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ORIGINAL ARTICLE

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Murine osteoblasts respond to LPS and IFN-γ similarly to macrophages

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Abstract Osteoblasts are bone-forming mesenchymal cells, while macrophages are cells of hematopoietic origin responsible for innate immunity. Lipopolysaccharide (LPS) can induce tolerance in macrophages, whereas interferon (IFN)-γ can activate macrophages to produce cytokines, exert bactericidal effects, and present antigens. In this study, we examined such macrophagic phenotypes regulated by LPS and IFN-γ in murine osteoblasts. In both primary calvarial osteoblasts and osteoblastic MC3T3-E1 cells, LPS pretreatment resulted in reduced production of IL-6 in response to a subsequent LPS stimulation or to *Salmonella* infection, indicating the existence of LPS-induced tolerance in osteoblasts. Furthermore, IFN-γ treatment of MC3T3-E1 cells resulted in both enhanced IL-6 production in response to LPS and upregulation of major histocompatibility complex class II (MHC II). Following infection, *Salmonella*-containing vacuoles (SCVs) were formed in MC3T3-E1 cells, and IFN-γ pretreatment enhanced bactericidal effects on intracellular *Salmonella*. Taken together, these observations indicate that osteoblasts can exhibit a subset of phenotypes reminiscent of macrophages in the course of bacterial infection.

Key words LPS · IFN-γ · tolerance · IL-6 · MHC class II

Introduction

Osteomyelitis is bone inflammation resulting from infection [1,2]. Bone can be invaded by bacteria and other microorganisms derived from open wounds or through the bloodstream. The most common causative bacteria of osteomyelitis by either route is *Staphylococcus aureus*. In addition, *Mycobacteria tuberculosis*, *Salmonella* spp., and *Pseudomonas aeruginosa* can infect bones and bone marrow. Bones contain several cell types, including chondrocytes, osteoblasts, and osteoclasts, all of which can be exposed to pathogens in osteomyelitis. Involvement of *Salmonella* spp. is often associated with patients suffering from sickle cell anemia [3]. If untreated, infection can become chronic, cause loss of the blood supply to the affected area, and lead to destruction and death of the bone.

In infected bone, macrophages kill bacteria by phagocytosis and produce proinflammatory cytokines via signaling pathways downstream of Toll-like receptors. For example, lipopolysaccharide (LPS) activates NF-κB through Toll-like receptor 4 (TLR4) signaling, resulting in cytokine production and increased bactericidal activities [4]. On the other hand, in macrophages, cytokine production is suppressed after preexposure to low concentrations of LPS, a phenomenon known as LPS-induced tolerance. Such tolerance is thought to protect the host from pathological hyperactivation of the innate immune system during infection. Macrophages also activate T cells by secreting proinflammatory cytokines, presenting antigens, and providing costimulatory signals to T cells. Presentation of bacterial antigen is accomplished by molecules such as the major histocompatibility complex class II (MHC II), whereas members of the B7 family such as B7-1/CD80 and B7-2/CD86 function in T-cell costimulation [5].

Osteoblasts differentiate from mesenchymal precursors and form bone by producing bone matrix proteins, whereas osteoclasts differentiate from myeloid precursors and resorb bone [6]. Osteoblasts regulate bone resorption by expressing RANKL and macrophage colony stimulation factor (M-CSF), which are essential for osteoclast differentiation. Osteoblasts also secrete osteoprotegerin (OPG), a decoy RANKL receptor inhibiting its osteoclastogenic activity throughout the body [7–9]. Recently, however, it has been shown that osteoblasts can respond to inflammatory stimuli in a manner similar to macrophages. For example, murine osteoblasts express TLR4, which recognizes bacterial LPS [10,11]. Stimulation of Toll-like receptors on osteoblasts by bacteria results in interleukin (IL)-6 production [12]. Because IL-6 positively regulates osteoclast differen-

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tiation, increased IL-6 production in response to bacterial infection enhances osteoclast differentiation, leading to bone destruction [13]. Besides osteoblasts, activated T cells also affect bone resorption by expressing RANKL and interferon (IFN)-γ. In particular, IFN-γ strongly suppresses osteoclastogenesis [14]. However, litter is known about the effects of LPS and IFN-γ on osteoblast function. Recent in vitro studies show that bacteria can enter murine osteoblasts [15]. Moreover, few morphological studies of intracellular bacteria in osteoblasts or functional studies of bactericidal effects of osteoblasts have been reported. In this study, we show that osteoblasts exhibit a subset of macrophagic phenotypes when exposed to LPS, bacteria, or IFN-γ.

Materials and methods

Cells

MC3T3-E1 and RAW264.7 cells were obtained from American Type Culture Collection (ATCC). Primary calvarial osteoblasts and mouse embryonic fibroblasts were prepared as described [16,17]. Cells were grown in α-minimum essential medium (Sigma) supplemented with 10% fetal calf serum (FCS) and antibiotics and cultured in 48-well plates (Falcon).

Bacterial strain and infection

Overnight standing cultures in Luria–Bertani broth of *Salmonella enterica* serovar Typhimurium, strain χ3306 [18], were diluted and shaken, and midlog-phase bacteria were collected by centrifugation. Bacteria were washed with phosphate-buffcred saline (PBS), diluted in Hanks salt solution, and used to inoculate osteoblasts or macrophages at the indicated multiplicity of infection (MOI). Cultures were incubated at 37°C, washed with PBS to remove extracellular bacteria, and incubated at 37°C in complete media containing 25 μg/ml gentamycin (Gibco). Culture supernatants were then harvested for a cytokine assay. In some experiments, cells were homogenized in 0.1% sodium deoxycholate (Sigma) in water and plated on agar plates containing nalidixic acid (Nacalai) to count colony-forming units (CFU).

Cytokine assays

MC3T3-E1 cells and primary osteoblasts were plated at a density of $3–5 \times 10^4$/well in 48-well plates. Cells were stimulated with LPS (S. Minnesota Re595; Sigma), *Salmonella*, or IFN-γ. IL-6 levels in culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) sets (BD). In some experiments, supernatant OPG and prostaglandin E_2 (PGE_2) levels were also measured using ELISA kits (R&D). The COX-2 inhibitor NS-398 was purchased from Cayman Chemical.

LPS-induced tolerance

Cells were pretreated with different concentrations of LPS for 24 h. Cultures were subsequently washed twice with PBS, and then cells were stimulated with LPS or *Salmonella* (MOI = 10) as indicated. After incubation at 37°C, culture supernatants were harvested for a cytokine assay.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Cells were collected and homogenized in Isogen (Nippon Gene), and total RNA was isolated. cDNA was synthesized using the Enhance Avian HS RT-PCR kit (Sigma). Quantitative PCR for IL-6 and *Gapdh* was performed on an ABI PRISM 7000 using TaqMan Assay-on-demand (Applied Biosystems).

IFN-γ stimulation assay

Cells were pretreated with or without indicated concentrations of IFN-γ (Sigma) for 24 h. Cultures were subsequently washed twice with PBS and then stimulated with LPS or *Salmonella* (MOI = 100) as indicated. After incubation at 37°C, culture supernatants were harvested for an IL-6 assay.

Transmission electron micrographs

For transmission electron microscopy, cells were infected for 3 h and then fixed for 1 h with 2.5% glutaraldehyde/PBS. Cells were then washed three times with PBS and postfixed in 1% OsO_4 in Sorensen's buffer for 1 h. Samples were subsequently dehydrated in ethanol and flat-embedded in epoxy resin (Agar100). Thin sections (60–80 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate. Sections were viewed with a 120 kV in JEM1230 electron microscope (JEOL).

FACS analysis

Fluorescein isothiocyanate (FITC)-labeled antimouse TLR4-MD2 complex rat monoclonal antibody (clone MTS510, MBL), FITC-labeled antimouse MHC II rat monoclonal antibody (clone ER-TR3; BMA Biomedicals), phycoerythrin (PE)-labeled antimouse CD80 Armenian hamster, and CD86 rat antibodies (BD) were used. Cells were stained with FITC- or PE-conjugated antibodies in PBS-10% FBS, washed, and analyzed on a FACScan using the CELLQuest program (BD).

Results

OPG and IL-6 production by osteoblasts

To determine whether osteoblasts respond to activators of macrophages such as IFN-γ and LPS, we asked whether

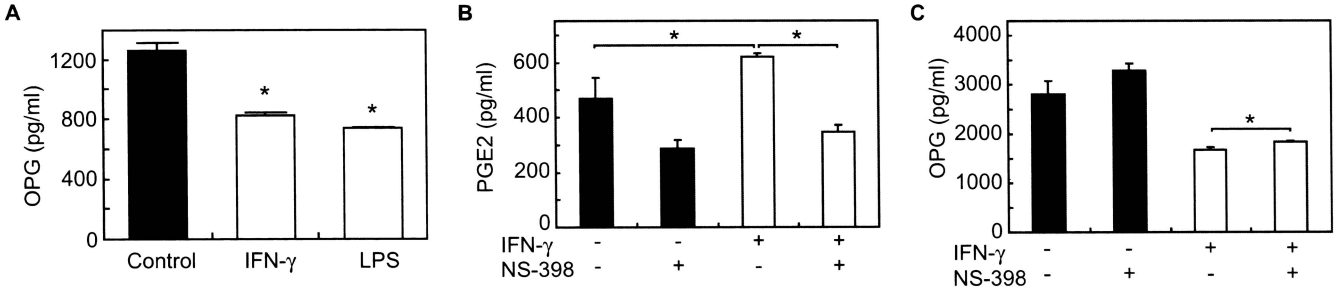

Fig. 1. Effect of interferon-γ (*IFN-γ*) or lipopolysaccharide (*LPS*) treatment on osteoprotegerin (*OPG*) production in osteoblasts. **A** MC3T3-E1 cells were stimulated with medium alone (control, *closed bar*), 100 ng/ml IFN-γ, or 500 ng/ml LPS for 24 h. Supernatants were harvested, and OPG levels were quantified by enzyme-lined immunosorbent assay (ELISA). **B, C** MC3T3-E1 cells were treated with 300 ng/ml IFN-γ or 1 μM NS-398 where indicated for 28 h ($n = 3$ culture wells). Supernatants were harvested, and protaglandin E_2 (PGE_2) (**B**) and OPG (**C**) levels were quantified by ELISA. *Error bars* represent means ± SEM ($n = 3$ culture wells). *$P < 0.05$

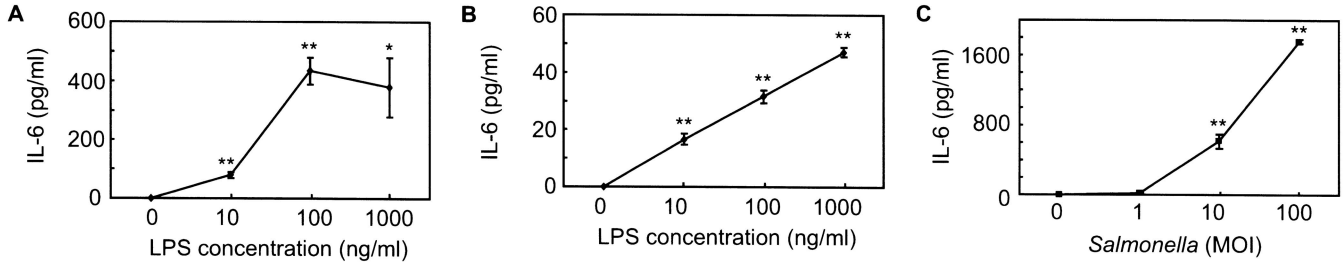

Fig. 2. Interleukin-6 (*IL-6*) production by osteoblasts. **A** MC3T3-E1 cells were stimulated for 6 h with increasing concentrations of LPS, and IL-6 levels in supernatants ($n = 2$) were measured. **B** Primary osteoblasts were analyzed as in **A**. **C** MC3T3-E1 cells were infected with *Salmonella* at the indicated multiplicity of infection (*MOI*) for 30 min. After 3 h, IL-6 levels in supernatants ($n = 3$) were measured as in **A**. *Error bars* represent means ± SEM. *$P < 0.05$, **$P < 0.01$

production of OPG, an antiosteoclastogenic cytokine secreted by osteoblasts, is affected by IFN-γ or LPS treatment in murine preosteoblastic MC3T3-E1 cells. To this end, we measured OPG levels in culture supernatants by ELISA after stimulating cells with IFN-γ or LPS for 24 h. In both cases, OPG levels were significantly lower than those seen in unstimulated controls (Fig. 1A). To examine possible involvement of PGE_2 in suppression of OPG levels [19], we measured PGE_2 levels in culture supernatants after IFN-γ stimulation. Consistent with previous studies [20], we found that PGE_2 levels were slightly elevated by IFN-γ and suppressed by NS-398, a selective inhibitor of cyclooxygenase-2 (COX-2) (Fig. 1B). Importantly, NS-398 treatment produced a small but statistically significant increase in OPG levels in MC3T3-E1 cells, suggesting that suppression of OPG production in osteoblasts is mediated in part by PGE_2 (Fig. 1C).

We next asked whether osteoblasts produce IL-6 in response to *Salmonella*-derived LPS stimulation by quantifying IL-6 levels in culture supernatants. We observed that MC3T3-E1 cells produced IL-6 in response to LPS in a dose-dependent manner (Fig. 2A). Primary calvarial osteoblasts also showed LPS-induced elevation of IL-6 levels (Fig. 2B). Furthermore, MC3T3-E1 cells showed increased levels of IL-6 as the MOI with *Salmonella* increased (Fig. 2C). These observations show that osteoblasts can produce IL-6 during bone infection.

LPS tolerance in osteoblasts

Next, we determined whether LPS-induced tolerance occurs in osteoblasts. We first pretreated MC3T3-E1 cells with 0, 1, or 10 ng/ml LPS for 24 h and then stimulated cells with 1 μg/ml LPS for another 3 h. LPS-induced IL-6 production in osteoblasts was suppressed both at the protein and RNA levels when cells were pretreated with 10 ng/ml LPS (Fig. 3A, B). LPS-induced tolerance was similarly observed in primary calvarial osteoblasts (Fig. 3C, D). Furthermore, LPS-induced tolerance was observed, although less prominently, when the second LPS treatment was replaced with *Salmonella* infection of MC3T3-E1 cells (Fig. 3E). Such LPS-induced tolerance was not limited to osteoblasts, because mouse embryonic fibroblasts exhibited similar tolerance (Fig. 3F). These data indicate that LPS-induced tolerance in terms of IL-6 production occurs in osteoblasts.

IFN-γ enhances IL-6 production and macrophagic phenotypes in osteoblasts

We next examined the effects of IFN-γ on IL-6 production in MC3T3-E1 cells. IFN-γ pretreatment resulted in enhanced production of IL-6 in response to secondary LPS stimulation (Fig. 4A). Similar effects were observed when LPS was replaced with *Salmonella* as the stimulus (Fig. 4B).

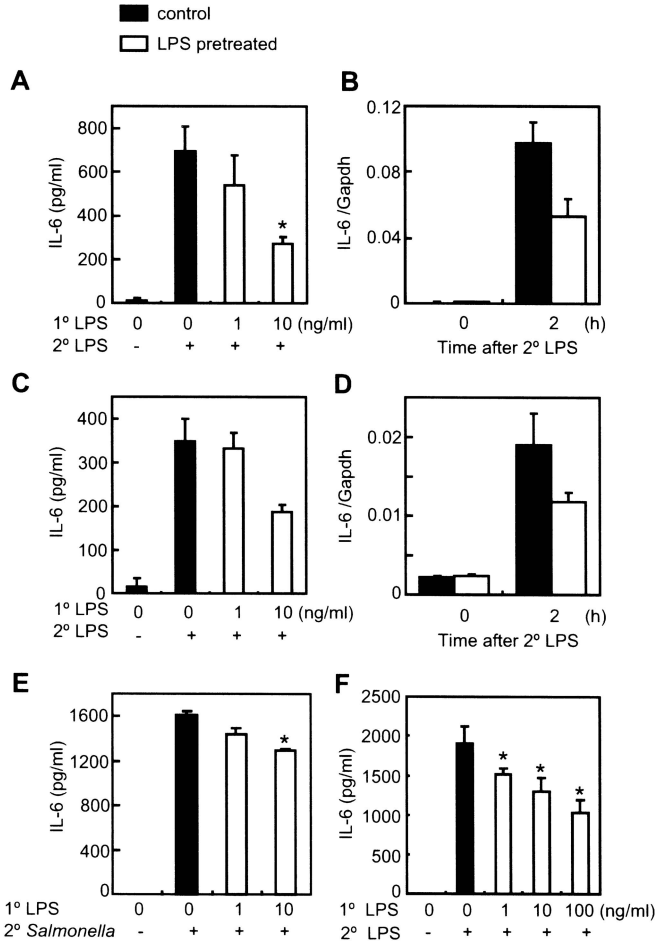

Fig. 3. LPS-induced tolerance in osteoblasts. **A** MC3T3-E1 cells were pretreated with indicated concentrations of LPS for 24 h (*1°*). Cultures were replaced with medium containing 1 μg/ml LPS (*2°*). After 3 h, IL-6 protein levels in culture supernatants ($n = 2$ culture wells) were measured. **B** MC3T3-E1 cells were pretreated without (*closed bars*) or with (*open bars*) 10 ng/ml LPS for 24 h, stimulated with 1 μg/ml LPS, and harvested at 0 and 2 h. IL-6 mRNA levels were determined by reverse transcription-polymerase chain reaction (RT-PCR). **C, D** LPS-induced tolerance in primary osteoblasts. The same experiments as in **A** and **B** were performed using primary osteoblasts. **E** MC3T3-E1 cells were pretreated with LPS as in **A** and infected with *Salmonella*. After 3 h, culture supernatants were harvested ($n = 2$) and IL-6 levels were measured as in **A**. IL-6 levels in culture supernatants ($n = 3$ culture wells) were determined. *Error bars* represent means ± SEM. At least two independent experiments gave similar results. $^*P < 0.05$

LPS-induced IL-6 production was also enhanced in mouse embryonic fibroblasts by IFN-γ pretreatment (Fig. 4C). These data indicate that IFN-γ enhances LPS-induced IL-6 production in osteoblasts and in fibroblasts, which, similar to osteoblasts, are differentiated from mesenchymal stem cells.

To examine the effect of IFN-γ on the number of bacterial CFU in MC3T3-E1 cells, cells were pretreated with medium alone or with 50 or 500 ng/ml IFN-γ for 24 h. Cells were then infected with *Salmonella* and the CFU number in MC3T3-E1 cells was determined. CFU counts decreased in response to IFN-γ treatment in a dose-dependent manner (Fig. 5A). Consistent with previous reports [21], IFN-γ enhanced *Salmonella* killing activity of RAW264.7 macrophages at lower doses than those required to activate osteoblasts (Fig. 5B). To assay for the presence of intracellular *Salmonella*, we examined infected MC3T3-E1 cells by transmission electron microscopy. We observed the presence of *Salmonella*-containing vacuoles (SCVs) [22] (Fig. 5C, D), as seen in macrophages (Fig. 5D, inset).

To examine the effect of IFN-γ and LPS on expression of MHCII, CD80, CD86, and the TLR4-MD2 complex, MC3T3-E1 cells were treated with medium alone, LPS, or IFN-γ for 1 or 4 days and analyzed by fluorescence-activated cell sorter (FACS). No significant difference was detected in expression levels of CD80, CD86, or the TLR4-MD2 complex following any of these treatments. However, IFN-γ treatment significantly upregulated MHC II expression levels in MC3T3-E1 cells, as is seen in macrophages (Fig. 6). These data suggest that osteoblasts have the potential to present antigens.

Discussion

In this study, we demonstrated that osteoblasts can produce significant levels of IL-6 in response to *Salmonella*-derived LPS or *Salmonella* infection. We show for the first time that LPS-induced tolerance occurs in osteoblasts. One proposed mechanism for LPS tolerance in macrophages is downregulation of the surface TLR4-MD2 complex [23]. However, in MC3T3-E1 osteoblasts, this complex was not downregulated by LPS. Therefore, tolerance observed in osteoblasts is possibly caused by other mechanisms, such as induction of the IL-1 receptor-associated kinase M (IRAK-M) or suppressor of cytokine signaling (SOCS), both of which inhibit signaling downstream of IL-1 receptor and Toll-like receptors [24–26]. Our preliminary data suggest that IL-1-induced IL-6 production was not suppressed by LPS pretreatment. Therefore, the mechanism of LPS-induced tolerance might be specific to signaling downstream of Toll-like receptors in osteoblasts. We also found that the number of viable intracellular *Salmonella* contained in MC3T3-E1 cells is reduced when cells are exposed to IFN-γ before infection and that MHC II expression levels are significantly upregulated by IFN-γ. These data suggest that murine osteoblasts have functions reminiscent of those seen in macrophages.

During bacterial infection of bone, osteoblasts are likely exposed to IFN-γ produced by activated T cells and NK cells. It is well known that IFN-γ upregulates MHC II expression in macrophages. Although we have not observed upregulation of costimulatory molecules such as CD80 and CD86 in MC3T3-E1 cells, osteoblasts exposed to IFN-γ significantly upregulate MHC II. Together with results obtained by others [27,28], our data suggest that IFN-γ enhances antigen presentation potential in osteoblasts as well as in macrophages.

It has been reported that IFN-β and IFN-γ suppress osteoclast formation by acting directly on osteoclast precursors [14,29]. In addition to such direct antiosteoclastogenic

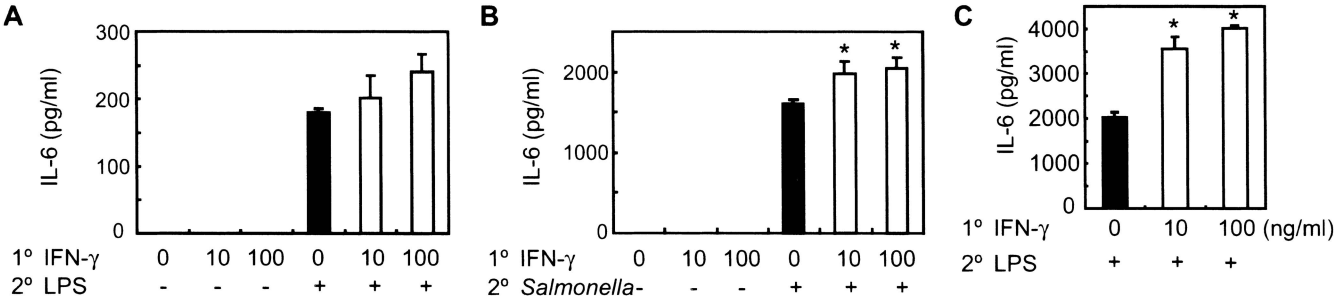

Fig. 4. IFN-γ pretreatment enhances IL-6 production in response to LPS and *Salmonella*. **A** Sequential treatment with IFN-γ and LPS stimulation. MC3T3-E1 cells were pretreated with increasing concentrations of IFN-γ for 24 h and then stimulated with 500 ng/ml LPS. After 3 h, IL-6 levels in supernatants (n = 2) were determined. **B** Sequential treatment with IFN-γ and *Salmonella*. MC3T3-E1 cells pretreated with IFN-γ as in **A** and then stimulated with *Salmonella* (MOI = 100). After 3 h, IL-6 levels in supernatants (n = 2) were measured as in **A**. **C** Mouse embryo fibroblasts were pretreated with increasing concentrations of IFN-γ for 24 h and then stimulated with 1 μg/ml LPS. *Closed bars*, without pretreatment; *open bars*, with pretreatment. *Error bars* represent means ± SD. *$P < 0.05$

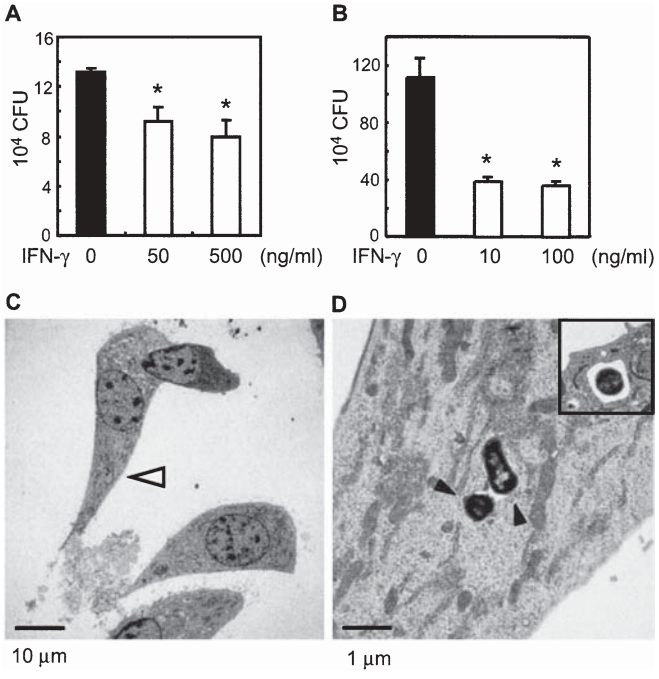

Fig. 5. Effect of IFN-γ on bactericidal function of osteoblasts. **A** MC3T3-E1 cells were pretreated with medium alone (*closed bar*), or 50 or 500 ng/ml IFN-γ (*open bars*) for 24 h. Cells were then infected with *Salmonella* (MOI = 10) for 2 h. After 3 h, cells were homogenized using sodium deoxycholate (n = 3) and plated to count colony-forming units (CFU). *Error bars* represent means ± SD. *$P < 0.05$, vs no IFN-γ pretreatment. **B** RAW264.7 macrophages were analyzed as in **A** using 10 or 100 ng/ml IFN-γ pretreatment. **C** Transmission electron microscopic analysis of *Salmonella*-containing MC3T3-E1 cells 3 h after infection. **D** A higher magnification of **C** at the *open arrowhead*. *Closed arrowheads* indicate *Salmonella* in a *Salmonella*-containing vacuole (SCV). *Top right inset* shows SCVs formed in RAW264.7 macrophages

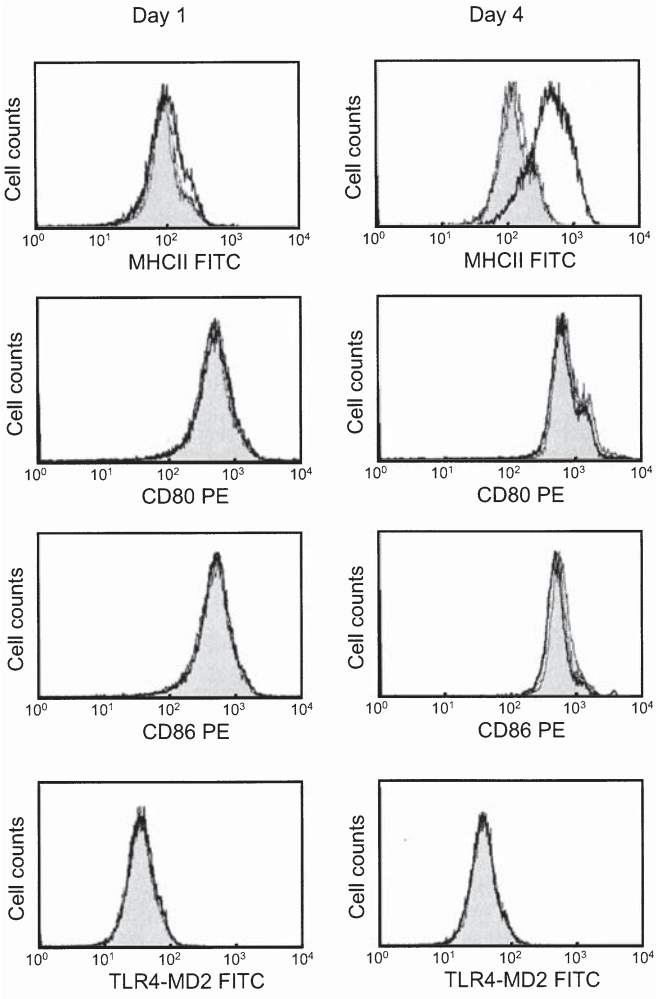

Fig. 6. Effect of IFN-γ on expression of major histocompatibility complex II (MHC II), the costimulatory molecules CD80 and CD86, and the TLR4-MD2 complex in osteoblasts. MC3T3-E1 cells were treated with medium alone (*gray filled line*), 10 ng/ml LPS (*broken line*), or 40 ng/ml IFN-γ (*bold line*) for 1 or 4 days. Cells were then analyzed for indicated protein expression by fluorescence-activated cell sorter (FACS). Experiments shown are representative of three assays

activity, our observations suggest that IFN-γ also stimulates osteoclast formation indirectly through osteoblasts. We observed that IFN-γ enhances IL-6 production in MC3T3-E1 cells in response to LPS or *Salmonalla* and that production of OPG in osteoblasts is downregulated by IFN-γ. IFN-γ induces PGE_2 production in osteoblasts [20], and the COX-2 inhibitor NS-398, which reduces PGE_2 production

[19], upregulates OPG levels. Therefore, downregulation of OPG by IFN-γ may be mediated by PGE_2. Both increased production of IL-6 and decreased production of OPG by osteoblasts can result in enhanced osteoclast formation and thereby excess bone resorption [30]. Therefore, differentiation of osteoclast-macrophage precursors may be modulated by both the positive effects of IFN-γ acting through osteoblasts and its negative effects directly on osteoclast precursors.

The dual role of IFN-γ is reminiscent of that of LPS. We observed that, similar to IFN-γ, LPS also downregulates OPG levels in osteoblasts. Interestingly, it has been reported that RANKL gene expression in synovial fibroblasts and osteoblasts is enhanced by LPS stimulation or *Salmonella* infection, and RANKL expression accelerates osteoclastogenesis [10,31]. By contrast, LPS strongly suppresses osteoclast differentiation by acting directly on precursors [32]. Thus, like IFN-γ, LPS has both pro- and antiosteoclastogenic functions. Taken together, both LPS and IFN-γ have proosteoclastogenic effects through osteoblasts and antiosteoclastogenic effects on osteoclast precursors. Although the antiosteoclastogenic activity of IFN-γ and LPS is widely accepted, proosteoclastogenic activities of IFN-γ and LPS may be dominant during bone infection, resulting in bone destruction. In osteomyelitis, it has been reported that IL-6 contributes to tissue damage occurring in chronic bone infection by protecting neutrophils from apoptosis [33]. Suppression of IFN-γ and LPS proosteoclastogenic activities could be an effective therapeutic strategy to combat infection-associated bone destruction.

We also observed that IFN-γ pretreatment reduces the number of intracellular bacteria seen in MC3T3-E1 cells, indicating that IFN-γ can increase the resistance of osteoblasts to *Salmonella* infection. It has been reported that osteoblasts express antimicrobial peptides [34] and inducible nitric oxide (NO) synthase, the latter resulting in NO production [35]. Decreased numbers of intracellular bacteria may be caused by induction of such bactericidal effectors induced by LPS and IFN-γ. By electron microscopy, we showed that *Salmonella* can enter osteoblasts, as has been previously reported [15]. We further demonstrated that most of these bacteria reside in membrane-bound components. To our knowledge, this is the first demonstration of so-called SCVs [22] formed in osteoblasts. Recent studies show that c-Fos, a positive regulator of osteclastogenesis, negatively regulates macrophage phenotypes [36,37]. It will be interesting to investigate whether and how c-Fos suppresses macrophagic function in osteoblasts, in which c-Fos overexpression has been shown to induce osteosarcoma formation [38].

In summary, we have demonstrated that osteoblasts share several phenotypes associated with macrophages, namely, regulation of cytokine production by IFN-γ and LPS, LPS tolerance, and upregulation of MHC II by IFN-γ. This study provides a basis for in vivo stusies investigating possible protective roles played by osteoblasts against bacterial infection in bone, including osteomyelitis.

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