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# IL-12 Stimulates the Osteoclast Inhibitory Peptide-1 (OIP-1/hSca) Gene Expression in CD4+ T Cells

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## ABSTRACT

Immune cell products such as interferon (IFN)-γ and interleukin (IL)-12 are potent inhibitors of osteoclast formation. We previously characterized the human osteoclast inhibitory peptide-1 (OIP-1/hSca), a Ly-6 gene family member and showed IFN-γ modulation of OIP-1 expression in bone marrow cells. Whether, IL-12 regulates OIP-1 expression in the bone microenvironment is unclear. Real-time PCR analysis revealed that IL-12 treatment significantly enhanced OIP-1 mRNA expression in human bone marrow mononuclear cells. Because IL-12 induces IFN-γ production by T cells, we tested whether IFN-γ participates in IL-12 stimulation of OIP-1 gene expression in these cells. IL-12 treatment in the presence of IFN-γ neutralizing antibody significantly increased OIP-1 mRNA expression, suggesting that IL-12 directly regulates OIP-1 gene expression. Interestingly, real-time PCR analysis demonstrated that IL-12 induces OIP-1 expression (3.2-fold) in CD4+ T cells; however, there was no significant change in CD8+ T cells. Also, IL-12 (10 ng/ml) treatment of Jurkat cells transfected with OIP-1 gene (−1 to −1,988 bp) promoter-luciferase reporter plasmid demonstrated a 5-fold and 2.7-fold increase in OIP-1 gene promoter activity in the presence and absence of antibody against IFN-γ, respectively. We showed that STAT-1,3 inhibitors treatment significantly decreased IL-12 stimulated OIP-1 promoter activity. Chromatin immunoprecipitation (ChIP) assay confirmed STAT-3, but not STAT-1 binding to the OIP-1 gene promoter in response to IL-12 stimulation. These results suggest that IL-12 stimulates the OIP-1 gene expression through STAT-3 activation in CD4+ T cells. J. Cell. Biochem. 107: 104–111, 2009. 

**KEY WORDS:** OSTEOCLAST INHIBITORY PEPTIDE-1; T CELLS; INTERFERON-γ; STAT; BONE MARROW CELLS

Osteoclast formation and bone resorption activity is regulated by a variety of cell types and its products in the bone microenvironment [Roodman, 1996]. We have previously identified and characterized a novel autocrine/paracrine inhibitor of osteoclast formation termed osteoclast inhibitory peptide-1 (OIP-1/hSca). OIP-1/hSca is also known as retinoic acid induced gene expression (*RIG-E*) or thymic shared antigen-1 (*TSA-1*) and is a *Ly-6* gene family member expressed on immature thymocytes and thymus epithelial cells. OIP-1/hSca is a glycophosphatidylinositol (GPI)-linked membrane protein (16 kDa) containing a 79 amino acid extracellular peptide and a 32 amino acid carboxy terminal GPI-linked peptide (c-peptide) [Koide et al., 2002]. OIP-1 has 65% homology with mouse *Sca-2* at the nucleotide level. High levels of Sca-2 expression in murine bone marrow cells and spleen cells have been reported [Classon and Coverdale, 1996]. Sca-2 is a useful marker in early T cell development and activation, and seems to play a regulatory role in thymocyte differentiation. Sca-2 is expressed in human lymphoid tissues as well as in various nonlymphoid tissues [MacNeil et al., 1993]. Sca-2 has been shown to function as a modulator of the T cell receptor (TCR) signaling pathway. Recently, it has been reported that Sca-2 is physically and functionally associated with CD3 ζ chains of the TCR complex [Kosugi et al., 1998]. Moreover, Ly-6A (Sca-1) knock-out mice had decreased bone mineral density and bone mineral content, implicating an essential role for the LY-6 gene family in normal bone remodeling [Bonyadi et al., 2003].

Immune cell products such as interferon's (IFNs) that are released in response to inflammatory stimuli have been reported to be important local negative regulators for bone remodeling [Roodman, 1999]. We have shown that IFN-γ significantly enhances OIP-1/hSca mRNA expression in osteoclast precursor cells and that OIP-1/

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hSca c-peptide specific antibody partially neutralized IFN-γ inhibition of osteoclast formation [Koide et al., 2003]. T cell production of IFN-γ strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway [Takayanagi et al., 2000]. These studies suggested there is cross-talk between TNF and IFN families of cytokines, through which IFN provides a negative link between T cell activation and bone resorption. Furthermore, activated CD8+ T cell subsets have been shown to inhibit osteoclast formation while CD4+ subsets induce osteoclast formation in the presence of M-CSF [Choi et al., 2001]. However, it has been reported that the T cells exerts both stimulatory and inhibitory actions on osteoclastogenesis based on mode of activation and cytokine production by these cells [Grcevic et al., 2001]. IL-12 is primarily produced by macrophages and dendritic cells and has been shown to induce IFN-γ production by T cells [Robinson and O'Garra, 2002]. It has been reported that IL-12 alone and in synergy with IL-18 has been shown to inhibit osteoclast formation in co-cultures of murine osteoblast and spleen cells [Horwood et al., 2001]. Also, the inhibitory effects of these cytokines was shown to be blocked by the addition of an anti-IFN-γ neutralizing antibody suggesting that IFN-γ plays an important role in the IL-12 and IL-18 inhibition of the bone resorbing activity of osteoclasts [Yamada et al., 2002]. In this study, we examine IL-12 transcriptional regulation of OIP-1 expression in T cells and show that IL-12 directly regulates OIP-1 gene expression in CD4+ T cells.

## MATERIALS AND METHODS

### REAGENTS

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). STAT-1 inhibitor, fludarabine was purchased from Sigma (St. Louis, MO) and the STAT-3 specific peptide inhibitor that contains a C-terminal mts (membrane translocating sequence) which acts as a highly selective, potent blocker of STAT-3 activation (Cat. log # 573096) was obtained from Calbiochem (Gibbstown, NJ). IL-12 recombinant protein and anti-IFN-γ antibody were obtained from R&D systems, Inc. (Minneapolis, MN). Peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Super signal-enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Piscataway, NJ) and nitrocellulose membranes were purchased from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI).

### OIP-1/hSCA GENE PROMOTER ACTIVITY ASSAY

We have previously developed an OIP-1 gene promoter (−1 to −1,988 bp relative to transcription start site)-luciferase reporter plasmid construct (OIP-1 pGL2 Basic#3) as described [Srinivasan et al., 2006]. Jurkat T-cells ($2 \times 10^6$) plated in a six-well culture plate were transiently transfected with the OIP-1 pGL2 Basic#3 plasmid DNA (2 μg) by using the nucleofector kit V (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions. The cells were cultured in the presence/absence of IL-12 (10 ng/ml) for 48 h. The cells were washed twice with phosphate buffered saline and incubated at room temperature for 15 min with 0.3 ml cell lysis reagent (Promega). A 20 μl aliquot of each sample was mixed with 100 μl of the luciferase assay reagent and the light emission was measured for 10 s of integrated time using Sirius Luminometer. The transfection efficiency was normalized by co-transfection with pRSV β-gal plasmid (0.2 μg) and measuring the β-galactosidase activity in these cells.

### QUANTITATIVE REAL-TIME RT-PCR

OIP-1 mRNA expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) as described previously [Srinivasan et al., 2006]. Briefly, total RNA was isolated from human bone marrow mononuclear cells or Jurkat T cells stimulated with and without IL-12 (10 ng/ml) for 48 h, using RNAzol reagent (Tel-Test Inc., Friendswood, TX). A reverse transcription reaction was performed using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) in a 25 μl reaction volume containing total RNA (2 μg), 1× PCR buffer and 2 mM $MgCl_2$, at 42°C for 15 min followed by 95°C for 5 min. The real-time PCR was performed using SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real Time-PCR detection system; Bio-Rad, Hercules, CA). The primer sequences used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5′-CCTACCCCCAATGTATCCGTTGTG-3′ (sense) and 5′-GGAGGAATGGGAGTTGCTGTTGAA-3′ (anti-sense); OIP-1 mRNA 5′-TTTATTCACCCAGGAGGAGCTGAAGGTTCC-3′ (sense) and 5′-CCAACATGAGAGTCTTCCTGCCTG-3′ (anti-sense) and for IFN-γ mRNA were 5′-GCGTCATTGAATCACACCTG-3′ (sense), 5′-TGAGCTCATTGAATGCTTGG-3′ (anti-sense). Thermal cycling at 94°C for 3 min, followed by 40 cycles of amplifications at 94°C for 30 s, 66°C for 1 min, 72°C for 1 min and 72°C for 5 min as the final elongation step. Relative levels of OIP-1 mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification.

### PREPARATION OF HUMAN T CELL SUBSETS

Peripheral blood mononuclear cells (PBMC) obtained following the IRB approved protocol at the Medical University of South Carolina were separated from the heparinized blood by standard Ficoll-Hypaque (Sigma, MO) gradient centrifugation. CD4+ and CD8+ T cell subsets at >95% purity were isolated by negative selection with Dynal CD8 or CD4 magnetic beads (Dynal-Invitrogen, CA). Briefly, the PBMC ($2 \times 10^7$/ml) were incubated with either CD4 or CD8 beads at the concentration recommended by the manufacturer for 20 min at 4°C. The cell suspensions were then placed on a magnetic plate for 2–3 min. The unbound cells were transferred to another tube and washed three times with PBS. The cells depleted of CD4+ or CD8+ T cells were then suspended in complete medium.

### OLIGONUCLEOTIDE PULL-DOWN ASSAY

Jurkat T cells were treated with and without IL-12 (10 ng/ml) and in the presence/absence of excess concentration of anti-IFN-γ neutralizing antibody (50 U/ml) for the indicated time period. Total cell lysates were subjected to nuclear extraction (NE) using a nuclear extraction kit (Pierce, CA). The extracts were incubated for 2 h with biotinylated oligonucleotides from the OIP-1 gene promoter containing STAT binding sequence (−1,629 to −1,639 bp position), sense 5′-GGTGAGCAAGCGTTCCTCTAACAGTGGGAA-3′

and antisense 5′-TTCCCACTGTTAGAGGAACGCTTGCTCACC-3′. The STAT-1 binding site was mutated as underlined (Mut.STAT oligo sense 5′-GGTGAGCAAGCGTTAATTTAACAGTGGGAA 3′). The complexes were isolated by streptavidin conjugated Dynal magnetic beads and analyzed by immunobloting with anti-pSTAT-1 or pSTAT-3 antibodies as described [Srinivasan et al., 2006].

### CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP was performed using the ChIP Assay Kit (Upstate, Temecula, CA). The Jurkat T cells were treated with or without IL-12 (10 ng/ml) and in the presence/absence of excess anti-IFN-γ antibody (50 U/ml). Cells were cross-linked with 1% final concentration of formaldehyde at 37°C for 10 min before harvest. Soluble chromatin was prepared following sonication with a Branson-250 digital sonifier (Branson Ultrasonics, Danbury, CT) to an average DNA length of 200–1,000 bp. Approximately $5 \times 10^5$ cell equivalent (1/6th) of the sheared soluble chromatin was precleared with blocked Protein G agarose, and 10% of the precleared chromatin was set aside as input control. Immunoprecipitation was carried out with 5 μg of pSTAT-1 and pSTAT-3 antibodies, overnight at 4°C. Immune complexes were pulled down using Protein G agarose, washed and eluted twice with 250 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) and cross-linking reversed in 200 mM NaCl at 65°C overnight with 20 μg RNase A (Sigma). DNA was purified following proteinase K treatment (Invitrogen Life Technologies, Carlsbad, CA) with the Qiagen PCR purification kit (Qiagen, MD). Then, PCR was performed using the OIP-1 gene specific primers for STAT binding region, sense 5′-CCAGAGGCCTGGTGAGCAAG-3′ and antisense 5′-AGACTGCGATAGACGTCCAT-3′. DNA samples or input DNA fractions were analyzed by 35 cycles of PCR (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). PCR products were subjected to electrophoresis using 2% agarose gels and visualized by ethidium bromide.

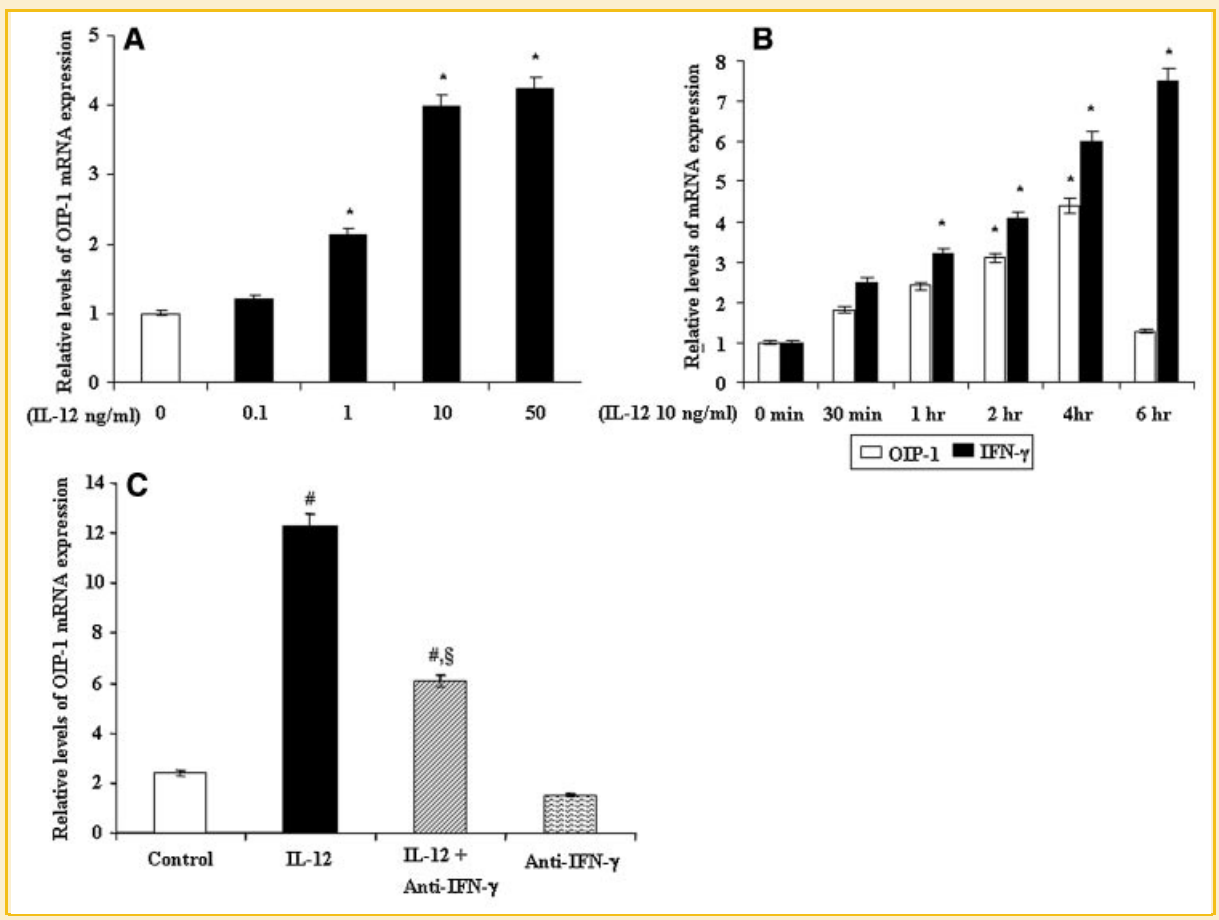


Fig. 1. IL-12 stimulation and OIP-1 mRNA expression in human bone marrow cells. A: The human bone marrow mononuclear cells were stimulated with IL-12 (0–50 ng/ml) for 4 h and total RNA isolated was subjected to quantitative real-time PCR analysis for OIP-1 mRNA expression. B: IL-12 stimulation of OIP-1 and IFN-γ expression in human bone marrow cells. Bone marrow mononuclear cells were stimulated with IL-12 (10 ng/ml) for indicated time period (0–6 h) and total RNA isolated was subjected to real-time RT-PCR analysis for OIP-1 and IFN-γ mRNA expression. C: IL-12 stimulation of OIP-1 expression in human bone marrow cells cultured in the presence of anti-IFN-γ neutralizing antibody. Cells stimulated with IL-12 (10 ng/ml) for 4 h with/without IFN-γ antibody (50 U/ml) and total RNA isolated was subjected to quantitative real-time PCR analysis for OIP-1 expression. Relative levels of OIP-1 and IFN-γ gene expression were normalized with respect to the levels of GAPDH amplification. Values expressed as mean ± SE for three independent experiments. $^{*}P < 0.05$ level, $^{\#}P < 0.05$ versus control and $^{\S}P < 0.05$ versus IL-12 treated group.

### STATISTICAL ANALYSIS

Results are presented as mean ± SE for three independent experiments and were compared by Student's *t*-test or one-way ANOVA. Values were considered significantly different for $P < 0.05$.

## RESULTS

### IL-12 ENHANCES THE OIP-1 mRNA EXPRESSION IN HUMAN BONE MARROW MONOCYTES

We examined IL-12 stimulation of OIP-1 mRNA expression in human bone marrow derived mononuclear cells by quantitative real-time RT-PCR analysis. As shown in the Figure 1A, IL-12 treatment (0–50 ng/ml) to human bone marrow mononuclear cells demonstrated a significant increase in OIP-1 mRNA expression in a dose-dependent manner. We further examined the IL-12 stimulation of OIP-1 expression in a time (0–6 h) dependent manner. Time course study identified that IL-12 treatment for 4 h period induced high-level expression of OIP-1 in these cells; however this declined by 6 h period of treatment (Fig. 1B). Relative levels of OIP-1 expression were normalized with respect to GAPDH amplification in RT-PCR analysis. We therefore used IL-12 treatment (10 ng/ml) for a 4 h period for subsequent experiments unless indicated.

IL-12 is primarily produced by macrophages and dendritic cells, and has been shown to potently induce the production of IFN-γ by T and NK cells [Horwood et al., 2001]. Real-time RT-PCR analysis of total RNA isolated from human bone marrow mononuclear cells stimulated with IL-12 further confirmed a significant increase in IFN-γ mRNA expression in a time-dependent (0–6 h) manner (Fig. 1B). We have previously reported IFN-γ stimulation of OIP-1 expression in bone marrow cells [Koide et al., 2003]. We further determined if IL-12 stimulation of OIP-1 expression is mediated by IFN-γ. Since OIP-1/hSca and IFN-γ expression is abundant in T lymphocytes, we quantified the levels of OIP-1 mRNA expression in human bone marrow cells in response to IL-12 treatment in the presence and absence of IFN-γ neutralizing antibody. Real-time PCR analysis demonstrated that bone marrow cells treated with IL-12 showed a significant increase (10-fold) in the level of OIP-1 expression. However, IL-12 treatment in the presence of a neutralizing antibody against IFN-γ showed a 4-fold increase in OIP-1 mRNA expression compared to control unstimulated cells (Fig. 1C). These results suggest that IL-12 may have direct effect in stimulating OIP-1 gene expression and confirms our previous results that elevated levels of IFN-γ are in part responsible for IL-12 stimulation of OIP-1 gene expression.

### IL-12 SPECIFIC STIMULATION OF OIP-1 EXPRESSION IN CD4+ T CELLS

To further delineate the IL-12 stimulation of OIP-1 gene expression in lymphocytes, we isolated the T cell subsets of CD4+ and CD8+ cells from human peripheral blood as described in Materials and Methods Section. Purified CD4+ and CD8+ cells were stimulated with IL-12 in the presence/absence of IFN-γ antibody for 4 h and total RNA isolated was subjected to real-RT-PCR analysis for OIP-1 mRNA expression. As shown in Figure 2A, IL-12 modulation of OIP-1 expression is specific to CD4+ cells and in contrast IL-12 has no significant effect on OIP-1 expression in CD8+ cells. Furthermore,

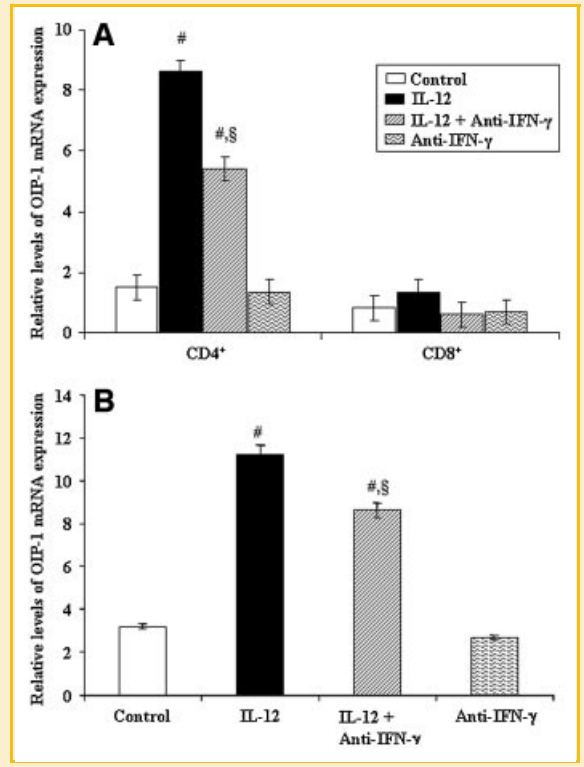


Fig. 2. IL-12 regulation of OIP-1 expression in the T cell subsets cultured in the presence of anti-IFN-γ neutralizing antibody. The peripheral blood mononuclear cells (PBMC) were subjected for CD4+ and CD8+ cells isolation and stimulated with IL-12 (10 ng/ml) for 4 h with/without anti-IFN-γ antibody (50 U/ml) and total RNA was isolated. A: Real-time RT-PCR analysis of OIP-1 mRNA expression in CD4+ and CD8+ T cells. B: Real-time RT-PCR analysis of OIP-1 mRNA expression in Jurkat T cells stimulated with IL-12 (10 ng/ml) for 4 h with/without anti-IFN-γ antibody (50 U/ml). The levels of OIP-1 mRNA expression were normalized with respect to the levels of GAPDH amplification. Values expressed as mean ± SE for three independent experiments. $^{\#}P < 0.05$ versus control and $^{\S}P < 0.05$ versus IL-12 treated group.

real-time PCR analysis of total RNA isolated from Jurkat T CD4+ cells stimulated with IL-12 in the presence IFN-γ neutralizing antibody revealed significantly increased (2.6-fold) OIP-1 expression, which suggests IL-12 specific modulation of OIP-1 expression in these cells. IFN-γ neutralizing antibody alone, however, has no significant effect on OIP-1 expression in control cultures (Fig. 2B). These results further suggest that IL-12 stimulation of OIP-1 expression is restricted to CD4+ T cells.

### IL-12 REGULATION OF OIP-1 GENE PROMOTER ACTIVITY

We also examined the transcriptional regulatory mechanism operative in CD4+ T cells with respect to IL-12 modulation of OIP-1 gene expression. We transiently transfected OIP-1 pGL2 Basic#3 luciferase reporter plasmid containing the OIP-1 gene promoter sequence (−1 to −1,988 bp) into Jurkat T cells and measured the luciferase activity levels in response to IL-12 treatment. IL-12 treatment (10 ng/ml) significantly increased (5-fold) in OIP-1 gene promoter activity in these cells (Fig. 3A). Jurkat cells cultured in the presence of excess concentration of

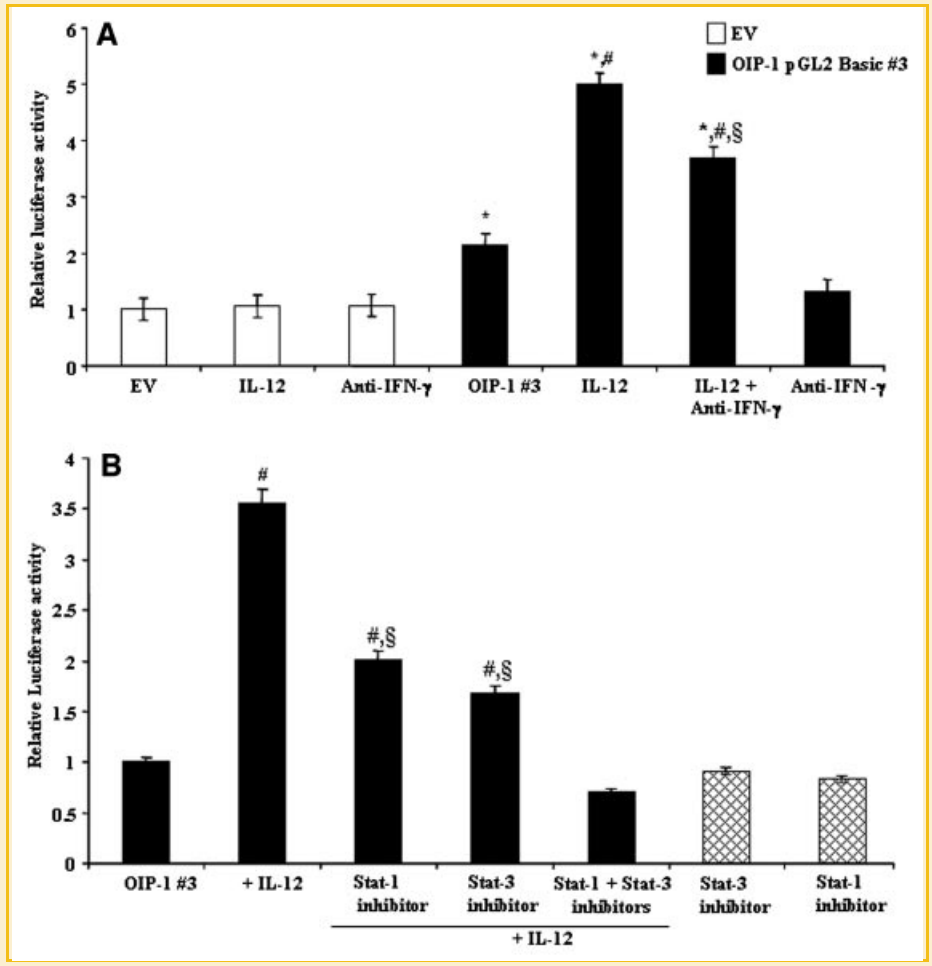


Fig. 3. IL-12 Stimulation of OIP-1 gene promoter activity. A: OIP-1 pGL2 Basic#3 promoter-luciferase reporter plasmid was transiently transfected into Jurkat T cells ($2 \times 10^6$ cells) in a six-well plate using a nucleofector kit V (Amaxa). The cells were stimulated with IL-12 (10 ng/ml) and with/without anti-IFN-γ antibody for 48 h and total cell lysates obtained were assayed for luciferase activity. pGL2 basic empty vector (EV) transfected cells were served as control. B: Effect of STAT-1 and STAT-3 inhibitors on IL-12 stimulated OIP-1 gene promoter activity. OIP-1 pGL2 Basic#3 promoter plasmid was transiently transfected into Jurkat T cells and cultured in the presence/absence of IL-12 (10 ng/ml). STAT-1 specific inhibitor, fludarabine (50 μM) and STAT-3 inhibitor peptide (1 mM) was applied to the cells for 30 min prior to IL-12 stimulation. Total cell lysates prepared after 48 h were assayed for luciferase activity. Transfection efficiency was normalized by measuring β-galactosidase activity co-expressed in these cells. Values expressed as mean ± SE for three independent experiments. $^{*}P < 0.05$ versus EV, $^{\#}P < 0.05$ versus OIP-1 #3 and $^{\S}P < 0.05$ versus OIP-1 #3 stimulated with IL-12.

neutralizing antibody against IFN-γ showed partial inhibition of IL-12 stimulated OIP-1 gene promoter activity, a finding that further confirms IL-12 specific modulation of OIP-1 gene promoter activity in these cells. The increase in the OIP-1 mRNA expression maximum at 4 h period (Fig. 1B) and stimulation of promoter activity at 48 h period could be explained by experimental conditions of transient transfections using the OIP-1 promoter-luciferase construct.

IL-12 has been shown to regulate gene expression through STAT signaling [Bright and Sriram, 1998]. We previously identified the presence of a potential STAT binding motif at −1,629 to −1,639 bp position in the OIP-1 gene promoter region [Srinivasan et al., 2006]. We therefore tested the ability of a STAT-1 specific inhibitor, fludarabine and a STAT-3 specific inhibitor to block the IL-12 stimulation of OIP-1 gene promoter activity. Jurkat T cells transfected with the OIP-1 pGL2 Basic#3 promoter (−1 to −1,988 bp)-luciferase reporter construct were cultured in the presence of STAT-1 inhibitor, fludarabine (50 μM) or a STAT-3 inhibitor for 48 h prior to IL-12 stimulation. Then, total cell lysates obtained were assayed for luciferase activity. As shown in Figure 3B, both STAT-1 and STAT-3 inhibitors significantly decreased IL-12 stimulated OIP-1 gene promoter activity in these cells. Furthermore, cultures treated with both STAT-1 and STAT-3 inhibitors abolished IL-12 stimulated OIP-1 gene promoter activity in Jurkat cells. However, STAT inhibitors in the absence of IL-12 did not significantly effect on OIP-1 promoter activity in control cultures. We further confirmed that STAT-1 specific inhibitor fludarabine and a STAT-3 specific inhibitor peptide suppressed IL-12 stimulated OIP-1 expression in peripheral blood derived CD4+ T cells. Real-time RT-PCR analysis revealed that fludarabine has a modest effect; however, the STAT-3 inhibitor significantly decreased IL-12 induced OIP-1 mRNA expression in isolated CD4+ T cell (Fig. 4). These results implicate

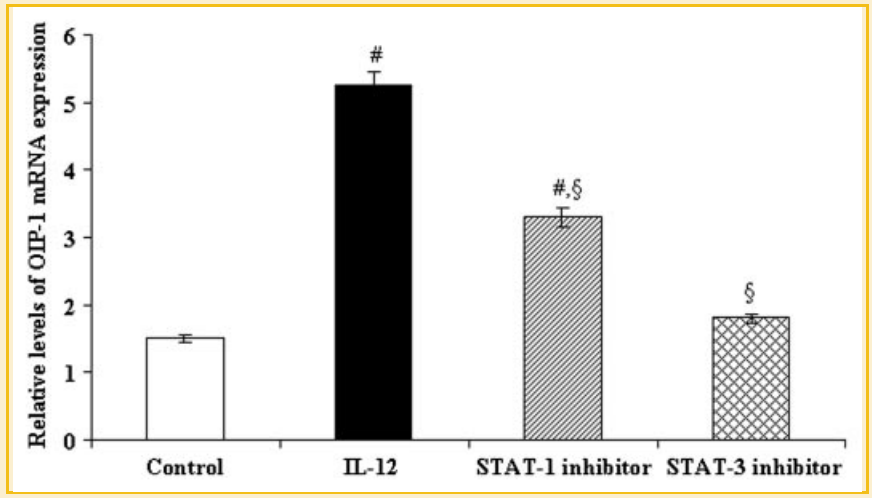


Fig. 4. Effect of STAT inhibitors on IL-12 stimulation of OIP-1 expression in CD4+ T cells. The CD4+ cells from PBMC were treated with STAT-1 specific inhibitor, fludarabine (50 μM) or STAT-3 inhibitor peptide (1 mM) for 30 min prior to IL-12 (10 ng/ml) treatment. Total RNA isolated was subjected to real time PCR analysis of OIP-1 mRNA expression in these cells. Relative levels of OIP-1 gene expression were normalized with respect to the levels of GAPDH amplification. Values are expressed as the mean ± SE for three independent experiments. $^{\#}P < 0.05$ versus control and $^{\S}P < 0.05$ versus IL-12 treated group.

STAT participation in IL-12 stimulation of OIP-1 gene expression in CD4+ T cells.

### IL-12 STIMULATION OF STAT-3 BINDING TO OIP-1 PROMOTER SEQUENCE

We also examined the status of STAT binding to the OIP-1 gene promoter element in response to IL-12 treatment of Jurkat T cells by oligonucleotide pull-down assay as previously described [Ikeda et al., 2004]. Total cell lysates prepared from the Jurkat T cells stimulated with IL-12 in the presence/absence of IFN-γ neutralizing antibody were immunoblot analyzed for pSTAT-3 expression. IL-12 stimulation of Jurkat cells induced expression of pSTAT-3, which was modestly decreased (0.92 ± 0.03) in cells cultured in the presence of IFN-γ neutralizing antibody relative to IL-12 treatment alone. However, IFN-γ antibody treatment alone did not significantly effect on pSTAT-3 and STAT-3 expression in these cells (Fig. 5A). We then examined STAT binding to the OIP-1 promoter element in response to IL-12 stimulation by oligonucleotide pull-down assay as described. The nuclear extracts (NE) obtained from Jurkat cells showed both STAT-1 and STAT-3 binding with biotinylated oligonucleotides containing the intact STAT binding sequence present in the OIP-1 gene promoter. NE obtained from cultures stimulated with IL-12 in the presence of excess anti-IFN-γ antibody demonstrated a partially inhibited STAT-3, but completely inhibited STAT-1 binding to these oligomers. In contrast, mutant oligo (Mut.STAT oligo) which lacks the STAT recognition site did not demonstrate specific affinity binding to pSTAT-3 and pSTAT-1 (Fig. 5B).

We further confirmed STAT-3 binding to the OIP-1 gene promoter element using the ChIP assay. Jurkat T cells stimulated with IL-12 in the presence/absence of anti-IFN antibody subsequently were subjected to chromatin immune precipitation with anti-pSTAT-1 and anti-pSTAT-3 antibodies as described. PCR analysis of chromatin immune complexes was then performed using OIP-1 gene specific primers for the STAT binding region. As shown in Figure 5C, treatment with IFN-γ antibody partially inhibited pSTAT-3 binding and completely inhibited pSTAT-1 binding to the OIP-1 gene promoter element. These results suggest that STAT-3 signaling play an important role in IL-12 modulation of OIP-1 gene expression in CD4+ T cells.

## DISCUSSION

We have previously reported that IFN-γ enhances OIP-1 expression in bone marrow cells and in osteoclast precursors [Koide et al., 2003]. Also, it is known that immune regulatory cytokines such as IL-12 and IL-4 are known to be potent inhibitors of osteoclastogenesis [Abu-Amer, 2001; Horwood et al., 2001]. IL-12 has been shown to inhibit osteoclast formation and bone resorption activity alone and/or in synergy with IL-18 [Horwood et al., 2001; Kitaura et al., 2002]. Also, IL-12 and IL-18 were shown to synergistically elevate IFN-γ production [Yamada et al., 2002]. The present study delineates IL-12 specific regulation of OIP-1 expression in T cells. We show that IL-12 stimulates OIP-1 expression in both bone marrow derived monocytes and Jurkat T cells. Partial inhibition of IL-12 stimulated OIP-1 expression in the presence of IFN-γ neutralizing antibody is consistent with that IL-12 increasing IFN-γ production by T cells. The modest decrease in OIP-1 expression observed in bone marrow cells treated with IFN-γ antibody alone suggest an autocrine/paracine effect of basal levels of IFN-γ present in these cultures. It has also been shown that IFN-γ produced from non-T cell population of bone marrow contributes to IL-12 inhibition of osteoclastogenesis [Nagata et al., 2003]. IL-12 has been shown to modulate IFN-γ production in both CD4 and CD8+ T cells [Gerosa et al., 1996]. However, our results suggest that IL-12 specific stimulation of OIP-1 expression is restricted to CD4+ T cells.

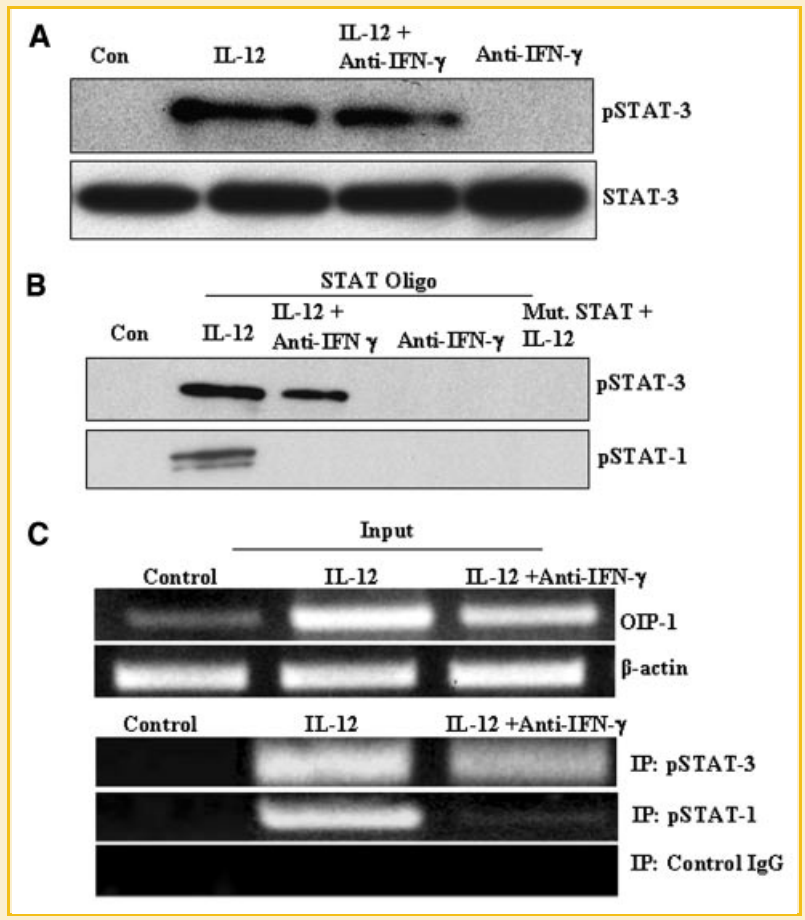


Fig. 5. Oligonucleotide pull-down and ChIP assay for STAT-3 binding to OIP-1 gene promoter sequence. A: Jurkat T cells stimulated with IL-12 (10 ng/ml) for 30 min in the presence/absence of anti-IFN-γ antibody. pSTAT-3 and STAT-3 expression in the total cell lysates were confirmed by Western blot analysis. Data represent three independent experiments ($P < 0.05$). B: The nuclear extracts (NE) prepared from Jurkat cells were incubated for 2 h with biotinylated oligonucleotides containing the intact STAT binding sequence present in OIP-1 gene promoter and STAT Mut-oligo; analyzed by immunoblotting with specific anti-p-STAT-3, anti-p-STAT-1 antibody. (C) ChIP assay for STAT binding to OIP-1 promoter region. The input sample was an aliquot of nuclear extract (NE) from Jurkat T cells stimulated with IL-12 and in the presence/absence of anti-IFN-γ antibody. The input samples from Jurkat T cells were cross-linked, lysed, sonicated, and subjected to immunoprecipitation using polyclonal antibodies against pSTAT-1 and pSTAT-3. Rabbit IgG was used as a negative control antibody. The precipitated DNA fragments, input DNA, were subjected to PCR analysis using specific primers for the STAT binding region in the OIP-1 gene promoter. Data represent three independent experiments.

Therefore, CD4+ T cells may be involved in IL-12 inhibition of osteoclatogenesis. In contrast, CD8+ cells have been shown to regulate osteoclast differentiation by a mechanism not mediated by IL-4 and TGF-β [John et al., 1996]. Although T cells have been shown to mediate IL-4 inhibition of osteoclast formation, IL-4 does not affect TSA-1 (thymic sheared antigen-1) and osteoclast inhibitory lectin (OCIL) expression [Mirosavljevic et al., 2003]. IL-12 stimulation of OIP-1 gene promoter activity in Jurkat T cells implicated an IL-12 specific transcriptional regulatory mechanism in these cells. Modest decrease in OIP-1 promoter activity in cells cultured in presence of anti-IFN antibody indicates low level basal IFN production. However, IL-12 significantly stimulated OIP-1 gene promoter activity in the presence of excess concentrations of anti-IFN neutralizing antibody, which suggests IL-12 specific regulation of OIP-1 expression.

STAT signaling molecules have been implicated in IL-12 and IFN-γ signaling to modulate gene expression [Watford et al., 2003]. We previously identified the potential STAT binding element in the OIP-1 gene promoter region and further demonstrated STAT-1 binding in response to IFN-γ treatment of osteoclast progenitor cells. This study further demonstrates that a combination treatment of STAT-1 and STAT-3 inhibitors treatment to Jurkat T cells completely abolished IL-12 stimulated OIP-1 gene promoter activity and suggests a functional role for STAT signaling in IL-12 modulation of OIP-1 expression in CD4+ T cells. Also, our results show that IL-12 upregulated the level of pSTAT-1 and pSTAT-3 expression in Jurkat T cells, implicating STAT signaling in IL-12 modulation of OIP-1 expression. Both the oligonucleotide pull-down assay and the ChIP assay revealed that IFN-γ neutralizing antibody completely abolished binding of pSTAT-1 to the OIP-1 gene promoter element in IL-12 stimulated cells. This confirms our previous findings that STAT-1 is primarily involved in IFN-γ regulation of OIP-1 expression. However, pSTAT-3 binding to the OIP-1 promoter element in response to IL-12 stimulation in the presence of anti-IFN-

γ antibody indicates the STAT-3 specific signaling in IL-12 modulated OIP-1 gene expression. Similarly, IL-12 has been shown to upregulate T-bet (Th1-specific T box transcription factor) expression independently of IFN-γ in human CD4+ cells [Ylikoski et al., 2005]. The partial inhibition of OIP-1 promoter activity and abolished STAT-1 binding to the OIP-1 promoter in response to IL-12 stimulation in the presence of anti-IFN-γ antibody implicates a role for STAT-1 in IL-12 induced IFN-γ expression. These results suggest that IL-12 stimulates the OIP-1 gene expression through STAT-3 activation and binding to OIP-1 promoter element in CD4+ T cells.

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