Staphylococcus aureus Infection of Mouse or Human Osteoblasts Induces High Levels of Interleukin-6 and Interleukin-12 Production

Kenneth L. Bost,¹ Warren K. Ramp,² Natalie C. Nicholson,² Jennifer L. Bento,¹ Ian Marriott,¹ and Michael C. Hudson¹

Staphylococcus aureus is the principal causative agent of the inflammatory bone disease osteomyelitis. Unfortunately, the pathogenesis of this often chronic infection is poorly understood and is complicated by the recent observation that bone-forming osteoblasts can harbor S. aureus. Such an infection presents a significant challenge for the host immune response, because osteoblasts are not known to initiate protective cell-mediated immune responses. Cultured mouse and human osteoblasts infected with S. aureus were found to express high levels of interleukin (IL)–6 and IL-12p75, on the basis of complementary investigations demonstrating both S. aureus–induced up-regulation of expression of IL-6 and IL-12p40 mRNA and secretion of IL-6 and IL-12p75 by these cells. Additionally, a quantitative bioassay demonstrated that IL-12p75 secreted after infection was biologically active. These studies are the first to demonstrate induced IL-12p75 expression by osteoblasts and suggest a previously unrecognized role for osteoblasts in initiating immune responses after S. aureus infection.

Osteomyelitis is an infection characterized by progressive inflammatory destruction of bone [1]. Staphylococcus aureus is the most frequent causative organism in all types of osteomyelitis [2]. S. aureus has a propensity to colonize broken skin such that a history of trauma or skin infection is a significant risk factor for bone and joint infections caused by this organism [3, 4]. S. aureus initially adheres to bone by expressing receptors for bone matrix components, including collagen, bone sialoprotein, fibronectin, and laminin [5–7]. Once the bacteria adhere to and colonize bone matrix, they produce several virulence factors, such as proteases, that can break down matrix components. The resultant bone destruction facilitates bacterial invasiveness.

Importantly, S. aureus not only colonizes bone matrix but also can be internalized by osteoblasts [8–10]. Internalization may provide a means of protection against antibody- or neutrophil-mediated destruction and may also confound effective antibiotic therapy. The observation that S. aureus is internalized by osteoblasts and can persist intracellularly may begin to explain why many staphylococcal infections that tend to become chronic (e.g., osteomyelitis and mastitis) are associated with multiple reurrences and do not resolve even in the presence of what seems to be an adequate humoral immune response. Hence, S. aureus sequestered from the host immune system in the cytoplasm of the osteoblast may provide a reservoir of bacteria in recurring osteomyelitis and may be more relevant to chronic disease than are bacteria associated with the bone matrix.

The immune response against an intracellular bacterial pathogen is complex and is influenced by the expression of numerous signaling molecules, including cytokines. It has previously been shown that secretion of interleukin (IL)–12 by macrophages or dendritic cells is critical for optimal cell-mediated immune responses against a variety of intracellular pathogens (reviewed in [11]). In particular, endogenous production of IL-12 or exogenous administration of recombinant (r) IL-12 can augment immunity to a variety of intracellular pathogens, such as Salmonella species [12, 13], Leishmania species [14–17], and Mycobacterium tuberculosis [18, 19]. The mechanism of protection appears to be due in large part to the ability of IL-12 to promote the production of interferon (IFN)–γ from NK cells and T lymphocytes [20–23]. Increased IFN-γ production can then activate macrophages and T lymphocytes to more effectively clear the invading intracellular pathogen [11]. The production of other cytokines, such as IL-6, can further augment the T lymphocyte and macrophage response.

Although the major functions of the osteoblast are to synthesize the components of bone matrix (mainly type I collagen) and to catalyze mineralization of the matrix [24], recent observations suggest that osteoblasts might also have the ability to augment an inflammatory response by producing cytokines. Cultured osteoblast-like cells have the potential to secrete cytokines such as IL-1 [25–27], IL-6 [26–30], tumor necrosis factor–α [31, 32], and IL-18 [33], and this secretion has often been
associated with the ability of these cells to modulate osteoclast activity [28, 29, 33]. However, there is currently little evidence for the ability of infectious agents to elicit such responses from osteoblasts, and there are no prior reports of IL-12 production from this cell type.

In the present study, we have investigated the ability of normal mouse and human osteoblasts to express cytokines after infection with S. aureus.

Materials and Methods

Isolation and culture of mouse osteoblasts. Primary osteoblast cell cultures were prepared from mouse neonates according to a method described elsewhere for chick embryos [34]. Bone-forming cells were isolated from mouse neonate calvariae by sequential collagenase-protease digestion. The periosteum were removed, and the frontal bones were harvested free of the suture regions and incubated for 10 min at 38°C in 10 mL of digestion medium containing collagenase (375 U/mL, type VII; Sigma, St. Louis) and protease (7.5 U/mL; Sigma). The digestion medium and released cells were removed and discarded. Next, 10 mL of fresh digestion medium was added, and the incubation was continued for 20 min. Cells were harvested by centrifugation and rinsed 3 times in 25 mM HEPES-buffered Hank's balanced salt solution (pH 7.4). The digestion step was repeated twice, and the 3 cell isolates were pooled in osteoblast medium consisting of Dulbecco’s modified Eagle medium containing 25 mM HEPES, 10% Nu-serum IV (Collaborative Biomedical Products, Bedford, MA), 75 μg/mL glycine, 100 μg/mL ascorbic acid, 40 ng/mL vitamin B12, 2 μg/mL p-aminobenzoic acid, 200 ng/mL biotin, and 100 U/mL 100 μg/mL 0.25 μg/mL penicillin-streptomycin-Fungizone (pH 7.4) [35]. Cells were seeded in 6-well cluster plates and incubated at 37°C in a humidified incubator until they reached confluence (6–7 days). Cells were then infected with S. aureus as described below.

Normal human osteoblast cultures. Normal human osteoblasts (Clonetics, San Diego) were purchased and propagated according to the guidelines provided by the vendor. Cells were seeded in 25-cm² flasks and incubated at 37°C in a humidified incubator in medium supplied by the manufacturer that contained 10% fetal calf serum, vitamin C, and gentamicin. After cells reached ~80% confluence (5–9 days), they were removed from flasks by use of 0.025% trypsin-0.01% EDTA, washed in medium, and seeded into 6-well plates. After cells reached ~80% confluence (6–7 days), they were infected with S. aureus as described below. These commercially available cells have been extensively characterized as osteoblasts [36, 37].

Characterization of isolated mouse osteoblasts. Mouse osteoblasts were grown on glass coverslips in 24-well plates until confluent and were then fixed and permeabilized according to the methods recommended by the manufacturer (CytoFix/CytoPerm; PharMingen, San Diego). Rabbit antibodies specific for osteocalcin (1:100 dilution; Peninsula Laboratories, Belmont, CA), type I collagen (1:40 dilution; Chemicon, Temecula, CA), alkaline phosphatase (1:40 dilution; Sigma), or keyhole limpet hemocyanin (1:40 dilution) were incubated on cell preparations for 45 min at 4°C. After unbound antibody was washed off, a phycoerythrin-conjugated goat anti-rabbit IgG antibody (1:50 dilution; Sigma) was added for 45 min at 4°C. After washing, at least 500 cells were scored for positive fluorescence by use of a fluorescence microscope (model BX60; Olympus, Lake Success, NY). Osteocalcin, type I collagen, and alkaline phosphatase were selected for analysis because expression of these proteins has been used to define osteoblasts as such [37–39].

Intracellular infection of cultured mouse and human osteoblasts by S. aureus strain UAMS-1 (ATCC 49230). Intracellular infection of isolated osteoblasts was done essentially as described elsewhere [9], and colony counts of infected osteoblast cultures were taken at varying times after infection to demonstrate the presence of viable intracellular S. aureus at all time points. In parallel experiments, osteoblasts were exposed to UV-killed S. aureus to question the importance of bacterial viability on the induction of IL-6 and IL-12 expression. Further, the viability of cultured osteoblasts after infection was determined by means of both trypan blue and propidium iodide exclusion assays. Mouse and human osteoblasts were determined to be >97% or >93% viable at 24 or 48 h after infection, respectively.

RNA isolation, reverse transcription, and semiquantitative PCR. At the indicated times, RNA was extracted from cultured mouse osteoblasts, reverse-transcribed, and subjected to semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) as described elsewhere [40, 41]. Briefly, total RNA was isolated by use of TRIzol reagent (Gibco-BRL, Gaithersburg, MD), and 2 μg of total RNA was reverse-transcribed in the presence of random hexamers with use of 200 U of RNase H-Moloney murine leukemia virus reverse transcriptase (Superscript II; Gibco-BRL) in the buffer supplied by the manufacturer. PCR was done on 5% of the total cDNA to quantify expression of the mRNAs encoding IL-6, IL-12p40, IL-12p35, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) by use of 95°C denaturation, 60°C annealing, and 72°C extension temperatures (Robocycler 40; Stratagene, La Jolla, CA), with the first 3 of 27 total cycles having extended denaturation and annealing times. PCR primers were derived from the published sequences of IL-6 [42], IL-12p40, IL-12p35 [43], and G3PDH [44], as follows: GATGCTACCAAACTGGATAATC and GTGGCCTTGGACCTTTCTGTG to amplify IL-6; CCACTCATATCCTGCTTCCACAAG and ACTTCTCATAGTCAGGACGGGACCAAACCAAG and GGTCCTTAGCCACTCCTTCTGTG to amplify IL-12p40; AAGACATCACACGGGACCAAACCA and CGGCAGAGTCTCGCCATATTATGTTGCT to amplify IL-12p35; and CCATCACCATTCTCCAGGAGCGGCGG and CACAGTCCTCGGTTGGCAGGTG to amplify G3PDH. After PCR, 15% of each amplified sample was electrophoresed on ethidium bromide-stained agarose gels and visualized under UV illumination. Densitometric analysis of the RT-PCR product bands was done by use of NIH Image obtained from the National Institutes of Health Web site: http://rsb.info.nih.gov/NIH-image). Each gel image was imported into NIH Image by use of Adobe Photoshop (Adobe Systems, San Jose, CA), a gel-plotting macro was used to outline the bands, and the intensity was calculated on the uncalibrated optical density setting.

To ensure that similar amounts of input RNA were reverse-transcribed, RNA was quantified by use of DNA dipsticks (Invitrogen, San Diego) before the cDNA reactions. In addition, PCR amplification of the housekeeping gene, G3PDH, was done on cDNA from each sample to ensure equal input of RNA and similar efficiencies of RT. The identities of the amplified fragments were
verified by size comparison with DNA standards (Promega, Madison, WI) and by direct DNA sequencing of representative fragments as described elsewhere [40, 41]. Furthermore, the sensitivity and linearity of RT-PCR amplification for mouse IL-6 and IL-12 gene expression under these conditions has been described elsewhere [40, 41]. The results reported here are in the linear range of amplification for each of these genes.

Quantification of IL-6, IL-12p40, and IL-12p75 secretion in culture supernatants. Capture ELISAs were done to quantify IL-6, IL-12p40, and IL-12p75 secretion essentially as described elsewhere [40, 45]. Briefly, monoclonal capture antibodies (PharMingen) were coated onto microtiter plates (Maxisorp; Nunc, Naperville, IL) at 15 μg/mL for 18 h. Capture antibodies used included anti-mouse IL-6 (clone MP5-20F3), anti-mouse IL-12p40 (clone C15.6), anti-mouse IL-12p35 (clones Red-T and G297-289), anti-human IL-6 (clone MQ2-39C3), and anti-human IL-12p75 (clone 20C2). After washing, coated plates were blocked with 2% bovine serum albumin in PBS for 2 h, followed by addition of supernatants taken from osteoblast cultures at the indicated times after infection. After 2.5 h of incubation, unbound material was washed off, and biotinylated detection antibodies were added at 10 μg/mL for 2 h. Biotinylated detection antibodies used included anti-mouse IL-6 (clone MP5-32C11), anti-mouse IL-12p40 (clone C17.8), anti-human IL-6 (clone MQ2-39C3), and anti-human IL-12p40 (clone C8.6). After washing, bound antibody was detected by addition of streptavidin–horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) for 45 min, followed by addition of the substrate tetramethylbenzidine (Promega). Colorimetric reactions were stopped by the addition of 0.5 M H2SO4, and absorbances at 450 nm were measured (model 550 microplate reader; Bio-Rad Laboratories, Hercules, CA). Cytokine levels in culture supernatants were quantified by extrapolation from standard curves generated by determining absorbances with use of limiting dilutions of mouse rIL-6, mouse rIL-12p40 homodimer, mouse rIL-12p75, human rIL-6, or human rIL-12p75, respectively (PharMingen).

Bioassay for detection of IL-12p75. A bioassay based on the ability of IL-12p75 to induce secretion of IFN-γ by mononuclear cells in a quantitative manner was done as described elsewhere [45]. Monoclonal antibodies (PharMingen) that recognize mouse IL-12p35 (clone Red-T and G297-289) or human IL-12p75 (clone 20C2) were used to coat microtiter wells overnight at a concentration of 12 μg/mL. Wells were washed, and varying dilutions of recombinant human or mouse IL-12p75 or osteoblast culture supernatants were added to the antibody-coated wells for 3 h. After washing off unbound material, splenic leukocytes from BALB/c mice (2 × 10^6/well) or human peripheral blood mononuclear cells (2 × 10^6/well) were added, and plates were incubated at 37°C for 48 h. For quantification of mouse or human IFN-γ secretion into culture supernatants, a capture monoclonal antibody (clone R4-6A2 or NIB42, respectively) was coupled with a biotinylated detection monoclonal antibody (clone XMG1.2 or 4S.B3, respectively) by use of the ELISA procedure described above. IFN-γ levels in culture supernatants were quantified by extrapolation from standard curves generated by determining absorbances with use of limiting dilutions of mouse or human recombinant IFN-γ (PharMingen). Quantification of IL-12p75 was determined from standard curves of IFN-γ produced versus amount of recombinant IL-12p75 added. Of note, the coating antibodies were not able to bind IL-12p40; therefore, it was not possible for excess free IL-12p40 to interfere with this bioassay.

Statistical analyses. Results were tested statistically by means of Student’s paired t test or one-way analysis of variance as appropriate (GraphPad Prism; GraphPad Software, San Diego).

Results

Characterization of isolated mouse osteoblasts. Isolated mouse osteoblasts displayed characteristic morphology, having an abundant endoplasmic reticulum, a polygonal appearance, and the ability to elaborate an extensive extracellular matrix (data not shown). Furthermore, immunofluorescent staining of these cells for the presence of osteocalcin, type I collagen, and alkaline phosphatase demonstrated >99% of the cells being positive, whereas staining with the control, anti–keyhole limpet hemocyanin antibody, demonstrated <3% of the cells being positive. These markers have been used to identify this cell population as osteoblasts [37–39] and demonstrated the purity of the isolation procedure.

Induction of IL-6 and IL-12 expression by S. aureus–infected mouse osteoblasts. The ability of S. aureus to infect osteoblasts [8–10] creates a problem for the host, because osteoblasts are not recognized for their ability to initiate a cell-mediated immune response. To begin to address how osteoblasts might aid in the generation of a protective immune response, S. aureus–induced expression of IL-6 and IL-12 mRNAs was investigated. Figure 1 demonstrates a dramatic and rapid upregulation of IL-6 and IL-12p40 mRNA expression after S. aureus infection. Increases in IL-6 and IL-12p40 mRNA expression were as great as 6- and 40-fold, respectively, compared with uninfected controls, by 12 h after infection, whereas IL-12p35 mRNA expression was never >2-fold over constitutive expression.

![Figure 1](Image)

**Figure 1.** Kinetics of interleukin (IL)-6, IL-12p40, and IL-12p35 mRNA expression in *Staphylococcus aureus*–infected mouse osteoblasts. RNA was isolated at 6, 12, and 24 h from either 10^7 uninfected cultured osteoblasts or 10^7 osteoblasts infected with *S. aureus* at ratio of 250 : 1. *S. aureus* to osteoblasts, and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was done. Amplified product electrophoresed on ethidium bromide–stained agarose gels is shown. PCR amplification of housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), was done to ensure that similar amounts of input RNA and similar efficiencies of RT were being compared. These studies were done 3 times with similar results.
levels. These time-dependent increases in cytokine message expression could not be ascribed to differences in input RNA or to differences in the efficiency of reverse transcription, as evidenced by RT-PCR amplification of the housekeeping gene G3PDH for each sample (figure 1).

The induction of IL-6 and IL-12p40 mRNA expression in osteoblasts after *S. aureus* infection demonstrated a dose-dependent relationship, corresponding to the amount of *S. aureus* cells exposed to mouse osteoblasts. As shown in figure 2, when the inoculum of *S. aureus* used to infect the osteoblasts was decreased from a ratio of 250:1 to 25:1, *S. aureus* to osteoblasts, there was a concomitant decrease in IL-6 and IL-12p40 mRNA expression. However, it should be noted that an initial exposure for only 45 min with a ratio of 25:1, followed by removal of extracellular bacteria, was sufficient to induce significant cytokine mRNA expression. These data, coupled with the secretion data shown below, demonstrate that this pathogen provides a potent stimulus for the induction of IL-6 and IL-12 expression.

The dramatic up-regulation of IL-12p40 mRNA expression (figures 1 and 2) may not correlate with IL-12p75 secretion, because it has been observed that excess IL-12p40 monomers and homodimers may be preferentially secreted [45–47]. This is an important consideration, because IL-12p40 monomers have no known biologic activity, whereas IL-12p40 homodimers can actually function as antagonists of IL-12p75 [48–50]. It was therefore necessary to determine what fraction of the total IL-12 secreted was actually heterodimeric IL-12p75. The ELISA to quantify IL-12p40 secretion actually detects both IL-12p40 and IL-12p75, because both forms of IL-12 contain the IL-12p40 subunit. Therefore, the ELISA used to quantify IL-12p40 secretion represents “total” IL-12 present in the culture supernatants. Comparing the quantity of IL-12p40 secreted (i.e., “total” IL-12) with the quantity of IL-12p75 secreted showed that the majority of *S. aureus*-induced IL-12 was in the form of the agonist, IL-12p75 (figure 3). In addition, the quantity of IL-12p75 secreted increased in a time-dependent manner and routinely exceeded 1 ng/mL for 10⁶ mouse osteoblasts within 48 h (mean ± SD, 2.1 ± 0.2 ng/mL; figure 3).

Further, *S. aureus*-induced IL-12p75 secretion could be detected as early as 6 h after infection, suggesting that this induction was an early and direct response. In a similar manner, *S. aureus* infection was a potent stimulus for the induction of IL-6 secretion, because a mean of 3.9 ng/mL (± 0.3) was routinely observed as early as 6 h after infection (figure 3).

In contrast, exposure of mouse osteoblasts to equal numbers of UV-killed *S. aureus* was not a potent stimulus for cytokine secretion (figure 3). Viable *S. aureus* routinely induced 10- to 15-fold more IL-6 secretion (mean ± SD, 27.3 ± 2.1 ng/mL at 48 h after infection) than did a similar number of killed bacteria (2.2 ± 0.1 ng/mL at 48 h after infection). Further, UV-killed *S. aureus* was unable to induce detectable IL-12 secretion (figure 3).

Figure 4 demonstrates a dose-response relationship between cytokine secretion and the inoculum of *S. aureus* used to infect cultures. It is noteworthy that exposure to as few as 25 viable *S. aureus* cells/osteoblast, for as little as 45 min, followed by elimination of extracellular bacteria was sufficient to induce substantial IL-6 (mean ± SD, 1.2 ± 0.1 ng/mL at 48 h after infection) and IL-12p75 (0.3 ± 0.02 ng/mL at 48 h after infection) secretion.

To confirm the quantity of bioactive IL-12p75 that was being secreted by *S. aureus*-infected osteoblasts, a bioassay was also done. This bioassay is based on the ability of IL-12p75 to induce IFN-γ secretion by mouse T lymphocytes in a dose-dependent manner. As shown in figure 5, *S. aureus*-infected mouse osteoblasts were potent inducers of bioactive IL-12 (mean ± SD, 1.17 ± 0.1 ng/mL at 48 h after infection), in quantities similar to that found by use of ELISA (compare figures 5 with figures 3 and 4). Taken together, these results (figures 1–5) demonstrate the surprising ability of infected mouse osteoblast cultures to secrete IL-12p75 and the potent nature of viable *S. aureus* as a stimulus for initiating this osteoblast response.

**Induction of IL-6 and IL-12 secretion by *S. aureus*-infected human osteoblasts.** The importance of viable *S. aureus*-induced cytokine secretion by mouse osteoblasts was further demonstrated by the observation that human osteoblasts behaved in a similar manner. Normal human osteoblasts were
Quantification of kinetics of interleukin (IL)-6, IL-12p40, and IL-12p75 secretion by infected cultured mouse osteoblasts. Osteoblasts (10^7/well) were infected with *Staphylococcus aureus* or UV-killed *S. aureus* (250 : 1 *S. aureus* to osteoblasts) for 45 min, followed by elimination of extracellular bacteria. At 6, 12, 24, and 48 h after infection, culture supernatants were taken and appropriate capture ELISAs were performed to quantify cytokine secretion. Sensitivities for each ELISA were determined by use of appropriate recombinant cytokine diluted in culture media and were 30 pg/mL for IL-6, IL-12p40, and IL-12p75. Results are presented as means of triplicate determinations ± SD for 1 representative experiment. *Significantly different (P < .01) from control (uninfected) cultures. These studies were performed 3 separate times.

Figure 3. Figure 3. Quantification of kinetics of interleukin (IL)-6, IL-12p40, and IL-12p75 secretion by infected cultured mouse osteoblasts. Osteoblasts (10^7/well) were infected with *Staphylococcus aureus* or UV-killed *S. aureus* (250 : 1 *S. aureus* to osteoblasts) for 45 min, followed by elimination of extracellular bacteria. At 6, 12, 24, and 48 h after infection, culture supernatants were taken and appropriate capture ELISAs were performed to quantify cytokine secretion. Sensitivities for each ELISA were determined by use of appropriate recombinant cytokine diluted in culture media and were 30 pg/mL for IL-6, IL-12p40, and IL-12p75. Results are presented as means of triplicate determinations ± SD for 1 representative experiment. *Significantly different (P < .01) from control (uninfected) cultures. These studies were performed 3 separate times.

Discussion

The ability of viable *S. aureus* to induce IL-12p75 expression by human and mouse osteoblasts was unexpected in light of the cells currently considered as potent secretors of this cytokine. Dendritic cells [51, 52], neutrophils [53], and macrophages [45, 54], including microglia [55–57], are leukocytes that express IL-12 after appropriate stimulation. There have also been isolated reports of induced expression of IL-12 in cells of nonleukocytic origin, such as astrocytes [56]; however, other investigations have not found such expression [55, 57]. The lack of reports demonstrating substantial IL-12 secretion by nonleukocytic cells makes the present report even more surprising. In fact, the amount of IL-12p75 secreted by *S. aureus*–infected osteoblasts (i.e., 1–3 ng/mL in 10^7 cultured cells) is similar to, or exceeds, those quantities of IL-12p75 secreted by cultured dendritic cells or cells of macrophage origin after induction [45, 46, 51–53, 56].

The majority of IL-12 secreted by *S. aureus*–infected mouse osteoblasts was in the form of IL-12p75 (figures 3 and 4). This is an important observation for two reasons. First, many stimuli of dendritic cells or macrophages induce a substantial excess of IL-12p40 secretion over that of IL-12p75 [45–47]. This result is observed in vitro and in vivo and suggests regulation of the secretion of different forms of IL-12. Further, it is not altogether...
clear which stimuli induce the highest levels of IL-12p75 in dendritic cells or macrophages. The present report demonstrates that S. aureus is a potent inducer of IL-12p75 in mouse and human osteoblasts that does not result in a substantial excess of IL-12p40 being secreted. Second, the secretion of high levels of IL-12p40 can result in the secretion of homodimers that may function as antagonists of IL-12p75 [48–50]. Therefore, the observation that S. aureus–infected osteoblasts secrete mostly IL-12p75 demonstrates that the majority of induced secretion is the agonist form.

S. aureus is the primary causative agent of osteomyelitis, the progressive inflammatory disease of bone [2]. Although staphylococci are typically regarded as noninvasive extracellular pathogens that damage host cells after adhering to the extracellular matrix [58], several recent studies have shown that S. aureus can be internalized in a number of cell types that are not generally considered to be phagocytic [59–62]. In addition, recent reports [8–10] have demonstrated that S. aureus can be internalized by osteoblasts and can persist intracellularly. Such observations take on additional importance with the finding in this study that UV-killed S. aureus was not a potent inducer
Figure 6. Dose-dependent *Staphylococcus aureus* induction of interleukin (IL)-6 and IL-12p75 secretion by cultured human osteoblasts. Normal human osteoblasts (3 × 10⁶) were infected with varying numbers of *S. aureus* or UV-killed *S. aureus* (25:1, 75:1, and 250:1 *S. aureus* to osteoblasts) for 45 min. Extracellular bacteria were then eliminated, culture supernatants were taken 48 h after infection, and specific capture ELISAs were done to quantify cytokine secretion. Sensitivities for each ELISA were determined by use of appropriate recombinant cytokine diluted in culture media and were 30 pg/mL for IL-6 and 30 pg/mL for IL-12p75. Bioassay was also done to quantify amount of IL-12p75 present in supernatants of 3 × 10⁶ osteoblasts infected with *S. aureus*. Results were quantified by extrapolation from standard curves generated by use of recombinant human IL-12p75. Results are presented as mean of triplicate determinations ± SD for 1 representative experiment. *Significantly different (P < .01) from control (uninfected) cultures. These studies were performed 4 separate times.

of cytokine secretion compared with viable intracellular bacteria. Further, it was clear that IL-6 and IL-12 secretion was induced and not merely the result of *S. aureus*-mediated cell lysis, because viability of osteoblasts in infected cultures exceeded 93% even at 48 h after infection. On the basis of these observations, it is tempting to speculate that one component of *S. aureus*-mediated bone disease would be internalization of viable bacteria, which induces osteoblasts to initiate secretion of proinflammatory cytokines such as IL-6 and IL-12.

The ability of *S. aureus*-infected osteoblasts to elaborate IL-12p75 has important implications for host defense against intracellular organisms. IL-12 can stimulate T lymphocytes and NK cells to secrete significant amounts of IFN-γ, which can then activate macrophages and T lymphocytes to augment a Th1 response. Such a mechanism would seem to be a significant advantage for elimination of intracellular *S. aureus*. Further, the ability of infected osteoblasts to secrete IL-12p75 would focus such a Th1 response to the site of infection. The experimental design used in the present study limited the extracellular exposure of viable *S. aureus* to 45 min, while intracellular infection persisted throughout the experimental protocol. It would be logical to assume that the presence of viable intracellular organisms provided the most potent stimulus for IL-12p75 secretion in these studies. Such a hypothesis is supported by our findings that UV-killed *S. aureus* was not able to induce IL-12p75 secretion by mouse or human osteoblasts (figures 3 and 6).

Because of the high level of IL-12p75 induced in osteoblasts by *S. aureus*, one must also consider the possible damaging effects this cytokine might have in inflammatory diseases such as osteomyelitis. IL-12 is known for its ability to protect against intracellular pathogens, but it is also becoming apparent that excess IL-12 production may contribute to the initiation or development of organ-specific autoimmune diseases [63]. *S. aureus* is known for its ability to induce IL-12 in antigen-presenting cells [64], and we demonstrate here that *S. aureus* has a similar effect on osteoblasts. Therefore, it is possible that induction of substantial levels of IL-12 by this pathogen may augment the host response, as well as contribute to the pathophysiology associated with *S. aureus*-mediated diseases.

The implications for IL-12p75 secretion by infected osteoblast cultures are significant; in addition, this study demonstrates for the first time that viable intracellular *S. aureus* is a potent stimulus for the induction of IL-6 secretion. This result is perhaps less surprising, because the secretion of IL-6 by osteoblasts under other stimuli has been noted elsewhere [26–30]. However, the presence of this cytokine can also augment the development of humoral, as well as cell-mediated, immune responses [65]. Furthermore, the presence of IL-6 produced by bone-forming osteoblasts can directly or indirectly modulate the activity of bone-resorptive osteoclasts [29, 66–68], resulting in induction of osteoclast differentiation or osteoclast-mediated bone demineralization. Thus, the ability of *S. aureus* infection
to augment IL-6 expression in osteoblasts may have important implications for bone formation or destruction during disease processes mediated by *S. aureus*.

In summary, the present study conclusively demonstrates high levels of IL-6 and IL-12 expression by *S. aureus*-infected human and mouse osteoblasts. This conclusion was based on early and dramatic mRNA expression, followed by substantial cytokine secretion. The majority of induced IL-12 secretion was in the form of IL-12p75, and the activity of this cytokine was confirmed by quantifying its ability to induce IFN-γ secretion. Taken together, these studies demonstrate an unexpected ability of human and mouse osteoblasts to respond to viable *S. aureus* and suggest a new role for these cells in the host response against this pathogen.

**References**

18. Cooper AM, Roberts AD, Rhodes ER, Callahan JE, Getzy DM, Orme IM. The role of IL-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology 1995;84:423-32.