL for 2 days (Huang and others 2003). Our first objective of the present study was to further validate the finding by independently examining the regulatory role of IFN- γ in osteoclastogenesis using BMMs, which represent authentic primary osteoclast precursors. Moreover, we also demonstrated that osteoclasts derived from RANKL-pretreated BMMs are functional in the presence of IFN- γ . These findings may provide an answer to the question of why IFN- γ is unable to prevent excessive bone resorption in rheumatoid arthritis and periodontitis. In healthy individuals, RANKL levels in the bone marrow or RANKL expression levels on stromal cell/osteoblasts are low and thus BMMs are pre-exposed to low levels of RANKL. Thus, when these osteoclast precursors are recruited into inflammatory sites rich in activated T cells, IFN- γ is able to prevent formation of osteoclastogenesis. However, in certain individuals, RANKL levels in the bone marrow may be elevated and BMMs are pretreated by RANKL in the bone marrow before they are recruited to inflammatory sites. These pretreated osteoclast precursors are able to form osteoclasts in presence of IFN- γ . Consistent with this notion, RANKL expression levels are elevated in bone marrow cells in postmenopausal women (Eghbali-Fatourehchi and others 2003) and periodontal bone loss is associated with postmenopausal osteoporosis. Moreover, it has been also shown that stromal cells/osteoblasts from aged mice (C57BL/6) express higher levels of RANKL (Cao and others 2003) and age is one of key risk factors for periodontal bone loss.

Interestingly, it is noted that although osteoclasts from 2-day RANKL pretreatment followed by addition of IFN- γ for the remaining 6 days exhibited much higher bone resorption activity than those from 8-day RANKL treatment control (Fig. 2), osteoclast formation in tissue culture dishes in response to 2-day RANKL pretreatment followed by addition of IFN- γ for the remaining 2 days is similar to that stimulated by 4 day RANKL treatment (Fig. 1C). This discrepancy may reflect the fact that the formation of osteoclasts in tissue culture dishes is primarily signified by only few features of osteoclasts such as TRAP expression and cell fusion (leading to a change in cell size and multinucleation), osteoclast differentiation involves the regulation of a large number of genes and many other cellular changes. Our data suggest that while IFN- γ does not significantly affect cell fusion, it may considerably impair differentiation of BMMs into functional osteoclasts by inhibiting the expression of osteoclast genes required for bone resorption as shown in Fig. 5.

Our second goal was to investigate the molecular mechanism by which IFN- γ exerts the biphasic effects on osteoclastogenesis. It was originally shown that IFN- γ inhibits osteoclast formation by causing degradation of TRAF6, which further leads to impaired activation of the JNK and NF- κ B pathways (Takayanagi and others 2000). This finding was subsequently challenged by 2 studies demonstrating that IFN- γ exerts no effect on the stability of TRAF6 (Huang and others 2003; Ji and others 2009). We also failed to ob-

serve an effect of IFN- γ on the stability of TRAF6 (Fig. 3). However, our data replicated the previous finding that IFN- γ suppresses the activation of the JNK and NF- κ B pathways (Takayanagi and others 2000). More importantly, we have further demonstrated that IFN- γ -mediated inhibitory effect on the RANKL-induced activation of the JNK and NF- κ B pathways is abrogated in BMMs pretreated by RANKL for 2 days, indicating that IFN- γ exerts the biphasic effects on osteoclastogenesis in part by suppressing the activation of the JNK and NF- κ B pathways.

NFATc1 has been established as a master regulator of osteoclastogenesis (Takayanagi and others 2002; Takayanagi 2007; Aliprantis and others 2008). Recently, we have demonstrated that lipopolysaccharide plays a bifunctional role in osteoclastogenesis by regulating the expression of NFATc1 (Liu and others 2009). In our present study, we demonstrate that IFN- γ potentially inhibits RANKL-induced NFATc1 expression in BMMs (Fig. 3A), indicating that IFN- γ inhibits osteoclastogenesis in part by suppressing the RANKL-induced activation of NFATc1 expression. Moreover, we show that NFATc1 expression levels in BMMs pretreated with RANKL for 2 days do not significantly decline with time (Fig. 3C), revealing that NFATc1 is relatively stable in BMMs. This finding explains why IFN- γ exhibits reduced ability to inhibit osteoclastogenesis from RANKL-pretreated BMMs, namely, NFATc1 levels in RANKL-pretreated BMMs are largely sufficient to support osteoclastogenesis. Interestingly, we also demonstrate that continued RANKL treatment after 2-day RANKL pretreatment further enhances NFATc1 expression and IFN- γ only moderately inhibits the RANKL-induced additional NFATc1 expression (lanes 3 and 4, Fig. 3C). These data support the notion that once the NFATc1 gene is primed by RANKL, it becomes somewhat refractory to the inhibitory effect of IFN- γ .

Moreover, microarray studies have revealed that RANKL mediates osteoclastogenesis ultimately by regulating the expression of a large number of genes (Cappellen and others 2002; Ishida and others 2002; Rho and others 2002), including those encoding well-known osteoclast markers such as *MMP9*, *Car2*, *Ctsk*, and *TRAP*. Our data demonstrate that although IFN- γ potentially inhibits the expression of the four osteoclast genes, it is no longer able to suppress the expression of these osteoclast genes in osteoclast precursors pretreated by RANKL for 2 days. Based on these findings, we conclude that IFN- γ inhibits osteoclastogenesis by preventing the activation of the expression of osteoclast genes.

Finally, we determined whether an TRAF-independent RANK cytoplasmic motif (IVVY⁵³⁵⁻⁵³⁸) is specifically involved in rendering osteoclast precursors refractory to the inhibition of IFN- γ since we previously showed that this motif plays an essential role in osteoclastogenesis by priming BMMs to the osteoclast lineage *in vitro* (Xu and others 2006). To this end, we used a chimeric receptor approach (Fig. 6). Our data indicate that the activation of the wild type chimeric receptor (WT) for 2 days efficiently rendered BMMs refractory to the inhibitory effect of IFN- γ as these cells were able to form osteoclasts in the presence of M-CSF, RANKL, and IFN- γ (Fig. 6C). In contrast, 2-day activation of the chimeric receptor bearing an inactivating mutation on the IVVY motif (Mu) failed to make BMMs refractory to the inhibitory effect of IFN- γ since the cells did not differentiate into osteoclasts in response to M-CSF, RANKL, and IFN- γ treatment (Fig. 6C). Although the precise mechanism by which the

IVVY motif mediates the refractory effect remains to be determined, a recent study has demonstrated that the IVVY motif regulates osteoclastogenesis by engaging Vav3 indirectly via an unknown adaptor protein (Kim and others 2009). Thus, one potential mechanism may involve the reorganization of cytoskeleton by the IVVY motif-mediated signaling, resulting in the constitutive activation of the RANK signaling important for osteoclastogenesis. Future studies aimed at delineating the molecular mechanism by which the IVVY motif mediates the refractory effect of IFN- γ on osteoclastogenesis may provide crucial mechanistic insights into the molecular mechanisms of osteoclastogenesis.

In summary, our key findings are that (a) IFN- γ exerts inhibitory effects on osteoclastogenesis by suppressing the activation of the JNK and NF- κ B pathways and suppressing the expression of NFATc1, (b) the inhibition of osteoclastogenesis by IFN- γ is ultimately regulated at the gene expression levels, and (c) the RANK IVVY⁵³⁵⁻⁵³⁸ motif plays a critical role in rendering osteoclast precursors refractory to the inhibition of IFN- γ . Our current findings provide important new insights into the molecular mechanism by which IFN- γ regulates osteoclastogenesis. Nonetheless, these observations have also raised additional questions to be addressed in the future. First, it was previously believed that IFN- γ suppresses the activation of the JNK and NF- κ B pathways through IFN- γ -induced degradation of TRAF6 (Takayanagi and others 2000). Since we and others have demonstrated that IFN- γ has no effect on the stability of TRAF6, it is likely that IFN- γ impairs the activation of the JNK and NF- κ B pathways by a mechanism(s) independent of TRAF6. Second, while IFN- γ potentially inhibits the RANKL-mediated NFATc1 expression, the IFN- γ -imposed inhibition on the activation of NFATc1 expression by RANKL was markedly reduced in BMMs pretreated by RANKL. The precise mechanism underlying the effects of IFN- γ on NFATc1 expression remains to be elucidated. Hence, future studies aimed at addressing these issues will not only provide additional insights into the molecular mechanism by which IFN- γ regulates osteoclastogenesis but may also reveal novel therapeutic targets for treating and preventing bone loss in various pathological conditions.

Acknowledgments

This work was supported by a grant (No. AR47830 to X.F.) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, a *Within Our Reach* Innovative Basic Research grant from Research and Education Foundation of American College of Rheumatology (to X.F.), a pilot grant from the Center for Metabolic Bone Disease at UAB (to S.W.), and the National Scholarship for Building High Level University from Chinese Ministry of Education (to J.C.).

Author Disclosure Statement

No competing financial interests exist.

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Received 4 March 2011 / Accepted 19 August 2011