The Journal of Immunology

RESEARCH ARTICLE | APRIL 15 2001

# IL-12 Alone and in Synergy with IL-18 Inhibits Osteoclast Formation In Vitro[1]

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*J Immunol* (2001) 166 (8): 4915–4921.
https://doi.org/10.4049/jimmunol.166.8.4915

# IL-12 Alone and in Synergy with IL-18 Inhibits Osteoclast Formation In Vitro[1]

**Nicole J. Horwood, Jan Elliott, T. John Martin, and Matthew T. Gillespie[2]**

**IL-12, like IL-18, was shown to potently inhibit osteoclast formation in cultures of cocultures of murine osteoblast and spleen cells, as well as in adult spleen cells treated with M-CSF and receptor activator of NF-κB ligand (RANKL). Neither IL-12 nor IL-18 was able to inhibit RANKL-induced osteoclast formation in cultured RAW264.7 cells, demonstrating that IL-12, like IL-18, was unable to act directly on osteoclastic precursors. IL-12, like IL-18, was found to act by T cells, since depletion of T cells from the adult spleen cell cultures ablated the inhibitory action of IL-12 and addition of either CD4 or CD8 T cells from C57BL/6 mice to RANKL-stimulated RAW264.7 cultures permitted IL-12 or IL-18 to be inhibitory. Additionally, IL-12 was still able to inhibit osteoclast formation in cocultures with osteoblasts and spleen cells from either GM-CSF $R^{-/-}$ mice or IFN-γ $R^{-/-}$ mice, indicating that neither GM-CSF nor IFN-γ was mediating osteoclast inhibition in these cultures. Combined, IL-18 and IL-12 synergistically inhibited osteoclast formation at concentrations 20- to 1000-fold less, respectively, than when added individually. A candidate inhibitor could not be demonstrated using neutralizing Abs to IL-4, IL-10, or IL-13 or from mRNA expression profiles among known cytokine inhibitors of osteoclastogenesis in response to IL-12 and IL-18 treatment, although the unknown inhibitory molecule was determined to be secreted from T cells.** ***The Journal of Immunology*****, 2001, 166: 4915–4921.**

Osteoclast formation is a multistep process involving complex interactions among a variety of cell types. Many hormones, cytokines, and growth factors, the majority of which act on osteoblastic stromal cells, are intricately involved in the development of osteoclasts from hematopoietic precursors. Two molecules expressed by osteoblasts are essential for the differentiation of hematopoietic cells to form osteoclasts. M-CSF is required for the proliferation of osteoclast precursors, while the TNF family member, receptor activator of NF-κB ligand (RANKL;[3] otherwise known as osteoclast differentiation factor, osteoprotegerin (OPG) ligand, and TNF-related activation-induced cytokine) commits precursors toward osteoclast formation (1, 2). The actions of RANKL can be ablated by OPG, a secreted member of the TNF receptor family. OPG acts as a decoy receptor for RANKL, antagonizing its biological actions by preventing it from binding to and activating its receptor, RANK. Consistent with this action, overexpression of OPG in transgenic mice resulted in severe osteopetrosis, with a failure to form marrow cavities and profound depletion of osteoclasts (3). A similar phenotype is also noted in the RANKL-deficient mice (4). Osteotropic agents such as parathyroid hormone, 1,25-dihroxyvitamin $D_3$, and IL-11 that can promote osteoclast formation in vitro use different signaling pathways (cAMP, vitamin D receptor, and gp130 signal transduction), and each elevates RANKL production by osteoblasts. Alteration of the RANKL:OPG ratio toward that of RANKL excess promotes a favorable environment for cells to support osteoclast formation (1, 5). This is particularly true for cells of the osteoblast lineage (5), fibroblastic cells (6), and T cells, which when activated result in RANKL production and may account for or participate in the bone destruction of rheumatoid arthritis (7, 8). Additionally cancer cells that metastasize in bone and induce osteolysis and hypercalcemia do so by enhancing osteoblastic RANKL production (9), the actions of which may be blocked by administration of OPG (10).

The actions of RANKL are not restricted to bone, since this molecule is essential for lymph node and breast development (4, 11), and due to its widespread distribution (12) is likely to have additional, as yet uncharacterized, functions.

IL-12 is an integral regulator of the immune response to antigenic challenge that promotes the Th1 while suppressing the Th2 (13–15). 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 (16, 17). In addition, macrophages, B cells, and dendritic cells have been reported to produce IFN-γ following IL-12 treatment (18–22). Recent studies have focused on the interaction between IL-12 and IL-18. IL-18, like IL-12, was initially described as an IFN-γ-inducing agent and these two cytokines have been shown to act in synergy, with combined treatment resulting in far greater IFN-γ production than with either IL alone (18, 23–25). In addition to their effect on IFN-γ, the combination of IL-12 and IL-18 has been shown to dramatically increase the proliferation and activation of T cells and NK cells (26–28). IL-12 up-regulates the production of the IL-18Rα chain (IL-1Rrp) on Th1 cells (21, 23), whereas IL-18 has been shown to up-regulate the IL-12Rβ2 chain on Th1 cells (29). This reciprocal up-regulation of receptor components provides a positive feedback mechanism, allowing these two factors to act synergistically. Other ILs, such as IL-1β, IL-4, and IL-15, have also been shown to synergize with either IL-12 alone or the combination of IL-12 and IL-18 (22, 28, 30).

We have previously shown that IL-18 is able to inhibit osteoclast formation via T cell production of GM-CSF, highlighting a

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Received for publication July 3, 2000. Accepted for publication February 2, 2001.

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[1] This work was supported by Program Grant 003211 from the National Health and Medical Research Council Australia (to T.J.M. and M.T.G.). M.T.G. is a Senior Research Fellow of the National Health and Medical Research Council Australia and N.J.H. is a Howard Florey Research Fellow.

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[3] Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; GM-CSF $R^{-/-}$, GM-CSF receptor-deficient mice; IFN-γ $R^{-/-}$, IFN-γ receptor type II-deficient mice; MNC, multinucleated cell; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; IL-1RA, IL-1R antagonist; OCL, osteoclast.



0022-1767/01/$02.00

role for cell types other than osteoblasts in the regulation of osteoclastogenesis (31). To investigate the role of IL-12 in osteoclastogenesis, we used recombinant IL-12 alone and in combination with recombinant IL-18 in a series of in vitro osteoclast formation assays. The ability of these cytokines to inhibit osteoclast formation, the cell types involved in this process and potential mediators of osteoclast inhibition have been examined.

## Materials and Methods

### *Animals, cell lines, and drugs*

Newborn (0- to 1-day-old) C57BL/6J mice and 3- to 4-wk-old male C57BL/6J mice were purchased from Monash University Animal Services Center (Clayton, Australia). We thank P. Tipping (Monash Medical Center, Australia) for access to the IFN-$\gamma$ type II receptor-deficient mice (32) and L. Corcoran and L. Robb (Walter and Eliza Hall Institute, Melbourne, Australia) for the Rag1 (33) and GM-CSF receptor-deficient mice (34), respectively. The murine macrophage-like cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA). 1$\alpha$,25-dihydroxyvitamin $D_3$ (1$\alpha$,25$(OH)_2D_3$) was purchased from Wako Pure Chemical (Osaka, Japan). $PGE_2$ was obtained from Sigma (St. Louis, MO). Recombinant murine IL-12 and IL-18 and polyclonal Abs to anti-murine IL-4, IL-10, and IL-13 were purchased from R&D Systems (Minneapolis, MN). Other chemicals and reagents were of analytical grade.

### *In vitro osteoclast formation assays*

For the coculture assays, osteoblastic cells were prepared from the calvaria of newborn mice by digestion with 0.1% collagenase (Worthington Biochemical, Freefold, Australia) and 0.2% dispase (Godo Shusei, Tokyo, Japan). Osteoblastic cells were cocultured with newborn spleen cells as described elsewhere (35). In short, primary osteoblastic cells ($5 \times 10^4$/well) and nucleated spleen cells ($1 \times 10^6$/well) were cocultured in 48-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) with 0.3 ml of $\alpha$MEM (Life Technologies, Grand Island, NY) containing 10% FBS (Cytosystems, Castle Hill, NSW, Australia) in the presence of test chemicals. Cultures were incubated in quadruplicate and cells were replenished on days 3 and 7 with fresh medium. Osteoclast formation was evaluated after culturing for 9–10 days.

Adult spleen cultures were performed using disaggregated spleen cells from 3- to 4-wk-old mice seeded at a concentration of $1 \times 10^6$/well in 48-well plates. Cells were incubated in 0.3 ml of $\alpha$MEM containing 10% FBS and were treated with RANKL (50 ng/ml) and M-CSF (25 ng/ml) unless otherwise stated. Cultures were incubated in quadruplicate with complete medium change on days 3 and 7. Osteoclast (OCL) formation was evaluated after culturing for 9–10 days. RAW264.7 cultures were performed using RAW264.7 cells at $1 \times 10^4$ cells/well in a 48-well plate. Cells were incubated in 0.3 ml of $\alpha$MEM containing 10% FBS and RANKL (50 ng/ml) unless otherwise stated. Cultures were incubated in quadruplicate with complete medium change at day 3. OCL formation was evaluated after culturing for 7 days.

Adherent cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), and the numbers of TRAP-positive OCLs were scored as described previously (35). For TRAP staining, adherent cells were fixed with 4% formaldehyde in PBS for 3 min. After treatment with ethanol-acetone (50/50, v/v) for 1 min, the well surface was air dried and incubated for 10 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma) as a substrate and 0.03% red violet B salt (Sigma) as a stain for the reaction product in the presence of 50 mM sodium tartrate. TRAP-positive cells appeared dark red.

The expression of calcitonin receptors was assessed by autoradiography using [$^{125}$I]salmon calcitonin as described elsewhere (36). The ability of these cells to resorb bone was examined by electron microscopy as described previously (36).

### *T cell depletion/repletion*

Total T cells were separated from adult mouse spleen cells using mouse pan T (Thy 1.2) dynabeads (Dynal, Oslo, Norway) for the isolation/depletion of all T cell subsets and CD4 and CD8 dynabeads were used to isolate their respective subsets, as previously described (31). T cells were immunomagnetically separated from the remaining spleen cells using a magnetic particle concentrator (Dynal) and rinsed three times in $\alpha$MEM to remove any contaminating spleen cells. T cells remained attached to the dynabeads and were resuspended in $\alpha$MEM + 10% FBS for addition to the various culture types. To investigate whether direct interaction between T cells and osteoclast precursors was required for inhibition of osteoclast formation, T cells were added to 0.4-$\mu$m pore transwells (Costar; Corning, Acton, MA) in the presence of test chemicals.

### *Semiquantitative RT-PCR*

Total cellular RNA was extracted from adult mouse spleen cells using guanidine thiocyanate-phenol-chloroform and used in RT-PCR essentially as described previously (5). Oligonucleotides were synthesized on an Oligo1000 M DNA Synthesizer (Beckman Coulter, Fullerton, CA). The following oligonucleotides were used in these experiments for GM-CSF; GM-CSF-1 (sense, 5′-AGAAAGGCTAAGGTCCTGAGGAGG-3′, nts 150–173 European Molecular Biology Laboratory (EMBL) accession number X02333), GM-CSF-2 (antisense, 5′-CCGCATAGGTGGTAACT TGTGTTTC-3′, nts 504–528 EMBL accession number X02333), and GM-CSF-3 (antisense, 5′-GGAGAACTCGTTAGAGACGAC-3′, nts 339–359 EMBL accession number X02333): IFN-$\gamma$; IFN-$\gamma$-1 (sense, 5′-TCTTGGCTTTGCAGCTCTTCC-3′, nts 130–150 GenBank number accession M282621), IFN-$\gamma$-2 (antisense, 5′-CGAATCAGCAGCGACTC CTTTTC-3′, nts 560–582 GenBank accession number M282621), and IFN-$\gamma$-3 (sense, 5′-ACCTTCTTCAGCAACAGCAAGG-3′; nts 411–432 GenBank accession M282621). Murine GAPDH primers were GAPDH-1, GAPDH-2, and GAPDH-4 as described elsewhere (5). Internal oligonucleotides (GM-CSF-3, IFN-$\gamma$-3, and GAPDH-1) were used for the hybridization studies as previously described (5).

## Results

### *IL-12 inhibits osteoclast formation in vitro*

IL-12 is primarily known for its ability to potentiate IFN-$\gamma$ secretion by and the cytolytic activity of both T cells and NK cells (14, 15, 19, 20). Since IFN-$\gamma$ is a potent inhibitor of osteoclastogenesis (37–39), we sought to identify whether IL-12 affects osteoclastogenesis through production of IFN-$\gamma$. Osteoclasts were formed in cocultures of newborn murine spleen cells and primary osteoblasts from normal C57BL/6J mice where $PGE_2$ and 1$\alpha$,25$(OH)_2D_3$ were added; without at least one of these agents, no osteoclasts were formed (Fig. 1*A*). The TRAP-positive ($TRAP^+$) multinucleated and mononuclear cells formed in these cocultures possessed calcitonin receptors and formed resorption lacunae on bone slices (data not shown). Recombinant murine IL-12 dose-dependently inhibited osteoclast formation with maximal inhibition observed at 0.1 ng/ml (Fig. 1*A*). This concentration is comparable to that required with other cell systems for IFN-$\gamma$ secretion (22, 24) and the development of the Th1 phenotype (40–42): IL-12 was subsequently used at 0.1 ng/ml unless otherwise stated. Treatment of cocultures revealed that the inhibitory actions of IL-12 on osteoclast formation occurred during the proliferation of osteoclast precursors (first 3 days of coculture), whereas IL-12 had no effect when added for the remainder (the differentiation phase of osteoclast formation) of the coculture period (Fig. 1*B*). In this respect, IL-12 differs from IFN-$\gamma$ that submaximally inhibits osteoclast formation during both the proliferative and differentiation phases of osteoclast formation (39).

### *IL-12 inhibits osteoclast formation in cocultures from IFN-$\gamma$ $R^{-/-}$ mice and GM-CSF $R^{-/-}$*

To establish whether IL-12 was inhibiting osteoclast formation by the production of IFN-$\gamma$, cocultures of primary osteoblasts and newborn spleen cells derived from IFN-$\gamma$ $R^{-/-}$ mice were treated with IL-12 for the entire culture period. We have previously shown that IFN-$\gamma$ was unable to influence osteoclast formation in this coculture system at 50 U/ml, a concentration that inhibits osteoclast formation in C57BL/6 cocultures (39) or at a 10-fold higher dose (500 U/ml; data not shown). However, IL-12 was able to inhibit osteoclast formation in a dose-dependent manner with a maximal effect at 1 ng/ml (Fig. 2*A*). There was negligible osteoclast formation ($<10$ $TRAP^+$ multinucleated cells/well), at a concentration of 0.1 ng/ml, similar to the result observed with the wild-type cocultures (Fig. 1*A*), indicating that IL-12 was mediating

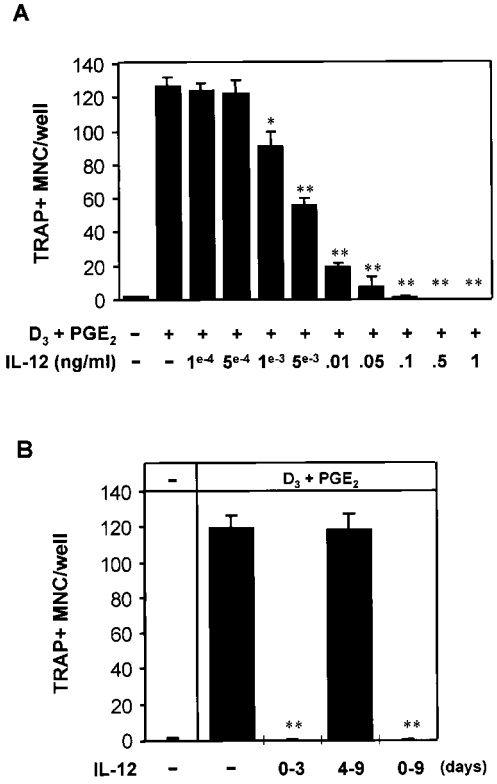


**FIGURE 1.** Effect of IL-12 on osteoclast formation in cocultures of neonatal C57BL/6J mouse spleen cells and osteoblastic cells. *A,* Newborn mouse spleen cells and primary osteoblasts were cocultured with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and $PGE_2$ ($10^{-7}$ M) in the presence of increasing concentrations of IL-12. *B,* IL-12 (0.1 ng/ml) was present for the first 3 days (days 0–3), after the first medium change (days 4–9), or for the entire culture period (days 0–9). Medium change occurred at days 3 and 6 of the coculture. For negative and positive controls, cocultures were performed in the absence and presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$, respectively. After culture for 9 days, $TRAP^+$ MNCs were counted. Data are expressed as the means ± SEM of quadruplicate cultures from three separate experiments. Statistical analysis was performed using a two-tailed *t* test comparing the numbers of $TRAP^+$ MNC to the values observed in the positive control wells. *, $p < 0.05$ and **, $p < 0.01$.

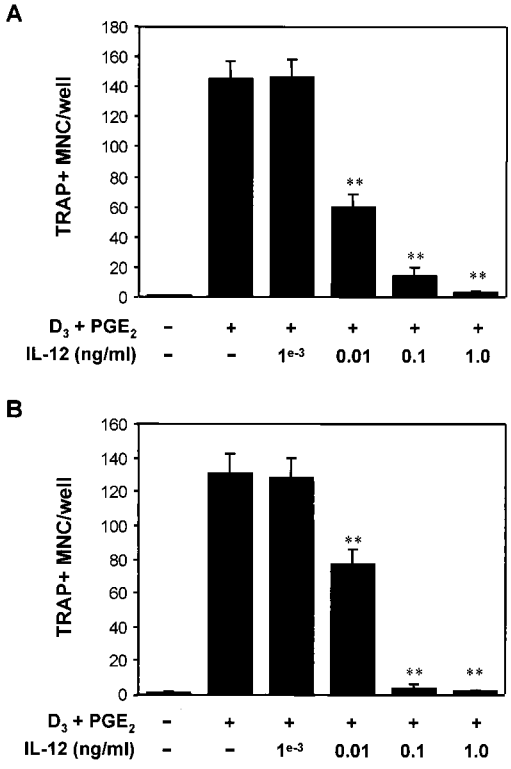


**FIGURE 2.** Osteoclast formation in cocultures of newborn spleen cells and osteoblastic cells derived from either IFN-$\gamma$ $R^{-/-}$ (*A*) or GM-CSF $R^{-/-}$ (*B*) mice. Spleen cells and primary osteoblasts were cocultured with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and $PGE_2$ ($10^{-7}$ M) in the presence of increasing concentrations of IL-12. For negative and positive controls, cocultures were performed in the absence and presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$, respectively. After culture for 9 days, $TRAP^+$ MNCs were counted. Data are expressed as the means ± SEM of quadruplicate cultures. This experiment was repeated three times. Statistical analysis was performed using a two-tailed *t* test comparing the numbers of $TRAP^+$ MNC to the values observed in the positive control wells. **, $p < 0.01$.

its inhibitory action on osteoclast formation through an IFN-$\gamma$-independent pathway.

Because of our previous observation that IL-18 elicited its inhibitory effect on osteoclast formation through T cell production of GM-CSF, the possibility that IL-12 was acting in a similar manner was investigated using cocultures of primary osteoblasts and newborn spleen cells from GM-CSF $R^{-/-}$ mice. GM-CSF was unable to inhibit osteoclast formation in these cocultures (Ref. 31; data not shown); however, IL-12 was completely inhibitory at a concentration of 1.0 ng/ml (Fig. 2*B*), a concentration of IL-12 which inhibits osteoclast formation in cocultures established from cells derived from wild-type C57BL/6 mice. Thus, in the absence of functional GM-CSF receptors, IL-12 still inhibited osteoclast formation, indicating that GM-CSF was not an intermediate for inhibition.

### *IL-12 and IL-18 act synergistically to inhibit osteoclast formation*

IL-12 and IL-18 have been widely reported to act in synergy in other cell systems (43). To assess the ability of these two cytokines to synergistically inhibit osteoclast formation, cocultures of newborn spleen cells and primary osteoblasts were treated with a non-inhibitory dose of one IL combined with increasing doses of the other IL. Cocultures were treated with IL-12 ($5 \times 10^{-4}$ ng/ml), a concentration previously shown to lack an inhibitory effect on osteoclast formation (Fig. 1*A*), in combination with increasing doses of IL-18 from 0.1 to 5 ng/ml. The doses of IL-18 added were below the concentration required for maximal osteoclast inhibition by this cytokine alone (10 ng/ml; Refs. 31 and 39). Under these culture conditions, maximal osteoclast inhibition was observed following the addition of IL-12 ($5 \times 10^{-4}$ ng/ml) and IL-18 (0.5 ng/ml), indicating that these two agents were able to act in synergy to inhibit osteoclast formation (Fig. 3*A*). The reciprocal experiment was performed using a constant dose of IL-18 (0.5 ng/ml) in combination with increasing doses of IL-12 from $10^{-4}$ to $5 \times 10^{-3}$ ng/ml, with similar results obtained (Fig. 3*B*). This synergistic effect was also observed when using cells from either the IFN-$\gamma$ $R^{-/-}$ or GM-CSF $R^{-/-}$ mice, verifying that the effect of the combined cytokine treatment was not due to the production of or signaling through either IFN-$\gamma$ or GM-CSF (data not shown). Furthermore, like the actions of IL-18 alone (39) and IL-12 alone (Fig. 1*B*), the synergistic actions of IL-12 with IL-18 were limited to the first 3 days of coculture of osteoblasts with spleen cells (Fig. 3*C*).

### *IL-12 and IL-18 exert their inhibitory action via T cells*

Since the inhibitory action of IL-12 was confined to the first 3 days of culture, IL-12 may act upon the proliferation of osteoclast precursors or through another target cell, such as T cells, as we have

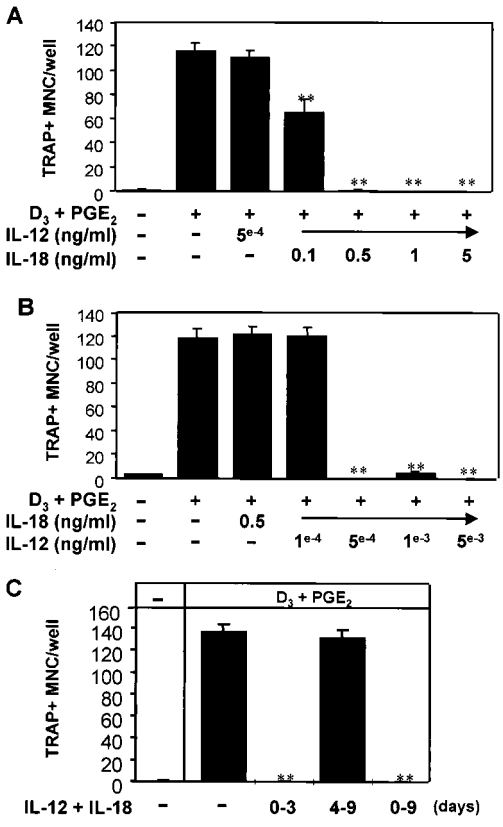


**FIGURE 3.** Effect of IL-12 and IL-18 on osteoclast formation in cocultures of newborn C57BL/6J mouse spleen cells and osteoblastic cells. Spleen cells and primary osteoblasts were cocultured with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and $PGE_2$ ($10^{-7}$ M) in the presence of either a constant dose of IL-12 ($5^{e-4}$ ng/ml, *A*) with increasing concentrations of IL-18, or a constant dose of IL-18 (0.5 ng/ml, *B*) with increasing concentrations of IL-12. For negative and positive controls, cocultures were performed in the absence and presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$, respectively. After culture for 9 days, $TRAP^+$ MNCs were counted. Data are expressed as the means ± SEM of quadruplicate cultures. This experiment was repeated four times. *C*, IL-12 ($5^{e-4}$ ng/ml) and IL-18 (0.5 ng/ml) was present for either the first 3 days (days 0–3), after medium change (days 4–9), or for the entire culture period (days 0–9). For negative and positive controls, cocultures were performed in the absence and presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$, respectively. After culture for 9 days, $TRAP^+$ MNCs were counted. Data are expressed as the means ± SEM of quadruplicate cultures from three separate experiments. Statistical analysis was performed using a two-tailed *t* test comparing the numbers of $TRAP^+$ MNC to the values observed in the positive control wells. **, $p < 0.01$.

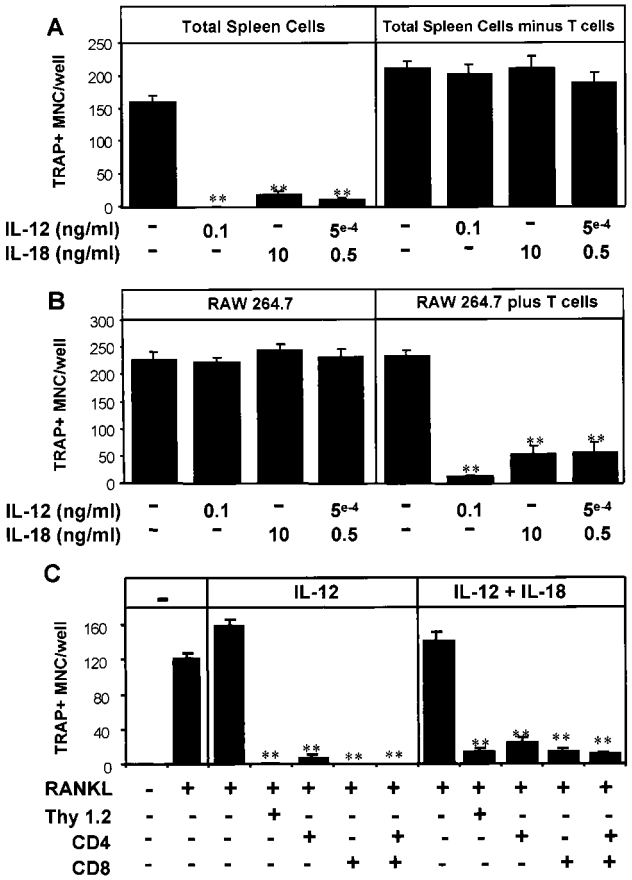


**FIGURE 4.** Effect of IL-12 and IL-18 on osteoclast formation in the presence and absence of T cells. *A*, Adult spleen cells treated with RANKL (50 ng/ml) and M-CSF (25 ng/ml). Total spleen cells and spleen cells minus T cells were cultured for a period of 10 days with either no additional treatment, IL-12 alone, IL-18 alone, or IL-12 and IL-18 in combination. *B*, RAW264.7 cells treated with RANKL (50 ng/ml). RAW264.7 cells alone and RAW 264.7 cells plus spleen cell-derived T cells were cultured for a period of 7 days with either no additional treatment, IL-12 alone, IL-18 alone, or IL-12 and IL-18 in combination. At the completion of the culture period, $TRAP^+$ MNCs were counted and the data are expressed as the means ± SEM of quadruplicate cultures. This experiment was repeated a minimum of three times. *C*, RAW264.7 cells treated with RANKL (50 ng/ml). RAW264.7 cells alone and RAW264.7 cells plus spleen cell-derived T (Thy1.2) or CD4 or CD8 cells were cultured for a period of 7 days with either no additional treatment, IL-12 alone, or IL-12 and IL-18 in combination. At the completion of the culture period, $TRAP^+$ MNCs were counted and the data are expressed as the means ± SEM of quadruplicate cultures. This experiment was repeated a minimum of three times. Statistical analysis was performed using a two-tailed *t* test comparing the numbers of $TRAP^+$ MNCs to the values observed in the positive control wells. **, $p < 0.01$.

previously observed for the inhibitory effects of IL-18 on osteoclastogenesis (31). The discovery of the osteoclast differentiation factor RANKL (1) has allowed the generation of new types of in vitro osteoclast formation assays. We examined $TRAP^+$ multinucleated cell (MNC) formation using three different in vitro osteoclast formation cultures: cocultures of newborn murine spleen cells and osteoblasts with osteotropic stimuli; adult spleen cells treated with a combination of RANKL and M-CSF; and the M-CSF independent macrophage-like cell line RAW264.7 treated with RANKL. Adult spleen cultures allow the investigation of osteoclastogenesis in the absence of osteoblasts, while retaining a variety of other cell types, such as T and B cells, along with the osteoclast precursor cells. RAW264.7 cultures contain the osteoclastic precursors in the absence of all other cell types, thereby allowing the examination of factors acting directly on osteoclasts and their precursors. The addition of either IL-12 alone, IL-18 alone, or IL-12 and IL-18 in combination resulted in the inhibition of osteoclast formation in the spleen cell cultures; however, the removal of total T cells from these cultures using mouse pan T (Thy 1.2) dynabeads ablated the effects of each of these treatments (Fig. 4*A*). Conversely, each of the cytokine combinations was unable to influence osteoclast formation in the RAW264.7 cultures (Fig. 4*B*); however, the addition of the T cells derived from the adult C57BL/6 mice spleens was able to confer the inhibitory effects of IL-12 alone, IL-18 alone, and the combination of IL-12 and IL-18 (Fig. 4*B*). In support of an essential role for T cells in the inhibitory cascade for IL-12, IL-18, or IL-12 along with IL-18, osteoclast formation was unaffected in cocultures established from

osteoblasts and spleen cells derived from Rag1 mice (a mouse strain that lacks the T and B cell populations) treated with each of these agents (data not shown). These results conclusively demonstrate that IL-12 and the combination of IL-12 and IL-18 are acting on T cells to induce the production of a factor which inhibits osteoclast formation.

To elucidate a particular subset of T cells responding to treatment with IL-12 and IL-12 in combination with IL-18, CD4 and CD8 T cells were immunomagnetically separated from adult spleen cells and added to RAW264.7 cultures treated with RANKL and IL-12 or IL-12 along with IL-18. Thy1.2, CD4, CD8, and the combination of both CD4- and CD8-captured T cells elicited IL-12- or IL-12- and IL-18-induced osteoclast inhibition when added to RAW264.7 cell cultures (Fig. 4*C*). In the absence of T cells, IL-12 or IL-12 with IL-18 was unable to inhibit RAW264.7 cell differentiation into osteoclasts (Fig. 4, *B* and *C*), therefore demonstrating that both the CD4 and the CD8 populations were capable of producing an osteoclast inhibitor(s) in response to IL-12 or IL-12 in combination with IL-18.

### *Nature of T cell-derived inhibitor*

The ability of IL-12, IL-18, and IL-12 with IL-18 to alter the expression of known osteoclast inhibitors (IL-1R antagonist (IL-1RA), IL-4, IL-10, IL-13, GM-CSF, IFN-γ, and OPG) was assessed. Spleen cells were treated with IL-12 (0.1 ng/ml), IL-18 (10 ng/ml), and IL-12 and IL-18 (0.1 and 10 ng/ml, respectively) over a 3-day time course (0.5, 1, 2, 4, 8, 12, 24, and 72 h), RNA was extracted, and regulation of known inhibitors was assessed by RT-PCR. The most striking regulation was observed with GM-CSF and IFN-γ (Fig. 5). GM-CSF mRNA levels increased within 2 h of exposure to IL-18 and continued through to a 6-fold increase at 72 h; however, there was no significant rise in GM-CSF mRNA following IL-12 treatment with the exception of the 72-h time point (7-fold; Fig. 5*A*). GM-CSF RNA levels in response to the combined treatment of IL-12 and IL-18 were equivalent to treatment with IL-18 alone (Fig. 5*A*). This expression profile was similar to that observed with IL-13 mRNA, where IL-18 induced a 3-fold increase in IL-13 mRNA levels at 2 h, and IL-12 alone or in combination with IL-18 did not enhance IL-13 mRNA levels (data not shown). In contrast, IFN-γ mRNA levels were markedly increased by 4 h of treatment with IL-12 and increased to 10-fold higher than control levels by 72 h; however, there was no effect on IFN-γ mRNA expression following IL-18 treatment (Fig. 5*B*). In response to IL-12 and IL-18 treatment, IFN-γ mRNA levels were equivalent to those observed with IL-12 alone (Fig. 5*B*). Similarly, IL-10 mRNA levels were increased 3-fold by IL-12 by 4 h and were unaffected by IL-18, and no additive or synergistic effect was observed in treatments with IL-12 and IL-18 (data not shown). Similarly, recent work by Nakamura et al. (44) confirmed that IL-10 protein levels were similar to the mRNA expression profile in the sera of BALB/c mice in vivo. IL-1RA and IL-4 mRNA levels were both elevated 2-fold in response to IL-12 or IL-18 with maximal effects at 4 and 2 h, respectively, but mRNA levels for IL-1RA or IL-4 were not enhanced further by the combination of IL-12 and IL-18 (data not shown). There was no effect on OPG mRNA expression in response to IL-12, IL-18, or IL-12 and IL-18 (data not shown).

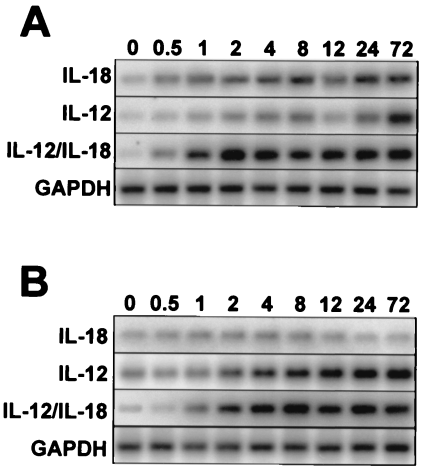


**FIGURE 5.** Semiquantitative RT-PCR analysis of GM-CSF and IFN-γ mRNA. Total spleen cells were treated with either IL-18 (10 ng/ml), IL-12 (0.1 ng/ml), or IL-12 and IL-18 (0.1 and 10 ng/ml, respectively) as indicated on the left-hand side of this figure. A time course was performed with total RNA extracted from the spleen cells at 0.5, 1, 2, 4, 8, 12, 24, and 72 h. Total RNA samples were reverse transcribed with oligo(dT) and subjected to PCR for either GM-CSF (*A*, 28 cycles) or IFN-γ (*B*, 20 cycles), which was in the log-linear range of amplification. Resultant PCR products were electrophoresed, transferred to nylon membrane, and hybridized with γ-$^{32}$P-labeled internal detection oligonucleotide specific to each of the cDNA products. RT-PCR analysis was repeated in triplicate.

The potential involvement of the secreted inhibitors IL-4, IL-10, and IL-13 in IL-12- and IL-12 in combination with IL-18-induced osteoclast inhibition were assessed using neutralizing Abs to each, independently and in combination, in RANKL- and M-CSF-treated spleen cultures. Neutralizing Abs to IL-4, IL-10, and IL-13 (each at 500 ng/ml) neither alone (data not shown) nor in combination were unable to rescue osteoclast formation inhibited by IL-12 or by IL-12 with IL-18 (Fig. 6*A*). Each Ab alone could restore osteoclast formation inhibited by their respective target IL (data not shown).

We have already excluded the potential involvement of GM-CSF and IFN-γ in IL-12- or IL-12 with IL-18- mediated osteoclast inhibition using GM-CSF $R^{-/-}$ or IFN-γ $R^{-/-}$ mice cocultures (Fig. 2.). Furthermore, since IL-12 in combination with IL-18 did not augment the production of any known osteoclast inhibitor and since neutralizing anti-IL-4, anti-IL-10, or anti-IL-13 Abs could not rescue osteoclast formation in IL-12 or the combination of IL-12 and IL-18-treated cultures, we suggest that IL-12 and IL-12 with IL-18 most likely inhibit osteoclast formation by increasing the production of an unknown inhibitor derived from T cells.

### *Transwell experiments*

To further elucidate the physical nature of the inhibitory factor produced by T cells in response to IL-12 alone and the combination of IL-12 and IL-18, coculture experiments were conducted using transwells. T cells were separated from the spleen population using mouse pan T (Thy 1.2) dynabeads, and the remaining spleen cells and primary osteoblasts were added to the lower chamber of a 24-well transwell culture plate in the presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$. The extracted T cells were added to the upper chamber that separates the two cell populations via a 0.4-μm filter and either IL-12 alone or IL-12 along with IL-18 were added to the upper culture chamber. Using these culture conditions, IL-12 alone and the combination of IL-12 and IL-18 were still able to inhibit osteoclast formation (Fig. 6*B*), indicating that the unknown inhibitor was a soluble factor. This could also be demonstrated using adult spleen cell cultures and RAW264.7 cultures with T cells added to the upper culture chamber (data not shown).

## Discussion

In this report, we establish that IL-12, a potent proinflammatory cytokine, can inhibit osteoclast formation in mouse cocultures and adult spleen cell cultures in vitro. This effect was dose dependent

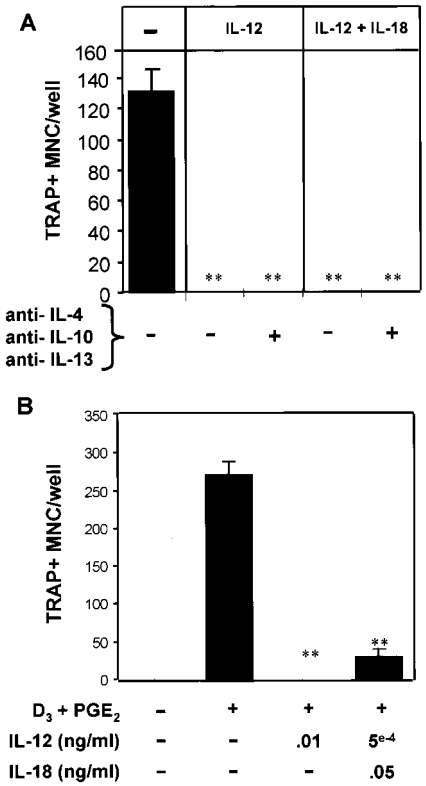


**FIGURE 6.** Nature of the T cell-induced inhibitor. *A*, Effect of neutralizing Abs against IL-4, IL-10, and IL-13 to rescue osteoclast formation in IL-12- or IL-12 and IL-18-treated spleen cell cultures. Adult spleen cells treated with RANKL (50 ng/ml) and M-CSF (25 ng/ml) were cultured for a period of 7 days with no additional treatment, IL-12 (0.1 ng/ml) alone, or IL-12 ($5^{e-4}$ ng/ml) and IL-18 (0.5 ng/ml) in combination. The effect of neutralizing Abs to IL-4, IL-10, and IL-13 (each at 500 ng/ml) in combination was determined. This experiment was repeated three times. *B*, Effect of physical separation of osteoclastic precursors from T cells. Osteoclast formation was assessed in cocultures of newborn C57BL/6J mouse spleen cells and primary osteoblasts where the T cells had been removed from the spleen population using mouse pan T (Thy 1.2) dynabeads. The remaining spleen cells and osteoblastic cells were added to the lower chamber of a 24-well Transwell plate with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and $PGE_2$ ($10^{-7}$ M), while the T cells were added to the upper chamber separating the two populations of cells by a 0.4-$\mu$m filter. Cultures were treated with either IL-12 (10 ng/ml) or IL-12 and IL-18 ($5^{e-4}$ and 0.5 ng/ml, respectively) by the addition of cytokines to the upper culture chamber. For negative and positive controls, cocultures were performed in the absence and presence of $1\alpha,25(OH)_2D_3$ and $PGE_2$, respectively. After culture for 9 days, $TRAP^+$ MNCs were counted. Data are expressed as the means ± SEM of quadruplicate cultures. This experiment was repeated four times. Statistical analysis was performed using a two-tailed *t* test comparing the numbers of $TRAP^+$ MNCs to the values observed in the positive control wells. **, $p < 0.01$.

and could be restricted to the first 3 days of the culture period. This time frame was consistent with IL-12 having actions on osteoclast precursors or through an intermediate cell type, such as T cells, such as we have described for the inhibitory action of IL-18 (31). Several lines of evidence established that IL-12-mediated inhibition of osteoclast formation was T cell-dependent and that this product(s) was a secreted factor(s): 1) IL-12 was able to inhibit osteoclast formation in M-CSF- and RANKL-stimulated splenic cultures, although removal of T cells from these cultures rendered IL-12 ineffective. 2) IL-12 was unable to inhibit osteoclast formation in RANKL-stimulated RAW264.7 cell cultures; however, IL-12 was inhibitory if T cells were added to these cultures. 3) IL-12 inhibited osteoclast formation in cocultures of osteoblasts and spleen cells derived from normal C57BL/6 mice, but was without action in cocultures established from Rag1 mice, which are devoid of T and B cells. 4) When T cells were separated from hematopoietic cells by a 0.4-$\mu$m filter, IL-12 was still inhibitory, indicating that the product was secreted.

Since IL-12 is known to induce the production of IFN-$\gamma$ by T and NK cells (16), IFN-$\gamma$ was a likely mediator of the inhibitory effects of IL-12. However, despite the known inhibitory actions of IFN-$\gamma$ on osteoclast formation in vitro (37–39), the present experiments exclude IFN-$\gamma$ as the major intermediate in the inhibitory effect of IL-12. In cocultures using spleen cells and osteoblasts from IFN-$\gamma$ $R^{-/-}$ mice, the complete ablation of osteoclast formation in response to IL-12 treatment was still evident. This finding negates IFN-$\gamma$ as the major intermediate inhibitor for IL-12 action, although it may exert a partial role. Furthermore, the time course of action of IFN-$\gamma$ that we have previously described (39) differs from that for IL-12. We have previously shown that IL-18, a cytokine with similar biological activities to IL-12, inhibited osteoclast formation by means of GM-CSF production by T cells and did not involve the production of IFN-$\gamma$ (31, 39). Unlike IL-18, IL-12 inhibited osteoclast formation in cocultures established from osteoblasts and spleen cells derived from GM-CSF $R^{-/-}$ mice. Thus, the osteoclast inhibitory action of IL-12 was independent of the IFN-$\gamma$ receptor or GM-CSF receptor signaling.

IL-12 and IL-18 are able to act synergistically in other cell systems, presumably via the reciprocal up-regulation of their receptor components (21, 23, 29), and their synergistic actions have been attributed to enhanced IFN-$\gamma$ production (18, 23–25). We describe herein that these agents act in synergy to inhibit osteoclast formation. Importantly, the synergistic action of IL-12 and IL-18 to inhibit osteoclast formation was independent of either IFN-$\gamma$ or GM-CSF signaling due to the combined actions of these agents to inhibit osteoclast formation in cocultures of osteoblast or spleen obtained from GM-CSF $R^{-/-}$ or IFN- $R^{-/-}$ mice. Recently, the treatment of BALB/c mice with IL-12 and IL-18 has demonstrated that although an IFN-$\gamma$-dependent pathway was evidenced in vivo, there was also evidence for an IFN-$\gamma$-independent pathway since this combination of cytokines was still able to induce toxic shock symptoms in IFN-$\gamma$-deficient mice (44).

The inhibitory effects of both IL-12 alone and IL-12 combined with IL-18 were shown to be T cell dependent, and could be facilitated through either CD4 or CD8 T cells. A number of cytokines expressed by T cells have the capacity to inhibit osteoclast formation (namely, IL-1RA, IL-4, IL-10, IL-13, GM-CSF, and IFN-$\gamma$); however, we were unable to demonstrate an increase at the mRNA level for any of these factors in response to the combination of IL-12 and IL-18 greater than that seen for IL-12 or IL-18 alone. Furthermore, neutralizing Abs to IL-4, IL-10, or IL-13 either alone or in combination were unable to rescue osteoclast formation inhibited by either IL-12 or IL-12 with IL-18. Transwell experiments in which T cells were separated from hematopoietic cells indicated that the inhibitory molecule was secreted and not membrane bound or membrane associated. Given that IL-12 alone induces the production of a hitherto unrecognized inhibitor of osteoclast formation of T cell origin, it is tempting to suggest that IL-18 augments the IL-12 production of this inhibitor.

IL-12 and IL-18 have been detected in the rheumatoid arthritis synovial membrane (45, 46), and from our studies such a finding may suggest that IL-12 and IL-18 may protect the joint from pathological destruction via osteoclast-mediated erosion. IL-18 has been described to be effective in limiting bone destruction in murine models of breast cancer metastasis in bone (47). Combined, these results suggest that IL-12 and IL-18 may be useful in therapy for physiological and

pathological bone loss. Ultimately, identification of the IL-12- and IL-12/IL-18-induced inhibitor could provide a novel inhibitor of osteoclastogenesis and be used to further examine the intriguing interactions between the immune system and bone metabolism.

## Acknowledgments

We thank Dr. Julian Quinn for performing bone resorption assays and for useful discussions.

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