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Locally administered interferon-γ accelerates lipopolysaccharide-induced osteoclastogenesis independent of immunohistological RANKL upregulation

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Background and Objective: Interferon-γ (IFN-γ) potently inhibits RANKL-induced osteoclastogenesis *in vitro*. In contrast, previous studies have shown that an increase in IFN-γ expression is correlated with an increase in lipopolysaccharide (LPS)-induced bone loss *in vivo*. However, it is not clear whether local IFN-γ accelerates osteoclastogenesis or not *in vivo*. Therefore, the aim of this study was to clarify the role of local IFN-γ in LPS-induced osteoclastogenesis.

Material and Methods: We induced bone loss in calvaria by injecting LPS. One group of mice received an IFN-γ injection together with LPS injection, while another group received IFN-γ 2 d after LPS injection. Bone resorption was observed histologically. Next, we stimulated murine bone marrow macrophages with macrophage-colony stimulating factor and RANKL *in vitro*. We added different doses of IFN-γ and/or LPS at 0 or 48 h time points. Cells were stained with tartrate-resistant acid phosphatase at 72 h.

Results: Local administration of IFN-γ together with LPS injection did not affect osteoclast formation. However, IFN-γ injected after LPS injection accelerated osteoclast formation. Also, we confirmed that IFN-γ added at 0 h inhibited RANKL-induced osteoclastogenesis *in vitro*. However, inhibition by IFN-γ added at 48 h was reduced compared with that by IFN-γ added at 0 h. Interestingly, IFN-γ together with a low concentration of LPS accelerated osteoclast formation when both were added at 48 h compared with no addition of IFN-γ.

Conclusion: The results suggest that local IFN-γ accelerates osteoclastogenesis in certain conditions of LPS-induced inflammatory bone loss.

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Osteoclasts, multinucleated giant cells that resorb bone, develop from hemopoietic cells of the monocyte/macrophage lineage. Osteoclast differentiation requires the presence of macrophage-colony stimulating factor (M-CSF) and RANKL. RANKL interacts with its receptor, RANK, which is expressed by mononuclear osteoclast precursors; this process is inhibited by osteoprotegerin, which is produced by bone stromal cells/osteoblasts (1).

Interferon-γ (IFN-γ) is secreted by T cells and natural killer (NK) cells and is intimately involved in innate and acquired immune responses (2). The specific role of this cytokine involved in osteoclast differentiation has not been fully elucidated in *in vivo* and *in vitro* studies. It has been shown in *in vitro* studies that IFN-γ exerts a potent inhibitory effect on RANKL-induced osteoclastogenesis (2–4). This mechanism involves a direct action on osteoclast precursors by accelerating degradation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6; 2). In contrast, others have reported that IFN-γ showed no inhibitory effect in RANKL prestimulated monocytes (5,6). This suggests that the effect of IFN-γ on pre-osteoclasts can be modified depending on the stage of osteoclast differentiation by RANKL.

Periodontitis is an infectious oral disease characterized by inflammatory destruction of soft periodontal tissues and alveolar bone resorption. Lipopolysaccharide (LPS) is one of the major constituents of the outer membrane of gram-negative bacteria and is thought to play an important role in the development of periodontal disease (7). Lipopolysaccharide is known to stimulate the production of many local factors, including TNF-α, interleukin (IL-1) and IFN-γ, from macrophages or other cells in inflamed tissues (8,9). These cytokines have been shown to be associated with LPS-involved bone destruction and osteoclast formation in *in vivo* and *in vitro* studies (10–16). Interferon-γ, found at high levels, has been shown to be associated with progressive lesions of periodontal disease (12,17). It has also been reported that the number of IFN-γ-positive cells was increased as assessed by histology of severely inflamed gingival samples from adult periodontitis patients (10). We have reported that IFN-γ-positive cells were increased significantly in relation to bone resorption induced by LPS injected into the gingiva (11). Moreover, it has been reported that an increase in IFN-γ is associated with enhanced *Actinobacillus actinomycetemcomitans*-specific RANKL-expressing CD4-positive Th cell-mediated alveolar bone loss (18). Furthermore, Gao *et al*. (19), who studied the systemic effect of IFN-γ using IFN-γ receptor knockout mice, reported that IFN-γ indirectly stimulates osteoclast formation and bone loss *in vivo*.

Thus, it has been shown that IFN-γ has a potent inhibitory effect on osteoclast formation *in vitro*. In contrast, many studies have shown that IFN-γ is associated with the severity of inflammatory bone loss *in vivo*. Based on these studies, it is not clear whether local IFN-γ increases osteoclast formation or not. Therefore, the aim of this study was to clarify the role of local IFN-γ in LPS-induced osteoclastogenesis.

Material and methods

Animals and reagents

Five- to 9-week-old male CB17/Icr + Jcl mice were purchased from Nihon Clea (Tokyo, Japan) and maintained in specific pathogenic-free conditions at the Biomedical Research Center for Frontier Life Sciences, Nagasaki University. Animal care and experiments proceeded according to the Guidelines for Animal Experimentation of Nagasaki University and with the approval of the Institutional Animal Care and Use Committee. Recombinant mouse M-CSF, recombinant mouse soluble RANKL, recombinant mouse IFN-γ, and anti-mouse TNF-α antibody and normal goat IgG were purchased from R&D Systems (Minneapolis, MN, USA). *Escherichia coli* 0111:B4 LPS (*E. coli LPS*) and a tartrate-resistant acid phosphatase (TRAP) kit were purchased from Sigma (St. Louis, MO, USA). Goat anti-mouse RANKL polyclonal antibody sc-7628 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal rabbit serum, biotinylated rabbit anti-goat polyclonal antibody and peroxidase-conjugated streptavidin were purchased from Dako (Glostrup, Denmark).

Bone resorption in mice calvaria

Bone resorption in mice calvaria was induced with one injection of 600 μg/60 μL of *E. coli* LPS. The injections were administered with a 30.5 gauge needle at a point on the midline of the skull located between the ears and the eyes. On day 5 after LPS injection, the mice were killed by cervical dislocation. Interferon-γ (40 μg/mL) was injected on days 0 and 2 after LPS injection. Anti-TNF-α antibody solution (1 mg/mL) was injected together with IFN-γ on day 2. Mice in the control group were injected with 60 μL of phosphate-buffered saline solution or IFN-γ on day 0.

Tissue preparation

After the mice had been killed, the entire calvarial bone was dissected and fixed in 4% paraformaldehyde overnight at 4°C. The specimens were then decalcified with 10% ethilenediaminetetraacetic solution (pH 7.6) for 5 d at 4°C. Following decalcification, the tissues were embedded in paraffin by the AMeX method (acetone, methyl benzoate and xylene; 20). Fifty serial sections of 4 μm thickness were obtained for each specimen. Every 10th serial section was stained with hematoxylin and eosin (H&E) for histopathological observations. The next serial section was stained for TRAP to determine TRAP-positive cells and osteoclasts, considered as TRAP-positive multinucleate (two or more nuclei) cells. We counted the osteoclasts attached to the bone inside the sagittal suture and the bone marrow area within the perimeter of 1 mm of the midline. These cells were counted using a light microscope (Nikon, Tokyo, Japan).

Histochemical and immunohistological staining

Serial sections were also immunohistologically stained to identify RANKL-expressing cells. Briefly, after

deparaffinization, endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min, followed by incubation with normal rabbit serum for 30 min at room temperature. These sections were then immersed in goat anti-mouse RANKL polyclonal antibody (Santa Cruz Biotechnology) or goat serum as a negative control at 4°C overnight, followed by biotinylated rabbit anti-goat polyclonal antibody (Dako) for 30 min and peroxidase-conjugated streptavidin (Dako) for 30 min. Positive reactions were visualized by diaminobenzidine tetraoxide and counterstaining with hematoxylin. Cells stained brown were considered to be RANKL-positive cells. We counted RANKL-positive cells that were present in the same area in which the osteoclast number count was performed.

Preparation of bone marrow macrophages

We obtained bone marrow macrophages according to previous reports (21,22). Briefly, tibiae and femurs were excised from mice, and bone marrow cells were flushed out with PBS, centrifuged at 210 *g* for 10 min, and resuspended in red blood cell lysis buffer (BD Biosciences Pharmingen , San Diego, CA, USA) at room temperature for 10 min. The cells were then plated at 1.5×10^7 to 2×10^7 cells in 10 cm dishes with 10 mL of minimal essential medium α medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured in the presence of 5 ng/mL M-CSF for 12 h. Nonadherent cells were collected and cultured with 30 ng/mL of M-CSF for 36 h. Finally, adherent cells were collected and used as bone marrow macrophages after the removal of nonadherent cells. By flow cytometry analysis, around 95% of the bone marrow macrophage population was found to be positive for RANK and CD11b. Cells positive for CD3 and CD19 accounted for approximately 5% of the total bone marrow macrophage population.

Osteoclast differentiation assays

Bone marrow macrophages were seeded at a concentration of 2×10^4 cells per well in a 96-well plate and cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) for 72 h. Interferon-γ or LPS at the indicated doses were added at 0, 24 and 48 h of culture. In other experiments, bone marrow macrophages were treated with RANKL at 1 ng/mL in the presence of M-CSF at 30 ng/mL for 48 h. Lipopolysaccharide, IFN-γ and anti-TNF-α antibody were added for an additional 24 h. At 72 h, the cells were fixed with 4% paraformaldehyde and stained with a TRAP kit for identification of osteoclasts. The TRAP-positive cells with three or more nuclei were considered to be osteoclasts.

Formation of resorption pits

After bone marrow macrophages (2×10^4 cells per well) were cultured with 30 ng/mL of M-CSF and 1 ng/mL of RANKL in BD BioCoat™ Osteologic™ Bone Cell Culture System (BD Biosciences, Bedford, MA, USA) for 48 h, LPS (0.1 ng/mL) was added alone or together with IFN-γ (0.1 ng/mL). After 48 h of incubation, the medium, reagents and cytokines were renewed. After 72 h, we stained with von Kossa staining. We counted the number of pits per disc by using a light microscope (Nikon).

ELISA for TNF-α protein level detection

Concentrations of TNF-α in culture supernatant fluids were determined by using commercially available murine ELISA kits (R&D Systems).

Reverse transcriptase-polymerase chain reaction

After bone marrow macrophages had been treated with RANKL at 1 ng/mL and M-CSF at 30 ng/mL for 48 h, they were stimulated with LPS and/or IFN-γ for an additional 3 h. Total RNA was prepared using an RNeasy Mini Spin Column (Quiagen, Germantown, MD, USA) according to the manufacturer's instructions, and the cDNA was reverse transcribed by AMV Reverse Transcriptase (Promega, Madison, WI, USA). Using TaKaRa Ex Taq™ (TAKARA BIO INC., Shiga, Japan), the reverse-transcribed cDNA was amplified with specific primers according to the manufacturer's instructions. The primer sequences used for amplification were as follows: TNF-α forward, 5′-ATGAGCACAGAAAGCATGAT CCGCGAC-3′ and reverse 5′- TCACA GAGCAATGACTCCAAAGTAGAC CTG; and β-actin forward, 5′-GAT GACGATATCGCTGCGCTG and reverse 5′-GTACGACCAGAGGCA TACAGG. The relative amounts of each mRNA were normalized to the β-actin expression. The sizes of PCR products for TNF-α and β-actin are 700 and 440 bp, respectively. The PCR products were separated by electrophoresis on a 2% agarose gel, and visualized by ethidium bromide staining and UV light illumination. After taking photographs and scanning, we measured the intensities of bands formed by the cytokine PCR products and compared them with the intensity of the band derived from the β-actin PCR product of the same sample by using ImageJ (Research Services Branch of the National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA).

Flow cytometry analysis

Flow cytometry analysis was performed with bone marrow macrophage cultures by first washing cells in buffer (0.2% bovine serum albumin in phosphate-buffered saline), followed by incubation with Fc receptor blocker (eBioscience, San Diego, CA, USA). The cells were then labeled with phycoerythrin-conjugated anti-mouse RANK (clone 9A725; IMGENEX, San Diego, CA, USA), fluorescein isothiocyanate-conjugated anti-mouse CD11b monoclonal antibody (clone 1/70; BD Biosciences Pharmigen), fluorescein isothiocyanate-conjugated anti-mouse CD3 monoclonal antibody (clone 145-2C11; BD Biosciences Pharmingen), fluorescein isothiocyanate-conjugated anti-mouse CD19 monoclonal antibody (clone MB19-1; eBioscience), or with isotype controls. The cells were washed, and 10,000 events were analyzed by flow cytometry using BDFACSCanto II (BD Biosciences, San Jose, CA, USA).

Statistics

Data were statistically analyzed using StatMate III software (ATMS, Tokyo, Japan). Differences among groups were assessed using one-factor ANOVA and Tukey's test. A value of $p < 0.05$ was considered statistically significant.

Results

Lipopolysaccharide induced osteoclast formation in mice calvaria

We first injected LPS into calvaria for examination of bone resorption and stained the tissues with H&E or TRAP. Animals were killed 2 or 5 d after LPS injection. On day 2 after LPS injection, there was a significant increase in the number of osteoclasts compared with that in the control group. The number of TRAP-positive mononucleated cells was also increased in the LPS day 2 group. The LPS day 5 group showed the greatest extent of inflammation and the largest number of osteoclasts among the LPS groups (Fig. 1A–C). We then checked RANKL expression through an immunohistochemistry study. Control tissues showed constitutively low levels of RANKL expression, while LPS day 2 group and day 5 group tissues showed an increase in RANKL expression (Fig. 1D).

Interferon-γ accelerated LPS-induced osteoclast formation in mice calvaria

We investigated the local effect of IFN-γ on LPS-induced osteoclast formation. We injected IFN-γ (40 μg/mL) into calvarial tissue at the same time as LPS or 2 d after LPS injection and then collected the tissues for examination of the bone condition. As shown in Fig. 2, control and IFN-γ groups showed very few TRAP-positive cells and

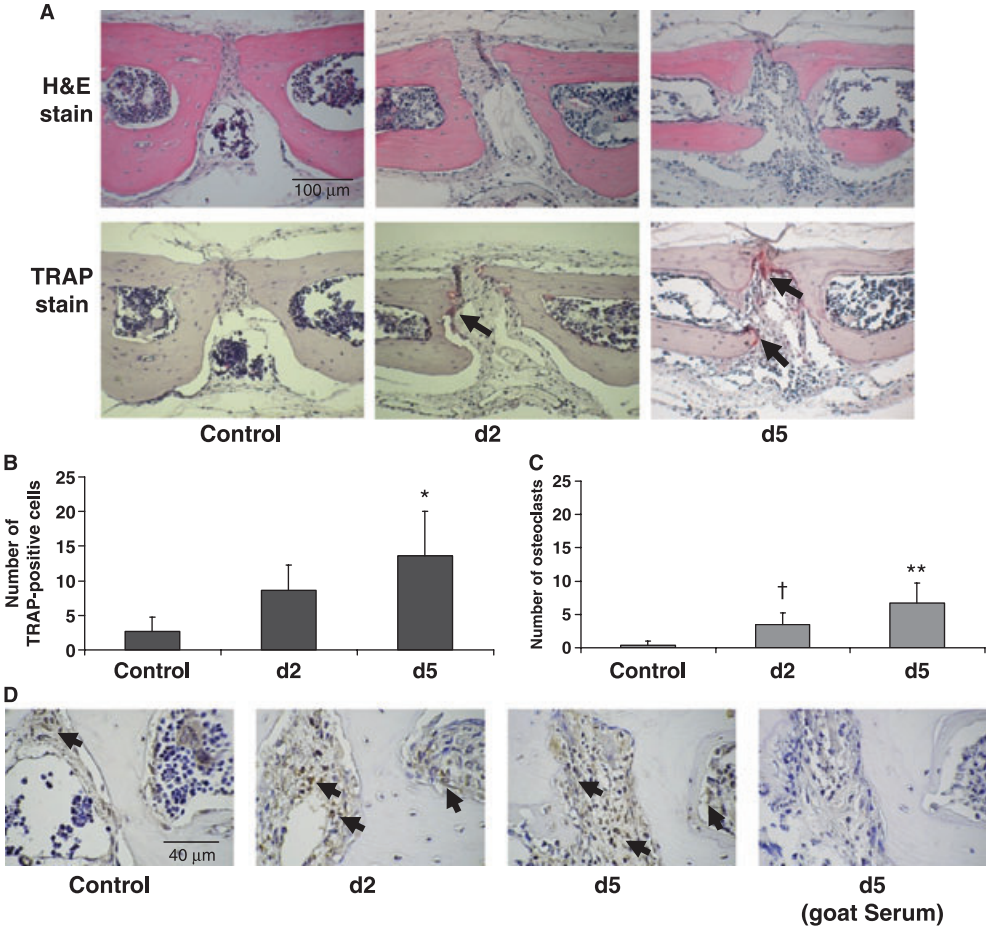

Fig. 1. Lipopolysaccharide (LPS) induced osteoclast formation *in vivo*. (A) Calvarial sections were obtained from control mice and from mice killed at 2 and 5 d after LPS injection, and the histological sections of calvarial bone were stained with hematolxylin and eosin (H&E) or TRAP. Cells stained red (indicated by arrows) are considered to be TRAP positive. Sections from days 2 and 5 after LPS injection show an increase of TRAP-positive cells, including mononucleated cells and osteoclasts. (B,C) Numbers of TRAP-positive cells (B) and osteoclasts (C) in control mice and mice 2 d and 5 d after LPS injection. Osteoclasts were considered to be TRAP-positive cells containing two or more nuclei. (D) Immunohistochemistry was performed to observe RANKL expression in control tissues and tissues on days 2 and 5 after LPS injection. The negative control (goat serum) was obtained on day 5 after LPS injection. Cells stained brown (indicated by arrows) are considered to be RANKL-positive cells, shown in representative photographs of part of the suture and bone marrow area. On days 2 and 5 after LPS injection there was an increase in RANKL-positive cells compared with controls. Bars represent means + SD; †$p < 0.05$, *$p < 0.01$, **$p < 0.001$ compared with the control.

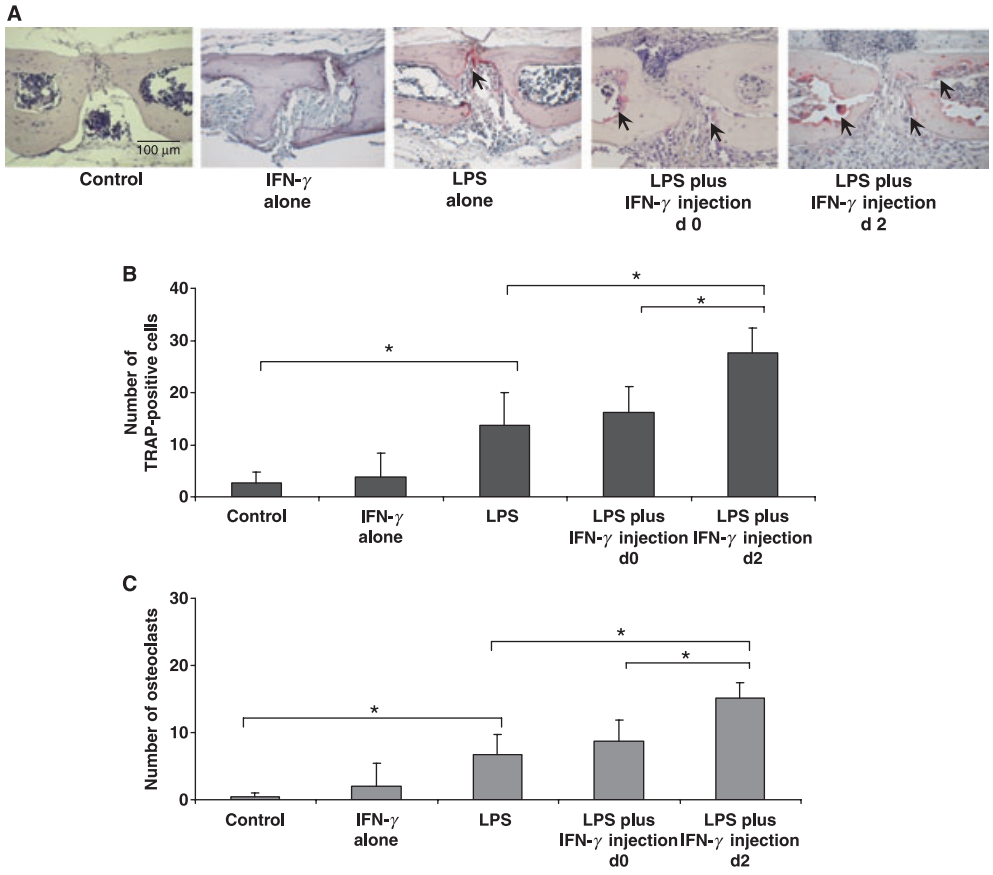

Fig. 2. Interferon-γ accelerates LPS-induced osteoclast formation *in vivo*. (A) Histopathological findings (staining with TRAP) in calvarial bone from mice without injection (control), injected with IFN-γ alone (without LPS), injected with LPS alone, or injected with IFN-γ on day 0 or 2 after LPS injection. Interferon-γ injected 2 d after LPS injection increased the numbers of TRAP-positive cells and osteoclasts induced by LPS. (B,C) Numbers of TRAP-positive cells (B) and osteoclasts (C) in mice without injection (control), injected with IFN-γ alone, injected with LPS alone, or injected with IFN-γ on day 0 or 2 after LPS injection. Osteoclasts were considered to be TRAP-positive cells containing two or more nuclei. Bars represent means + SD; $*p < 0.01$.

osteoclasts. Interferon-γ had no effect on osteoclast formation when it was injected at the same time as LPS injection, but had an accelerative effect on formation of both TRAP-positive mononucleated cells and osteoclasts when it was injected on day 2 (Fig. 2A–C).

Expression of RANKL stimulated in calvaria by injection of IFN-γ and/or LPS

RANKL is essential for osteoclast differentiation; therefore, we investigated whether IFN-γ had an effect on RANKL expression. Local injection of IFN-γ alone did not have any effect in the number of RANKL-positive cells compared with control conditions. Injection of LPS clearly increased RANKL expression. Injection of IFN-γ 2 d after LPS injection decreased the number of RANKL-positive cells in the calvarial tissue (Fig. 3). These findings are in agreement with previous studies in that RANKL expression was reduced in synovial fibroblasts by IFN-γ (23). Interferon-γ can accelerate osteoclastogenesis despite its inhibitory effect on RANKL expression.

Involvement of TNF-α in the acceleration of osteoclast formation induced by IFN-γ

Tumor necrosis factor-α is important for LPS-induced osteoclastogenesis and bone destruction (14–16). Moreover, IFN-γ-treated macrophages stimulate TNF-α production from T cells (19). We tested the effect of TNF-α on the acceleration of osteoclast formation by neutralizing its effect through a local injection of anti-TNF-α antibody. The use of antibody injection reduced the accelerated formation of TRAP-positive mononucleated cells and osteoclasts induced by IFN-γ (Fig. 4).

Effect of IFN-γ on RANKL-induced osteoclast formation in a time-dependent manner *in vitro*

In a different approach, we examined whether IFN-γ had an accelerative effect on LPS-induced osteoclastogenesis *in vitro*. We first checked time-dependent osteoclast formation induced by RANKL. We treated bone marrow macrophages with M-CSF and RANKL and stained these cells with TRAP at 24, 48 or 72 h. We used 1 or 10 ng/mL of RANKL. The TRAP staining revealed that RANKL at 1

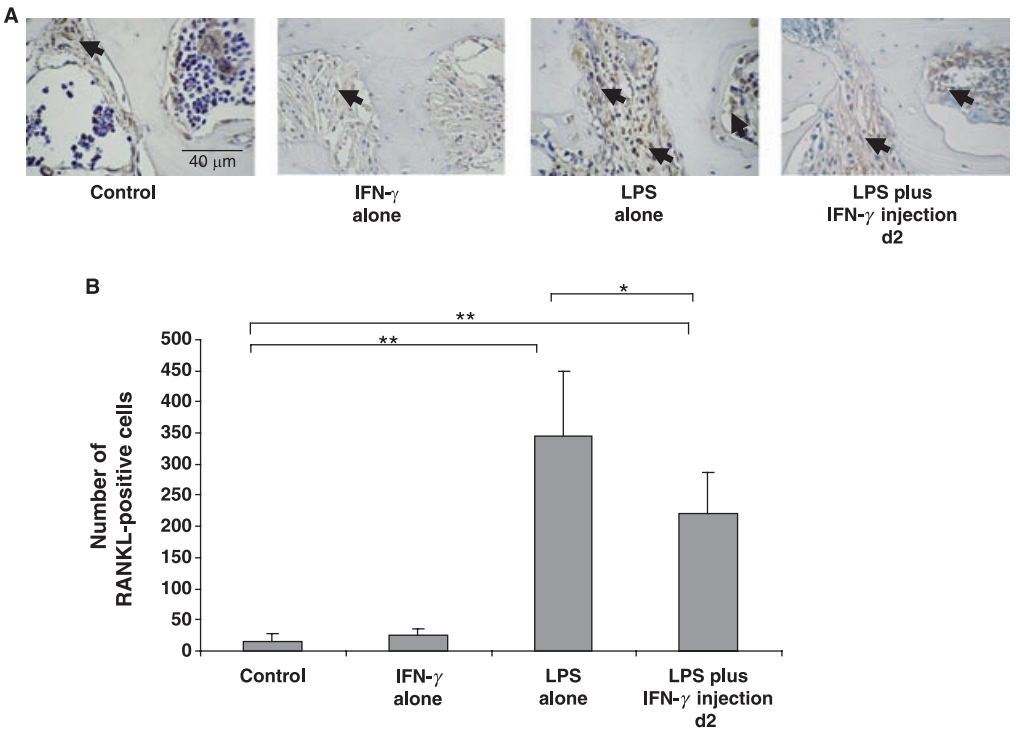

Fig. 3. Effect of IFN-γ on RANKL expression. (A,B) Representative photographs of calvarias (A) and numbers of RANKL-positive cells (B) from mice without injection (control), injected with IFN-γ alone (without LPS), injected with LPS alone, or injected with IFN-γ on day 2 after LPS injection. The LPS groups show an increase in the numbers of RANKL-positive cells compared with the control and IFN-γ groups. Interferon-γ injected on day 2 after LPS injection reduced the number of RANKL-positive cells induced by LPS. Bars represent means + SD; $*p < 0.05$, $**p < 0.001$.

and 10 ng/mL significantly induced formation of TRAP-positive mononucleated cells and induced little osteoclast formation at 48 h (Fig. 5A). At 10 ng/mL, RANKL induced significant formation of osteoclasts, whereas at 1 ng/mL RANKL induced the formation of only few osteoclasts at 72 h (Fig. 5B). Next, we investigated the effect of IFN-γ on RANKL-induced osteoclastogenesis by adding IFN-γ at 0, 24 or 48 h. At 0 h, IFN-γ completely inhibited osteoclast formation (Fig. 5C,D). This result is consistent with results of previous studies showing that IFN-γ potently inhibited osteoclast formation (2,4). It was also observed that IFN-γ-induced inhibition was decreased in a time-dependent manner. This decrease was clearly observed at 48 h; however, it was significantly different from the control cultures (Fig. 5C,D). These results are in agreement with previously reported results showing that IFN-γ did not inhibit osteoclastogenesis after RANKL stimulation (5,6). Then, we checked the effect of IFN-γ on osteoclastogenesis from bone marrow macrophages stimulated with LPS and/or IFN-γ in the presence of 30 ng/mL of M-CSF and 1 ng/mL of RANKL. The TRAP staining at 72 h revealed that LPS alone, IFN-γ alone and their combination decreased osteoclastogenesis (Fig. 5E,F). The inhibitory effect of LPS on osteoclastogenesis at the beginning of the culture *in vitro* has been reported in previous studies (13).

Interferon-γ accelerates LPS-induced osteoclast formation

In the following experiments, we decided to stimulate bone marrow macrophages with LPS and/or IFN-γ at 48 h because we found a reduction of the IFN-γ suppressive effect after 48 h of RANKL stimulation. We used RANKL at a concentration of 1 ng/mL because gingival crevicular fluids from patients with chronic periodontitis were found to contain approximately 1 ng/mL of RANKL (24). Osteoclast formation was accelerated by addition of LPS at concentrations above 0.5 ng/mL, but not by the addition of 0.1 ng/mL of LPS (Fig. 6A,B). These results are consistent with results of other studies showing that LPS up-regulated osteoclast formation from RANKL-pretreated osteoclast precursors (13). To determine the role of IFN-γ in LPS-induced osteoclast formation, we added IFN-γ alone or together with LPS at 48 h by performing dose-dependent experiments. We found that IFN-γ alone did not induce osteoclast formation even at a high dose (1000 ng/mL; data not shown). These results are in agreement with previous studies showing that IFN-γ did not have any effect on osteoclast formation from RANKL-stimulated osteoclast precursors *in vitro* (5,6); however, combination of LPS and IFN-γ was not tested in those studies. Interestingly, we found that the combined addition of IFN-γ and LPS resulted in very clear induction of osteoclast formation (Fig. 6C,D). When 1 or 10 ng/mL of LPS was used,

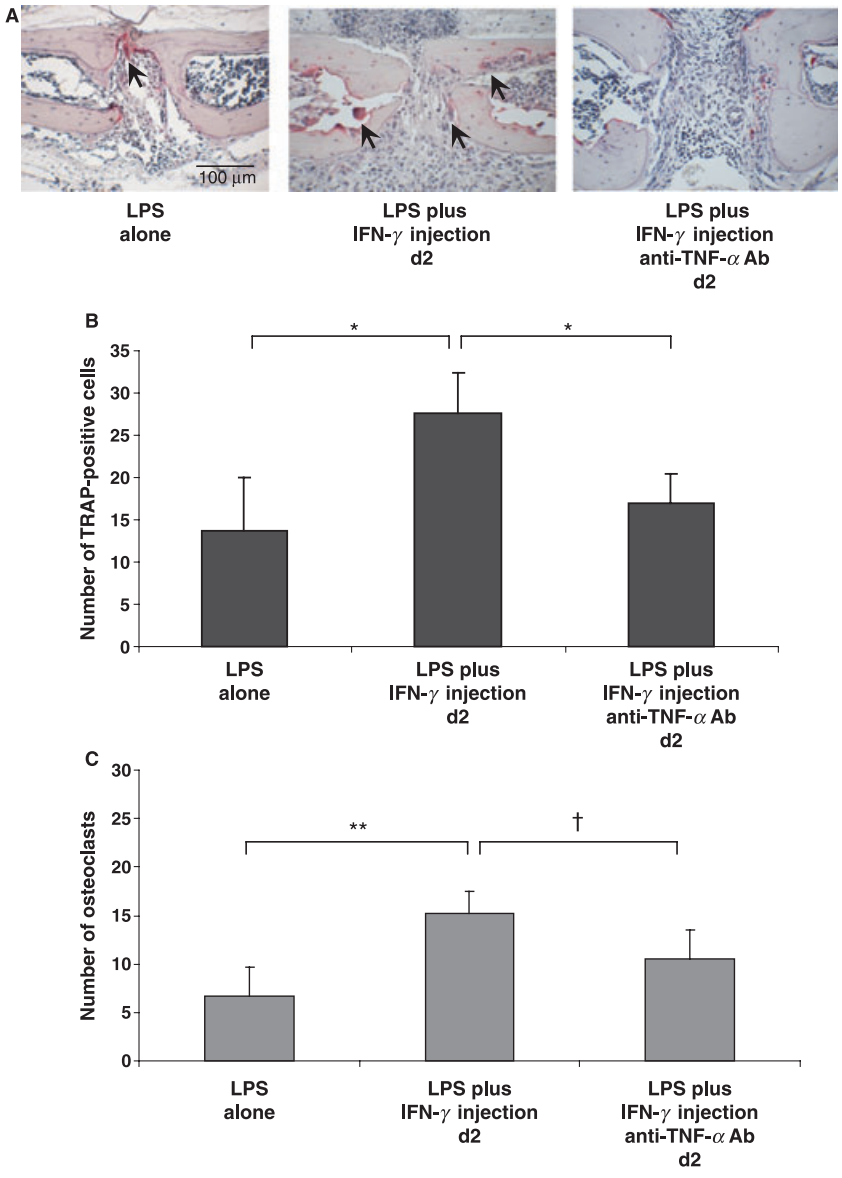

Fig. 4. Effect of anti-TNF-α antibody on acceleration of LPS-induced osteoclast formation by IFN-γ *in vivo*. (A) Histopathological findings (staining with TRAP) in calvarial bone from mice injected with LPS alone, mice injected with IFN-γ on day 2 after LPS injection, and mice injected with IFN-γ together with anti-TNF-α antibody on day 2 after LPS injection. Anti-TNF-α antibody reduced both the numbers of TRAP-positive cells and osteoclasts induced by IFN-γ. (B,C) Numbers of TRAP-positive cells (B) and osteoclasts (C) in mice injected with LPS alone, mice injected with IFN-γ on day 2 after LPS injection, and mice injected with IFN-γ together with anti-TNF-α antibody on day 2 after LPS injection. Osteoclasts were considered to be TRAP-positive cells containing two or more nuclei. Bars represent means + SD; †$p < 0.05$, *$p < 0.01$, **$p < 0.001$.

many osteoclasts were formed and reached an almost saturated condition in the well without IFN-γ. Therefore, we did not evaluate the effect of IFN-γ using a high concentration of LPS. Osteoclast formation was reduced by a high concentration (10 ng/mL) of IFN-γ when we used LPS at a concentration of 10 ng/mL. In order to determine the functionality of the osteoclasts induced by IFN-γ in combination with LPS, we performed a resorption activity assay. On microscopic observation it could be seen that osteoclasts from LPS conditions induced the formation of resorption pits; however, the number of pits was not significantly different from control conditions (RANKL alone). The combination of LPS and IFN-γ induced a significant formation of resorption pits compared with control conditions, but there was no significant difference compared with LPS alone (Fig. 6E,F).

Interferon-γ-accelerated osteoclast formation is blocked by anti-TNF-α antibody *in vitro*

In order to examine TNF-α involvement, we checked whether IFN-γ alone or in combination with LPS induced the expression of TNF-α mRNA and protein levels in bone marrow macrophages pretreated with RANKL for 48 h. After 3 h of stimulation, IFN-γ or LPS alone could induce a two- or 3.5-fold increase in TNF-α mRNA expression, respectively, compared with control cultures (RANKL alone). Interferon-γ together with LPS could induce a six-fold increase compared with control cultures (Fig. 7A). These data show that IFN-γ and LPS have a synergistic effect on TNF-α mRNA expression. In contrast, LPS could induce TNF-α protein levels, whereas IFN-γ did not cause any effect. Interferon-γ in combination with LPS failed to induce TNF-α protein levels (Fig. 7B). However, the addition of anti-TNF-α antibody (5 μg/mL) almost completely inhibited the acceleration of osteoclast formation by IFN-γ, similar to our *in vivo* experiment. Normal goat IgG used in control experiments did not affect osteoclastogenesis (Fig. 7C,D).

Discussion

Our *in vivo* data demonstrated that local injection of IFN-γ into mice calvaria 2 d after LPS injection, but not that at the same time as LPS injection, accelerated the formation of osteoclasts. We found that TRAP-positive mononucleated cells were also increased to a similar extent to the osteoclasts by IFN-γ stimulation. This finding suggests that IFN-γ not only accelerates osteoclast formation from TRAP-positive mononucleated cells, but also accelerates the formation of

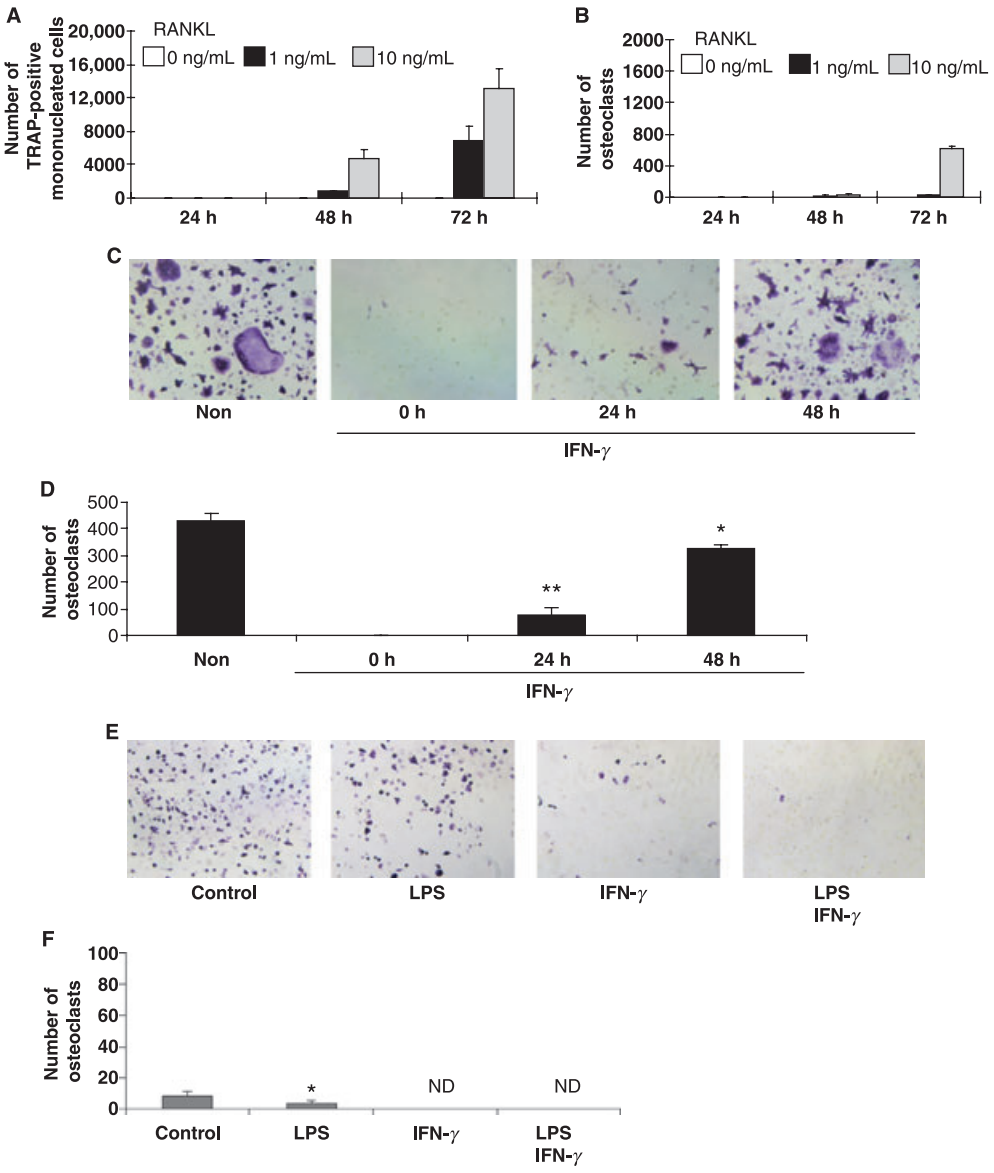

Fig. 5. Effect of IFN-γ on RANKL-induced osteoclast formation in a time-dependent manner *in vitro*. (A,B) Bone marrow macrophages were cultured with M-CSF (30 ng/mL) and RANKL (0, 1 or 10 ng/mL). TRAP staining was performed at 24, 48 and 72 h. TRAP-positive cells with three or more nuclei, considered as osteoclasts, were counted. (A) Numbers of TRAP-positive mononucleated cells. (B) Numbers of osteoclasts. (C,D) Bone marrow macrophages were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) without IFN-γ (non) and with IFN-γ (1 ng/mL) added at 0, 24 and 48 h. At 72 h, TRAP staining was performed to determine osteoclasts. The TRAP-positive cells with three or more nuclei, considered as osteoclasts, were counted. Representative photographs (C) and number of osteoclasts (D). Bars represent means + SD; $*p < 0.01$, $**p < 0.001$ compared with IFN-γ nontreated group. Data are representative of three independent experiments. (E,F) Bone marrow macrophages were stimulated with RANKL (1 ng/mL), LPS (0.1 ng/mL) and/or IFN-γ (1 ng/mL) in the presence of M-CSF (30 ng/mL). Lipopolysaccharide alone and together with IFN-γ inhibited osteoclast formation. At 72 h, TRAP staining was performed to determine osteoclasts. Representative photographs (E) and number of osteoclasts F). Bars represent means + SD; $*p < 0.05$ compared with control. ND, not detectable.

TRAP-positive cells from osteoclast precursors. We found an increase in RANKL expression with a subsequent increase in TRAP-positive mononuclear and multinucleated cells on day 2 after LPS injection. It has been reported that IFN-γ does not inhibit osteoclastogenesis in previously RANKL-stimulated pre-osteoclasts (5,6). These findings suggest that LPS induction in RANKL expression is involved in IFN-γ acceleration of pre-osteoclast and osteoclast formation. Promotion of osteoclastogenesis by IFN-γ is in agreement with results of previous studies showing an association between the expression of this cytokine and severe bone resorption in periodontitis patients (17). Interferon-γ has been shown to be associated with severe alveolar bone resorption in

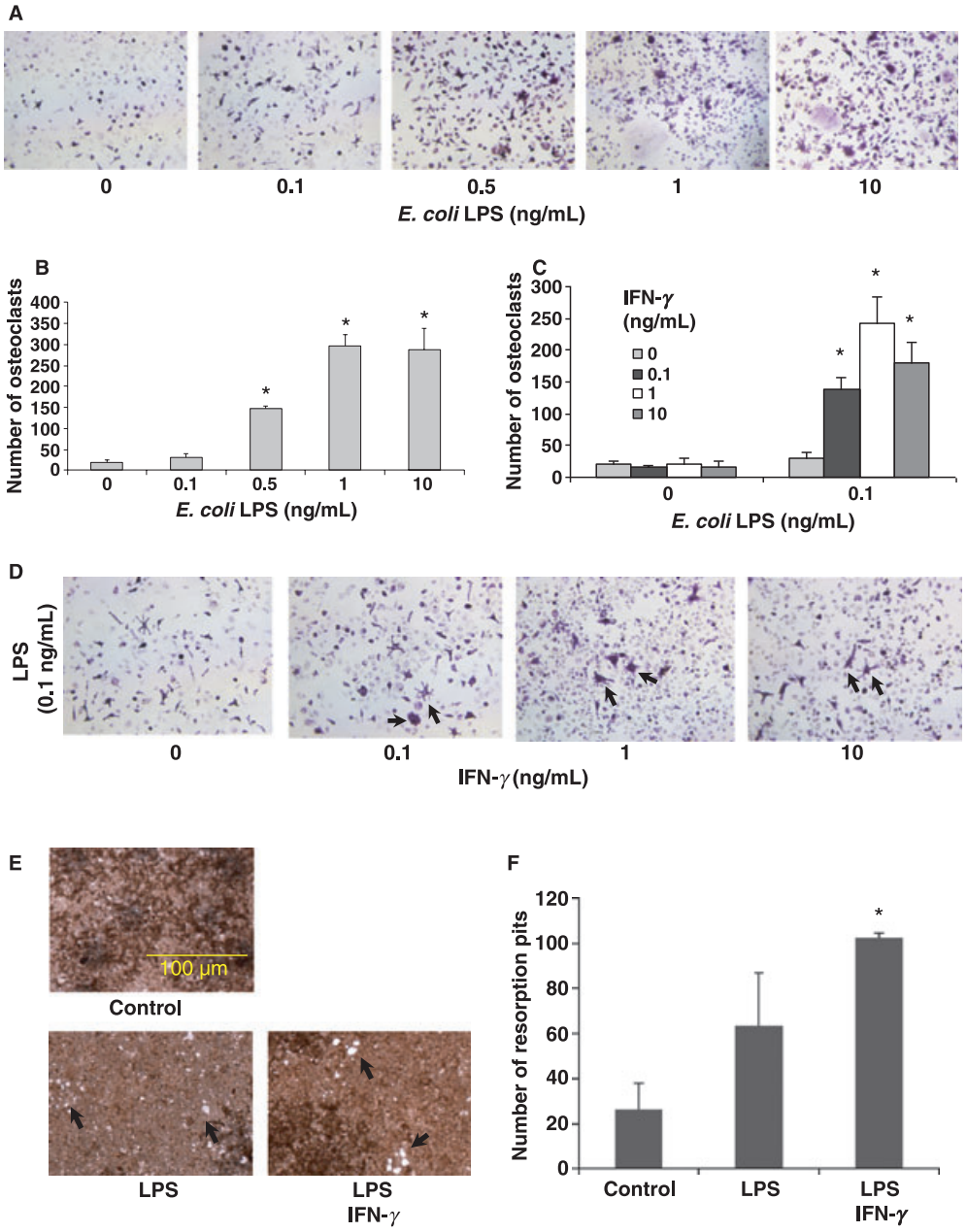

Fig. 6. Interferon-γ accelerates LPS-induced osteoclast formation. (A,B) Bone marrow macrophages were pretreated with RANKL (1 ng/mL) in the presence of M-CSF (30 ng/mL) for 48 h and then stimulated with LPS at different doses as indicated for 24 h. At 72 h, TRAP staining was performed to determine osteoclasts. Representative photographs (A) and vumbers of osteoclasts (B). *$p < 0.01$ compared with the group without LPS. (C,D) Bone marrow macrophages were pretreated with RANKL for 48 h and then stimulated with LPS alone or in combination with IFN-γ at different doses for 24 h. in the presence of LPS IFN-γ enhances the number of osteoclasts derived from RANK-treated bone marrow macrophages. At 72 h, TRAP staining was performed to determine osteoclasts. Numbers of osteoclasts (C) and representative photographs (D). Bars represent means + SD; *$p < 0.01$ compared with LPS alone. Data are representative of three independent experiments. (E,F) Bone marrow macrophages were pretreated with RANKL for 48 h (control; RANKL 1 ng/mL) and then stimulated with LPS at 0.1 ng/mL with or without IFN-γ at 0.1 ng/mL. After 48 h, medium and reagents were renewed. Von Kossa staining was performed after 72 h to count the number of pits per disc. Photographs (E) and numbers of resorption pits (F). Bars represent means + SD; *$p < 0.01$ compared with control.

LPS-induced or infection-induced bone loss *in vivo* (11,12,18). Bacterial infection and LPS have been reported to accelerate RANKL production (24,25). Therefore, we speculate that the acceleratory action of IFN-γ on day 2 is associated with an increase in RANKL expression induced by LPS.

We injected IFN-γ to observe its local biological effect on osteoclast formation in LPS-induced inflammatory bone loss *in vivo* and we found that

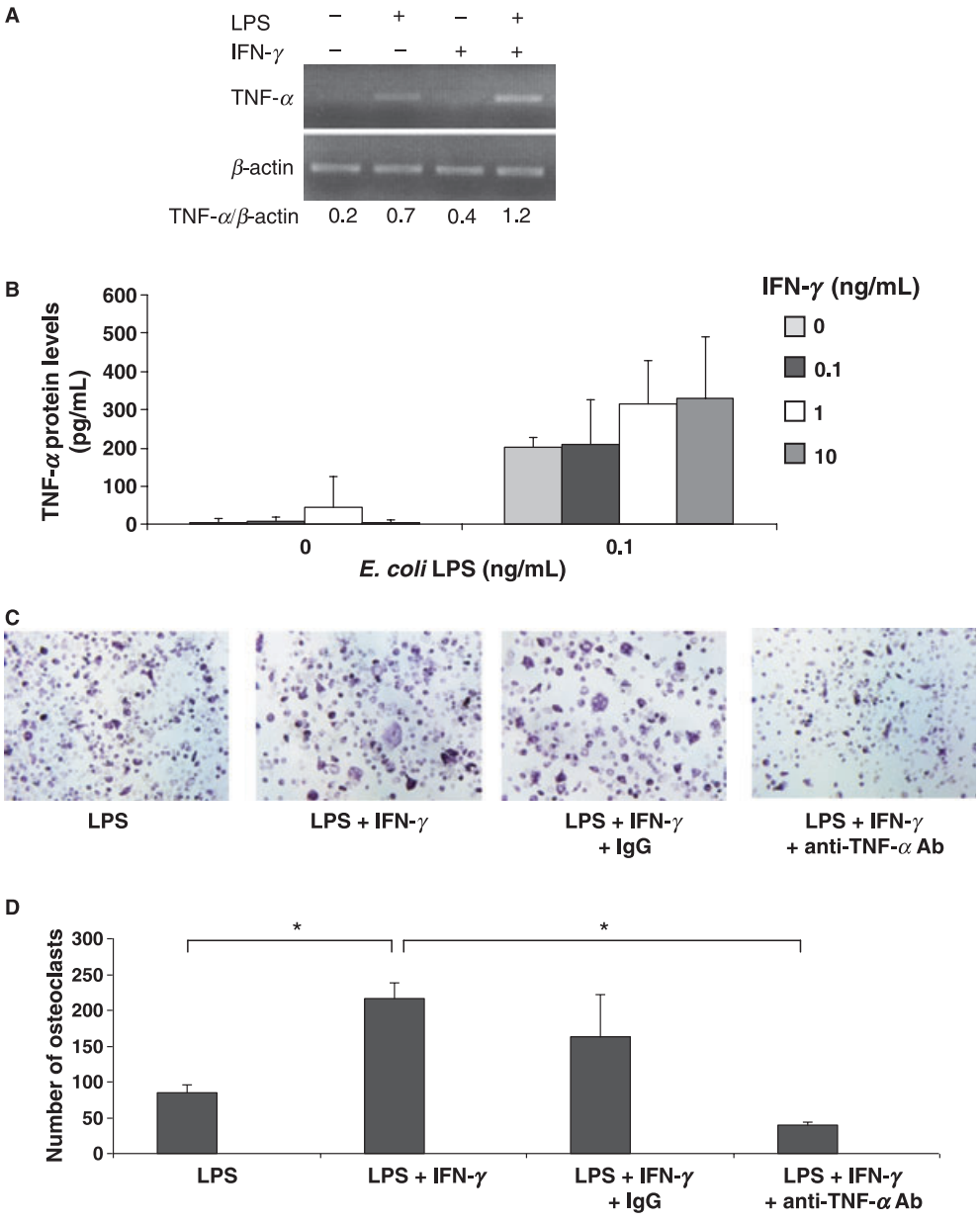

Fig. 7. Blocking TNF-α suppresses IFN-γ-induced osteoclast formation. (A) Tumor necrosis factor-α mRNA expression. Representative scanned photographs of TNF-α PCR products. The resulting ratios were normalized to β-actin mRNA, which is constitutively expressed. Total mRNA was collected after the lysis of bone marrow macrophages that were pretreated with RANKL at 1 ng/mL and M-CSF at 30 ng/mL for 48 h and stimulated with 0.1 ng/mL of LPS and/or 0.1 ng/mL of IFN-γ for an additional 3 h. Interferon-γ combined with LPS enhanced TNF-α mRNA expression compared with LPS alone. (B) Detection of TNF-α protein levels. Bone marrow macrophages were pretreated with RANKL and then stimulated with LPS alone or in combination with IFN-γ at different doses for 24 h. At 72 h, supernatants were collected to perform ELISA. Data are representative of two independent experiments. Bars represent means + SD. (C,D) After 48 h of RANKL (1 ng/mL) stimulation, bone marrow macrophages were treated with LPS (0.1 ng/mL) alone or in combination with IFN-γ (1 ng/mL) in the presence or absence of control goat IgG or anti-TNF-α antibody for 24 h. TRAP staining was performed at 72 h. Representative photographs (C) and number of osteoclasts (D). Bars represent means + SD; $*p < 0.01$. Data are representative of two independent experiments.

IFN-γ had an acceleratory action when injected at a later time but not at the beginning of bone resorption induced by LPS injection. In contrast to our results, Takayanagi *et al.* (2) found a protective role of IFN-γ from the beginning of bone loss induction by LPS in mice calvaria. The discrepancy in results might also be explained by the animal conditions. While Takayanagi *et al.* used IFN-γ receptor-deficient mice, we used normal mice. Knockout conditions could affect the response of macrophages to IFN-γ, leading to a defective elimination of LPS. This

may result in persistent production of cytokines, such as TNF-α, with a subsequent exacerbation of bone destruction (26). This effect was avoided in our *in vivo* experiment.

We observed that injection of IFN-γ at the same time as LPS did not inhibit the bone resorption induced by LPS *in vivo* even though IFN-γ completely inhibited osteoclastogenesis from untreated bone marrow macrophages *in vitro*. We believe this phenomenon occurs because osteoclast precursors in calvaria might be more differentiated toward osteoclasts compared with bone marrow macrophages. In fact, we detected a few mononucleated TRAP-positive cells in calvarial control tissues. TRAP is one of the osteoclast differentiation markers, and TRAP-positive mononucleated cells are defined as prefusion osteoclasts (27). Many TRAP-positive mononucleated cells were detected in calvaria tissue on day 2 after LPS injection *in vivo*. Many bone marrow macrophages treated for 48 h with RANKL also expressed TRAP *in vitro*. These *in vivo* and *in vitro* cells are thought to have similar conditions by judging from the increase in TRAP expression. Moreover, in these conditions, IFN-γ accelerated osteoclast formation *in vivo* and *in vitro*. Thus, the presence of many prefusion osteoclasts differentiated from osteoclast precursors is important for IFN-γ to exert its acceleratory action.

To investigate the role of IFN-γ in LPS-involved osteoclastogenesis further, we induced osteoclasts from bone marrow macrophages *in vitro*. Interferon-γ alone did not increase the number of osteoclasts from RANKL pretreated bone marrow macrophages. Our results show that stimulation with IFN-γ together with LPS at a low concentration accelerated osteoclastogenesis from bone marrow macrophages pretreated with RANKL. There was an approximately 2.5- to three-fold increase in osteoclast formation compared with that in the case of LPS alone. These osteoclasts showed functional activity, as they were able to induce significant formation of resorption pits compared the the control cultures, but there was no significant difference compared with LPS alone. It should be pointed out that LPS, IFN-γ or their combination did not generate osteoclasts from untreated bone marrow macrophages. Pretreatment with RANKL was necessary for acceleration in our culture system *in vitro*. In agreement with our *in vivo* results, RANKL also plays a key role in the accelerative effect of IFN-γ *in vitro*. It has been reported that RANKL-treated cells have increased mRNA expression levels of osteoclastogenic factors, such as RANK, TRAP, α_v integrin, nuclear factor of activated T cells c1, cathepsin K and interleukin-1 receptor compared with untreated cells (5). Zou & Bar-Shavit (13) reported that LPS induced RANKL-mediated osteoclastogenesis when it was added at a later period of culture, but not when it was added at the beginning of culture. Therefore, RANKL pretreatment of bone marrow macrophages would be important for IFN-γ-induced osteoclastogenesis *in vitro*.

The acceleration of LPS-involved osteoclastogenesis by IFN-γ may be explained by direct and indirect actions on osteoclast precursors *in vivo* and RANKL-treated bone marrow macrophages *in vitro*. Takayanagi *et al.* (2) reported that IFN-γ inhibits osteoclastogenesis via activation of signal transducer and activator of transcription 1 (Stat 1). However, LPS may interfere with the IFN-γ-induced inhibition of osteoclastogenesis, as LPS pretreatment has been reported to suppress IFN-γ-activated Stat1 in macrophages through the production of suppressor of cytokine signaling 3 (28). In addition, IFN-γ may enhance the activation of RANK or Toll-like receptor 4 (LPS receptor) signaling factors, such as p38MAPK and activator protein 1 (29–33), or the activation of additional osteoclastogenic factors directly in osteoclast precursors. Alternatively, IFN-γ may induce an endocrine TNF-α production from osteoclast precursors and TNF-α production may lead to an enhancement of osteoclast formation. An indirect action may involve the induction of TNF-α production from other cells, such as T cells or B cells, found to be < 5% of our bone marrow macrophage population *in vitro*. Fibroblasts, osteoblasts, lymphocytes and mature macrophages may also produce TNF-α in response to IFN-γ *in vivo* (19). Tumor necrosis factor-α is implicated in LPS-involved osteoclastogenesis and LPS-induced bone loss in calvaria (15–16). Furthermore, Lam *et al.* (34) reported that TNF-α could induce osteoclast formation from cells that were previously stimulated with RANKL. In the present study, we found that IFN-γ and LPS synergistically induced TNF-α mRNA expression compared with control conditions *in vitro*. In contrast, IFN-γ did not significantly increase the induction of TNF-α protein levels in response to LPS. However, we consider that the tendency to increase TNF-α protein levels may accelerate osteoclastogenesis in the conditions of close cell-to-cell contact *in vitro* even if there was no significant difference. In fact, anti-TNF-α antibody blocked the acceleration of osteoclast formation by IFN-γ *in vivo* and *in vitro*. Taken together, our data show that TNF-α may be involved in the acceleration of osteoclastogenesis by IFN-γ. The molecular mechanism of acceleration of osteoclastogenesis induced by LPS together with IFN-γ was not fully elucidated in the present study. Further experiments need to be performed to examine the activation of additional osteoclastogenic factors induced by IFN-γ in the presence of both RANKL and LPS.

In bone inflammatory diseases in which LPS plays an important role, such as periodontitis and osteomyelitis, a variety of immune cells migrate and produce cytokines, such as RANKL and IFN-γ, in response to LPS. Additionally, innate immune cells, including neutrophils and macrophages, that control infection by LPS internalization may modulate LPS local concentrations (35). Interferon-γ may have dual effects on osteoclastogenesis, an inhibitory effect and an acceleratory effect. The stimulation of osteoclasts precursors with RANKL and the local concentrations of LPS will determine the action of IFN-γ. Interferon-γ may exert an inhibitory effect on osteoclastogenesis from osteoclast precursors

that have not been stimulated with RANKL. In contrast, IFN-γ may also accelerate osteoclastogenesis from osteoclast precursors stimulated with RANKL and low levels of LPS. Thus, an LPS-associated inflammatory condition may facilitate the promotion of osteoclast formation and bone destruction by IFN-γ. Promotion of osteoclastogenesis is in line with results of previous studies showing an association between the expression of IFN-γ and severe bone resorption in periodontitis (17). Interferon-γ not only inhibits osteoclastogenesis; it can also accelerate it in certain conditions. Taken together, our results show that local IFN-γ plays an accelerative role in osteoclast formation when cells are stimulated with LPS and RANKL. Tumor necrosis factor-α may be involved in this acceleration. Thus, our findings suggest that the use of IFN-γ in bone loss inhibitor therapy for LPS-involved bone diseases should be reconsidered.

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