Osteoblasts Express the Inflammatory Cytokine Interleukin-6 in a Murine Model of *Staphylococcus aureus* Osteomyelitis and Infected Human Bone Tissue

Ian Marriott,* David L. Gray,*
Susanne L. Tranguch,* Vance G. Fowler Jr.,[†]
Martin Stryjewski,[†] L. Scott Levin,[‡]
Michael C. Hudson,* and Kenneth L. Bost*

From the Department of Biology,* University of North Carolina at Charlotte, Charlotte; and the Departments of Medicine[†] and Surgery,[‡] Duke University Medical Center, Durham, North Carolina

***Staphylococcus aureus* is the single most common cause of osteomyelitis in humans. Incidences of osteomyelitis caused by *S. aureus* have increased dramatically in recent years, in part due to the appearance of community-acquired antibiotic resistant strains. Therefore, understanding the pathogenesis of this organism has become imperative. Recently, we have described the surprising ability of bone-forming osteoblasts to secrete a number of important immune mediators when exposed to *S. aureus* *in vitro*. In the present study, we provide the first evidence for the *in vivo* production of such molecules by osteoblasts during bacterial infection of bone. These studies demonstrate the expression of the key inflammatory cytokine interleukin-6 by osteoblasts in organ cultures of neonatal mouse calvaria, and *in vivo* using a mouse model that closely resembles the pathology of trauma-induced staphylococcal osteomyelitis, as determined by confocal microscopic analysis. Importantly, we have established the clinical relevancy of these findings in infected human bone tissue from patients with *S. aureus*-associated osteomyelitis. As such, these studies demonstrate that bacterial challenge of osteoblasts during bone diseases, such as osteomyelitis, induces cells to produce inflammatory molecules that can direct appropriate host responses or contribute to progressive inflammatory damage. (Am J Pathol 2004, 164:1399–1406)**

The gram positive bacterium, *Staphylococcus aureus*, is the major causative agent of the bone disease osteomyelitis. This disease is an often severe infection that is characterized by progressive inflammatory destruction of bone,¹ and the incidence of this condition appears to be

increasing.² What is of particular concern is the changing nature of antibiotic resistance in *S. aureus*. While the incidence of methicillin-resistant *S. aureus* was once restricted to large university-based teaching hospitals, such infections are now emerging among patients with no identifiable risk factors.³ With the rapid increase in antibiotic resistance of bacteria long known to be associated with bone pathology, as is the case with *S. aureus*, it is becoming apparent that we must focus attention on the mechanisms that underlie bacterially induced inflammation in bone tissues to identify key therapeutic targets for the development of new treatment strategies. To date, the immune responses that develop during bone and joint disease are poorly understood. The inflammatory mediators elaborated during bone and joint infections are not well defined, nor is it clear what cell populations are responsible for such production.

Recent *in vitro* studies from our laboratory have indicated that bone-forming osteoblasts may play a previously unappreciated role in the development of inflammatory responses to bacterial challenges in bone tissue. While the major functions of osteoblasts are to synthesize the components of bone matrix and to control the bone-resorbing activity of osteoclasts, cultured murine and human osteoblasts have been shown by our laboratory to have a third function following bacterial challenge, namely, the ability to produce soluble inflammatory mediators. We have demonstrated that cultured osteoblasts exposed to bacterial species commonly associated with bone pathology secrete significant quantities of an array of inflammatory cytokines,⁴ colony-stimulating factors,⁵ and chemokines.^{6,7} Taken together, the pattern of inflammatory mediator production induced in osteoblasts following bacterial exposure could act in a protective manner by recruiting macrophages, neutrophils and activated T-lymphocytes to infected sites, and to sustain inflammatory responses by maintaining the activated status of such cells. Alternatively, because of the observed

Supported by National Institutes of Health grants AR47585 and AI48842 to I.M.

Accepted for publication January 6, 2004.

Address reprint requests to Ian Marriott, Ph.D., Department of Biology, 9201 University City Boulevard, University of North Carolina at Charlotte, Charlotte, NC 28223. E-mail: imarriot@email.uncc.edu.

high levels of immune molecules such as interleukin-6 (IL-6) produced by cultured osteoblasts in response to *S. aureus* exposure, one must also consider a role for such cytokines in initiating the inflammation that results in progressive bone destruction. However, the *in vivo* relevancy of these *in vitro* findings has remained unclear.

In the present study, we provide the first evidence for the *in vivo* production of inflammatory mediators by osteoblasts during bacterial infection of bone tissue. These studies demonstrate the expression of the key inflammatory cytokine IL-6 by murine osteoblasts in *S. aureus* infected bone under *in situ*-like conditions and in an *in vivo* mouse model that closely resembles the pathology of trauma-induced staphylococcal osteomyelitis. Importantly, we confirm the clinical relevancy of these findings by demonstrating the expression of IL-6 by osteoblasts in human bone tissue samples from patients with *S. aureus*-associated osteomyelitis. Taken together, these findings are in agreement with our earlier *in vitro* studies and strongly suggest a key role for osteoblasts in the initiation and/or maintenance of inflammation during bone diseases such as osteomyelitis.

Materials and Methods

Isolation and *In Vitro* Infection of Mouse Calvaria

Two-day-old neonatal BALB/c mice were euthanized and calvaria (skullcaps) removed and maintained in culture medium as described previously by our laboratory.⁶ Calvaria were exposed to *S. aureus* strain UAMS-1 (ATCC 49230), a clinical osteomyelitis isolate, for 3 hours followed by removal of media containing extracellular bacteria and replacement with media containing gentamicin. We have previously demonstrated that exposure of osteoblasts to bacteria in this manner results in the harboring of viable intracellular bacteria by these cells.⁵ After 48 hours in culture, calvaria were embedded in OCT solution (Sakura, Tokyo, Japan) and 5- μ m frozen sections were cut (Microm HM 505E, McBain Instruments, Chatsworth, CA). Sections were placed onto Superfrost Plus microscope slides (Fisher, Pittsburgh, PA) and fixed for 10 minutes at 4°C with 4% paraformaldehyde before staining.

Murine Model of Staphylococcal Osteomyelitis

A murine model of staphylococcal osteomyelitis has been used that has been modified from one previously shown to reproduce the clinical and gross pathological phases of inflammatory bone diseases such as human posttraumatic osteomyelitis.⁸ BALB/c mice were anesthetized with inhalant isoflurane and the femurs surgically exposed. A trough was drilled through the bone cortex by use of a high speed drill with a round burr. Damaged bone sites were either not treated or were inoculated with *S. aureus* (1×10^3) in agarose beads. Agarose beads containing *S. aureus* were prepared as follows. 1.4% low melt agarose (Invitrogen, Carlsbad, CA) was cooled to 40–42°C before the addition of bacteria. This mixture

was added to mineral oil, vigorously stirred, and cooled rapidly on ice. The resulting agarose beads were washed and stored on ice before bone application. This method of application induces local infection in bone tissue but markedly reduces the risk of systemic bacterial infection. The muscle fascias and surgical incision were closed and the disease was allowed to proceed for 2 or 4 days before femur removal and tissue analysis by RT-PCR and confocal microscopy. These times were selected based on empirical determination of the progression of infection at inoculated bone sites and on previously published findings in a similar murine model of staphylococcal osteomyelitis.⁹

Human Bone Tissue

Two clinical bone specimens were obtained from patients undergoing surgical debridement of *S. aureus*-associated osteomyelitis. A control bone sample was obtained from a subject undergoing hemiarthroplasty for reasons other than osteomyelitis. All bone samples were immediately fixed in 4% paraformaldehyde and stored at –70°C before tissue analysis.

Isolation of RNA and Semiquantitative RT-PCR

Murine femurs were cleaned free of connective tissue and marrow, quick-frozen in liquid N₂, and pulverized with a pestle and mortar. Total RNA was isolated from the ground tissue using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) as previously described.^{4–7} Poly(A)⁺ RNA was then isolated from total RNA using polystyrene latex-oligo dT beads (Oligotex-dT, Qiagen, Chatsworth, CA) as described previously.^{10,11} 100 ng of poly(A)⁺ RNA was reverse transcribed in the presence of random hexamers using 200 U of RNase H[–] Moloney leukemia virus reverse transcriptase (Promega, Madison, WI) in the buffer supplied by the manufacturer, as previously described by our laboratory.^{10,11}

PCR was performed on the reverse transcribed cDNA product to determine the expression of IL-6, as previously described.^{4,11} Positive and negative strand PCR primers used, respectively, were GATGCAACCAAACCTG-GATATAATC and GGTCTTAGCCACTCTTCTCTG to amplify mRNA encoding IL-6 (268 bp fragment), and CCATCACCATCTTCCAGGAGCGAG and CACAGTCT-TCTGGGTGGCAGTGAT to amplify mRNA encoding G3PDH (340 bp fragment). PCR primers were derived from the published sequences of IL-6¹² and G3PDH.¹³ These primers were designed by using Oligo 4.0 primer analysis software (National Biosciences Inc., Plymouth, MA) based on their location in different exons of the genomic sequences for IL-6 in addition to their lack of significant homology to sequences present in GenBank (MacVector Sequence analysis software, IBI, New Haven, CT).

The sensitivity and linearity of RT-PCR amplifications for each gene analyzed here were predetermined using limiting dilutions of RNA generated from *in vitro* transcription reactions as is routine in our laboratory.¹⁰ These

initial studies insured that the RT-PCR conditions used here were in the linear range of amplification for each mRNA species. To ensure similar input RNA amounts and efficiencies of reverse transcription, PCR amplification of the housekeeping gene, G3PDH, was performed on cDNA from each sample. The identity of the PCR amplified fragments were verified by size comparison with DNA standards (Promega), and by direct DNA sequencing of representative fragments as previously described.^{10,11} PCR products from the same series of experiments are shown throughout this manuscript for comparison purposes.

Immunohistochemical Analysis

Bone from the murine model or human tissue samples were decalcified using a procedure that has previously resulted in the preservation of cell surface markers for staining.¹⁴ Briefly, specimens were fixed for at least 6 hours at 4°C in 4% paraformaldehyde. After fixation, sequential 15-minute washes were performed using phosphate-buffered saline (PBS) containing 5% glycerol, PBS with 10% glycerol, and finally PBS with 15% glycerol. Specimens were then placed in a decalcifying solution (EDTA in 10% glycerol, pH 7.1) at 4°C for 5 and 14 days for murine and human bone samples, respectively. Specimens were washed sequentially in PBS containing 15% sucrose and 15% glycerol for 6 hours at 4°C, PBS containing 20% sucrose and 5% glycerol for 1 hour at 4°C, and PBS containing 20% sucrose and 5% glycerol for a further hour. Tissues were then embedded in OCT and quick-frozen in a mixture of ethanol and solid CO₂. Finally, 5-μm frozen sections were cut and sections were placed onto slides and allowed to air dry for 30 minutes at room temperature.

Murine bone slides were washed in PBS and exposed to blocking buffer (PBS containing 1% BSA and 0.3% nonimmune rabbit serum). Slides were stained using goat anti-mouse osteocalcin antibody (1:1000 dilution, Biomedical Technologies Inc., Stoughton, MA), or non-specific goat sera for 18 hours. An Alexa Fluor 488 conjugated chicken anti-goat IgG (Molecular Probes) was then added for 1 hour. Slides were co-stained with a monoclonal antibody directed against mouse IL-6 (Clone MP5-20F3, BD PharMingen, San Diego, CA), or species and isotype matched control monoclonal antibodies (Clone R3-34, BD PharMingen) for 18 hours before addition of Alexa Fluor 594 conjugated chicken anti-rat IgG (H+L) for 1 hour. For some sections, slides were not exposed to primary antibodies before addition of fluorochrome labeled secondary antibodies as an additional control.

Human bone tissue sections were stained in a similar manner using a goat polyclonal antibody for human osteocalcin (Diagnostic Systems Laboratories, Inc., Webster, TX), or nonspecific goat sera, for 18 hours, followed by addition of Alexa Fluor 488 conjugated chicken anti-goat IgG (Molecular Probes) for 1 hour. In addition, slides were co-stained with a PE-conjugated rat anti-human IL-6 monoclonal antibody (Clone MQ2-13A5, BD PharMin-

Figure 1. Induced expression of mRNA encoding the proinflammatory cytokine, IL-6, in bone tissue from a murine model of trauma-induced staphylococcal osteomyelitis. RNA was isolated from surgically damaged femur (lane 1), surgically damaged femur exposed to *S. aureus* (1×10^3) (lane 2), or untreated contralateral femur (lane 3) at 2 and 4 days postinfection (d.p.i.), and RT-PCR was performed for the presence of mRNA encoding IL-6. Amplified products electrophoresed on ethidium bromide-stained agarose gels are shown. In addition, PCR amplification of the housekeeping gene G3PDH was performed to ensure similar amounts of input RNA and similar efficiencies of reverse transcription. These experiments were performed twice with similar results.

gen), or PE-conjugated species and isotype matched control monoclonal antibodies (Clone R3-34, BD PharMingen) for 18 hours. For some sections, slides were not exposed to primary antibodies before addition of fluorochrome labeled secondary antibodies as an additional control. All slide preparations were illuminated and analyzed using an FLUOVIEW FV500 confocal laser scanning biological microscope (Olympus America Inc., Melville, NY).

Results

Elevated IL-6 mRNA Expression in Infected Bone Tissue in a Murine Model of Staphylococcal Osteomyelitis

Recent studies from our laboratory have indicated that cultured osteoblasts can express the key inflammatory cytokine IL-6 following exposure to bacterial pathogens associated with inflammatory bone disease.⁴ To begin to determine whether osteoblasts express IL-6 in response to challenge with *S. aureus* *in vivo*, we have used a murine model that reproduces the gross pathological phases of human posttraumatic osteomyelitis to investigate the expression of mRNA encoding this inflammatory mediator in infected bone tissue. At 2 and 4 days postsurgery, RNA was isolated from femurs that were untreated, mechanically damaged, or mechanically damaged and exposed to *S. aureus*, and RT-PCR was performed for the presence of mRNA encoding IL-6. As shown in the representative experiment in Figure 1, levels of mRNA encoding IL-6 were significantly elevated in bone tissue exposed to *S. aureus*, as compared to bone tissue that was damaged but uninfected, or bone tissue from the untreated contralateral limb. Differences in IL-6 mRNA levels could not be ascribed to differences in input RNA or to differences in the efficiencies of reverse transcription as evidenced by RT-PCR amplification of the housekeeping gene, G3PDH, for each sample (Figure 1). Rather, these findings are in agreement with previous demonstrations of local IL-6 production in bone tissue in both human and mouse osteomyelitis.^{9,15}

Figure 2. Expression of IL-6 in bone tissue from a murine model of staphylococcal osteomyelitis co-localizes with expression of the osteoblast cell marker osteocalcin. Tissue from *S. aureus*-infected bone (A–D) or uninfected sham-damaged bone tissue (E and F) was extracted and decalcified before staining. Samples were analyzed by confocal microscopy for the expression of osteocalcin (green fluorescence) and IL-6 (red fluorescence) (A–C, E, F) or irrelevant antigens (D) with light field reliefs. A shows osteocalcin signal alone, B and E show IL-6 fluorescence signals alone, while C and F show overlays of both signals with co-localized expression of both osteocalcin and IL-6 appearing yellow. D shows red and green signal overlays of infected murine bone tissue co-stained with isotype matched control antibodies. These experiments were performed on four separate animal preparations with similar results. Representative fields are shown under $\times 200$ magnification.

Expression of IL-6 by Murine Osteoblasts during In Vivo Infection as Determined by Confocal Microscopy

The presence of elevated mRNA encoding IL-6 in infected bone tissue does not establish the identity of the cell type(s) responsible for enhanced cytokine expression. Such expression may be induced in either resident bone cell types, such as osteoblasts and osteoclasts, or might result from the confounding presence of infiltrating leukocytes. Furthermore, elevated mRNA expression may not necessarily result in increases in IL-6 protein production. To determine whether IL-6 expression is elevated in osteoblasts, and to determine whether enhanced mRNA expression translates into IL-6 production, we have performed dual stained confocal immunohistochemical analyses of bone tissue from our murine model of osteomyelitis. Bone tissue from femurs that were untreated, mechanically damaged, or mechanically damaged and exposed to *S. aureus*, were processed and co-stained for IL-6 expression and the presence of the osteocalcin. Osteocalcin is an osteoblast-specific marker and has been widely used to define this cell type as such.^{4–7,16,17} As shown in the representative experiment

in Figure 2, sham infected bone tissue demonstrated orderly deposition of matrix and osteocalcin-positive osteoblasts (green fluorescence) (Figure 2F) and the absence of IL-6 expression (red fluorescence) (Figure 2, E and F). In contrast, bone tissue from femurs exposed to *S. aureus* demonstrated a high degree of disorganization characteristic of aberrant bone remodeling commonly associated with bacterial infections of bone tissue¹ (Figure 2A), and exhibited marked expression of IL-6 (Figure 2B). Importantly, IL-6 expression co-localizes with osteocalcin positive cells and is visualized as the yellow fluorescence in Figure 2C. As expected, this co-localization was particularly marked at the external surface of the bone cortex, an area known to contain a high density of osteoblasts (Figure 2C). Uninfected contralateral bone tissue in these animals failed to express detectable levels of IL-6 (data not shown), consistent with the localized application of *S. aureus* in these studies. Positive fluorescence could not be ascribed to nonspecific binding to murine or bacterial cells as determined by the absence of fluorescence seen in infected bone tissue stained with species and isotype matched control primary antibodies (Figure 2D) or in tissue exposed to the secondary antibody alone (data not shown). Taken together, these data

Figure 3. Expression of IL-6 in *S. aureus* infected neonatal murine calvaria bone tissue co-localizes with expression of the osteoblast cell marker osteocalcin. *S. aureus*-infected calvaria (A–D) or uninfected calvaria (E and F) were stained and analyzed by confocal microscopy for the expression of osteocalcin (green fluorescence) and IL-6 (red fluorescence) (A–C, E) or irrelevant antigens (D and F) with light field reliefs. A shows osteocalcin signal alone, B shows IL-6 fluorescence signal alone, while C and E show overlays of both signals with co-localized expression of both osteocalcin and IL-6 appearing yellow. D and F show red and green signal overlays of infected murine bone tissue co-stained with isotype matched control antibodies. These experiments were performed on three separate experimental series with similar results. Representative fields are shown under $\times 200$ magnification.

demonstrate that osteoblasts are a significant sources of the proinflammatory cytokine, IL-6, in a murine model of *S. aureus*-associated osteomyelitis.

S. aureus-Exposed Murine Osteoblasts Express IL-6 under in Situ-Like Tissue Culture Conditions

While the expression of IL-6 in infected murine bone tissue was predominantly associated with osteocalcin positive cells (Figure 2C), some cells expressing IL-6 were negative for the expression of this osteoblast marker. Such expression might be due to the presence of infiltrating immune cells or other resident bone cells, such as osteoclasts or their myeloid progenitors. To preclude the involvement of infiltrating leukocytes and to confirm that bone damage alone is not a sufficient stimulus for IL-6 expression in bone tissue, we have investigated the ability of *S. aureus* to initiate IL-6 expression in isolated murine calvaria under tissue culture conditions. These experiments allow the cell-cell interactions between resident bone cells that are likely to occur *in vivo* in the absence of immune cell involvement. As shown in a

representative experiment in Figure 3E, uninfected calvaria co-stained for the presence of osteocalcin and IL-6 demonstrated a high degree of organization and the absence of IL-6 production. In contrast, and in agreement with our findings using the *in vivo* model of bone infection, *S. aureus*-exposed calvaria display a disorganized appearance and the marked up-regulation of IL-6 production (Figure 3B). Again, expression of IL-6 showed marked co-localization with osteocalcin-positive osteoblasts (Figure 3C). Notably, few osteocalcin-negative cells expressing IL-6 are visible. While the identity of these rare IL-6 expressing cells is unclear, due to the absence of any infiltrating immune cells, it is likely that these cells are osteoclasts or their myeloid progenitors. Again, positive fluorescence could not be ascribed to nonspecific binding to murine or bacterial cells as determined by the absence of fluorescence seen in infected bone tissue stained with species and isotype matched control primary antibodies (Figure 3D) or in tissue exposed to the secondary antibody alone (data not shown). Taken together, these findings confirm the results seen in the *in vivo* model of murine bone infection and indicate that osteoblasts are a significant source of inflammatory cytokine production in bacterially infected bone tissue.

Human Osteoblasts Express IL-6 in *S. aureus*-Associated Osteomyelitis

To establish the clinical relevancy of our findings in murine bone tissue, we have analyzed human bone samples from uninfected individuals and debrided bone tissue from patients with *S. aureus*-associated staphylococcal osteomyelitis. Diseased and uninfected bone tissue samples were processed, co-stained for the presence of IL-6 and osteocalcin expression, and analyzed by confocal microscopy. As shown in the representative preparation in Figure 4D, uninfected bone tissue demonstrated orderly deposition of matrix and osteocalcin positive osteoblasts (green fluorescence) with no cell-associated IL-6 expression (red fluorescence). In contrast, diseased bone specimens demonstrated a high degree of disorganization and exhibited marked expression of IL-6 (Figure 4A). Importantly, IL-6 expression co-localized with osteocalcin positive cells as visualized by the yellow fluorescence in Figure 4B. Importantly, positive fluorescence could not be ascribed to nonspecific binding to human or bacterial cells as determined by the absence of fluorescence seen in infected bone tissue stained with species and isotype matched control primary antibodies (Figure 4C) or in tissue exposed to the secondary antibody alone (data not shown). Taken together, these findings are in agreement with earlier *in vitro* studies demonstrating the ability of cultured human osteoblasts to secrete IL-6 following exposure to bacterial pathogens associated with osteomyelitis.⁴ Furthermore, these findings mirror our *in vivo* and *in situ* experiments in murine bone tissue and strongly support the notion that bacterially exposed osteoblasts are a significant source of proinflammatory molecule production in response to bacterial challenges *in vivo*.

Discussion

Abnormal bone remodeling and chronic inflammation are commonly associated with bacterial infection of bone tissue, but the mechanisms responsible for such clinical observations are largely unclear.¹⁸ However, the generation of proinflammatory cytokines and chemokines is thought to be pivotal in the recruitment and activation of infiltrating immune cells and in the net balance between bone resorption and formation. Recent studies have demonstrated the local production of key inflammatory cytokines, such as IL-6, at sites of bacterial infection in human *S. aureus*-associated osteomyelitis¹⁵ and a murine model.⁹ While it is likely that infiltrating immune cells, such as monocytes/macrophages, are an important source of such molecules, our recent work has demonstrated the surprising ability of osteoblasts to produce significant amounts of an array of soluble immune mediators following *in vitro* exposure to clinically relevant bacterial pathogens. While osteoclasts might be anticipated to be capable of inflammatory mediator production due to their myeloid lineage, osteoblasts derive from a mesenchymal bone marrow precursor.^{19,20} As such, the ability of bacterially exposed osteoblasts to produce such mol-

ecules is unexpected. Importantly, the profile of immune mediator expression is highly consistent with a role for these cells in the initiation and/or maintenance of inflammation. Cultured human and murine osteoblasts respond to exposure to *S. aureus* and *Salmonella* with the production of chemokines, such as IP-10⁷ and monocyte chemoattractant protein-1,⁶ that can promote leukotaxis. In addition, bacterially challenged osteoblasts can express cytokines, including IL-6 and IL-12,⁴ that can direct appropriate immune responses by infiltrating leukocytes. Furthermore, infected osteoblasts produce growth factors, such as granulocyte-macrophage colony-stimulating factor,⁵ that can serve to enhance macrophage responses to bacterial pathogens. However, since these observations were made in isolated cultures of osteoblasts, the *in vivo* relevancy of these findings has remained in question.

In the present study we have extended our *in vitro* findings to a murine model that reproduces many of the pathological features of human trauma-induced staphylococcal osteomyelitis.⁸ We confirm the elevated expression of mRNA encoding the key proinflammatory cytokine, IL-6, in bacterially infected bone consistent with earlier studies in murine bone tissue¹⁵ and with our previous studies using isolated osteoblasts.⁴ Importantly, in the present study we provide the first *in vivo* evidence for osteoblasts as a significant source of such inflammatory mediator production in response to bacterial challenge. We have used confocal microscopy techniques to co-localize the expression of IL-6 in infected bone tissue with the presence of osteocalcin, a specific marker for osteoblasts. IL-6 production was observed in osteoblasts at infected sites in our *in vivo* murine model. Such production could not be attributed to nonspecific antibody binding due to the absence of positive staining in infected tissue stained with species and isotype matched control antibodies, and the absence of staining with the secondary antibody alone. This expression was not constitutive as assessed by analysis of the contralateral limb bone, and does not occur as a direct result of mechanical damage as uninfected sham damaged bone failed to display detectable IL-6 production. This conclusion was confirmed in studies using isolated murine calvaria that were infected *in vitro* but were otherwise undamaged. Furthermore, the use of isolated calvaria confirmed the production of IL-6 by bacterially exposed osteoblasts in a system that allows interaction between resident bone cells, but precludes the involvement of infiltrating leukocytes. Finally, and most importantly, we establish the clinical relevancy of our findings by demonstrating the *in vivo* production of IL-6 by osteoblasts in bone specimens from patients with *S. aureus*-associated osteomyelitis.

While a wide range of cell types, including non-leukocytic cells, have been shown to be capable of producing IL-6 (for review see ref. 21), the implications for the secretion of this cytokine by osteoblasts *in vivo* following exposure to this clinically important pathogen are significant. The production of inflammatory cytokines by infected osteoblasts may contribute to protective host immune responses to bone pathogens. The presence of IL-6 can directly augment the development of both hu-

Figure 4. Expression of IL-6 in human bone samples of patients with *S. aureus*-associated osteomyelitis co-localizes with expression of the osteoblast cell marker osteocalcin. Bone tissue from patients with *S. aureus*-associated osteomyelitis (A–C) or normal uninfected human bone tissue (D) were stained and analyzed by confocal microscopy for the expression of osteocalcin (green fluorescence) and IL-6 (red fluorescence) (A, B, D) or irrelevant antigens (C) with light field reliefs. A shows IL-6 fluorescence signal alone, while B and D show overlays of both osteocalcin and IL-6 signals with co-localized expression of both appearing yellow. C shows red and green signal overlays of infected human bone tissue co-stained with isotype matched control antibodies. These experiments were performed on four separate preparations of two infected bone samples and one healthy tissue sample with similar results. Representative fields are shown under $\times 200$ magnification.

moral and cell-mediated immune responses.²² In addition, IL-6 has recently been reported to play a role in blocking the suppressive activity of regulatory CD4⁺ CD25⁺ regulatory T-cells,²³ thereby enabling the progression of immune responses. Alternatively, immune molecule production by bacterially exposed osteoblasts may contribute to the development of the progressive inflammatory damage and aberrant bone remodeling associated with the pathology of staphylococcal osteomyelitis. IL-6 produced by osteoblasts can directly or indirectly modulate the activity of bone-resorptive osteoclasts,^{24–26} resulting in induction of osteoclast differentiation or osteoclast-mediated bone demineralization. As such, the ability of *S. aureus* to induce IL-6 production by osteoblasts *in vivo* may have important implications for bone formation or destruction in osteomyelitis.

The increasing incidence in bacterial infections caused by *S. aureus* and the emergence of strains of this organism that are resistant or show reduced susceptibility to antibiotics such as methicillin³ and vancomycin²⁷ have made understanding the pathogenesis of *S. aureus*-associated disease imperative. The present demonstration that bone-forming osteoblasts produce a key inflammatory mediator in both an animal model of staphylococcal osteomyelitis and in clinical specimens from patients suffering from this condition represents a potentially critical new role for osteoblasts during infection of bone tissues, namely, the ability to orchestrate immune responses in inflammatory bone diseases.

References

1. Lew DP, Waldvogel FA: Osteomyelitis. *N Engl J Med* 1997, 336:999–1007
2. Jensen AG, Espersen F, Skinhoj P, Rosdahl VT, Frimodt-Moller N: Increasing frequency of vertebral osteomyelitis following *Staphylococcus aureus* bacteraemia in Denmark 1980–1990. *J Infect* 1997, 34:113–118
3. Heseltine P: Has resistance spread to the community? *Clin Microbiol Infect* 2000, 2:11–16
4. Bost KL, Ramp WK, Nicholson N, Bento JL, Marriott I, Hudson MC: *Staphylococcus aureus* infection of mouse or human osteoblasts induces high levels of IL-6 and IL-12 production. *J Infect Dis* 1999, 180:1912–1920
5. Bost KL, Bento JL, Ellington JK, Marriott I, Hudson MC: Induction of colony stimulating factor expression following *Staphylococcus* or *Salmonella* interaction with mouse or human osteoblasts. *Infect Immun* 2000, 68:5075–5083
6. Bost KL, Bento JL, Petty CC, Schrum LW, Hudson MC, Marriott I: Monocyte chemoattractant protein-1 expression by osteoblasts following infection with *Staphylococcus aureus* or *Salmonella*. *J Interferon Cytokine Res* 2001, 21:297–304
7. Gasper NA, Petty CC, Schrum LW, Marriott I, Bost KL: Bacteria induced CXCL10 secretion by osteoblasts can be mediated, in part, through TLR4. *Infect Immun* 2002, 70:4075–4082
8. Spagnolo N, Greco F, Rossi A, Ciolli L, Teti A, Posteraro P: Chronic staphylococcal osteomyelitis: a new experimental rat model. *Infect Immun* 1993, 61:5225–5230
9. Yoshii T, Magara S, Miyai D, Nishimura H, Kuroki E, Furudoi S, Komori T, Ohbayashi C: Local levels of interleukin-1 β , -4, -6 and tumor necrosis factor α in an experimental model of murine osteomyelitis due to *Staphylococcus aureus*. *Cytokine* 2002, 19:59–65
10. Marriott I, Bost KL: Substance P diminishes lipopolysaccharide and interferon- γ -induced TGF- β 1 production by cultured murine macrophages. *Cell Immunol* 1998, 183:113–120
11. Marriott I, Bost KL, Mason MJ: Differential kinetics for induction of interleukin-6 mRNA expression in murine peritoneal macrophages: evidence for calcium-dependent and independent-signalling pathways. *J Cell Physiol* 1998, 177:232–240
12. Van Snick J, Cayphas S, Szikora JP, Renauld JC, Van Roost E, Boon T, Simpson RJ: cDNA cloning of murine interleukin-HP1: homology with human interleukin 6. *Eur J Immunol* 1988, 18:193–197
13. Tso JY, Sun XH, Kao TH, Reece KS, Wu R: Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* 1985, 13:2485–2502
14. Mori S, Sawai T, Teshima T, Kyogoku M: A new decalcifying technique for immunohistochemical studies of calcified tissue, especially applicable to cell surface marker demonstration. *J Histochem Cytochem* 1988, 36:111–114
15. Fullilove S, Jellis J, Hughes SP, Remick DG, Friedland JS: Local and systemic concentrations of tumour necrosis factor- α , interleukin-6 and interleukin-8 in bacterial osteomyelitis. *Trans R Soc Trop Med Hyg* 2000, 94:221–224
16. Rodan GA, Heath JK, Yoon K, Noda M, Rodan SB: Diversity of the osteoblastic phenotype. *Ciba Found Symp* 1988, 136:78–91
17. Marie PJ, Lomri A, Sabbagh A, Basle M: Culture and behavior of osteoblastic cells isolated from normal trabecular bone surfaces. *In Vitro Cell Dev Biol* 1989, 25:373–380
18. Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B: Bacterially induced bone destruction: mechanisms and misconceptions. *Infect Immun* 1996, 64:2371–80
19. Puzas JE: The osteoblast. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, ed 2. Edited by Favus MJ. New York, Raven Press, 1993, pp 15–21
20. Martin TJ, Ng KW: Mechanisms by which cells of the osteoblast lineage control osteoclast formation and activity. *J Cell Biochem* 1994, 56:357–366
21. Matsuda T, Hirano T: IL-6. *The Cytokine Reference*. Edited by Oppenheim, JJ, Feldmann M. London, Academic Press, 2000 (On-Line Database, DOI: 10.1006/rwcy.2000.0601)
22. Van Snick J: Interleukin-6: an overview. *Annu Rev Immunol* 1990, 8:253–578
23. Pasare C, Medzhitov R: Toll pathway-dependent blockade of CD4⁺CD25⁺ T cell-mediated suppression by dendritic cells. *Science* 2003, 299:1033–1036
24. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T, Hirano T, Kishimoto T, Suda T: IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol* 1990, 145:3297–3303
25. de la Mata J, Uy HL, Guise TA, Story B, Boyce BF, Mundy GR, Roodman GD: Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein *in vivo*. *J Clin Invest* 1995, 95:2846–2852
26. Greenfield EM, Shaw SM, Gornik SA, Banks MA: Adenyl cyclase and interleukin 6 are downstream effectors of parathyroid hormone resulting in stimulation of bone resorption. *J Clin Invest* 1995, 96:1238–1244
27. Tenover FC, Biddle JW, Lancaster MV: Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. *Emerg Infect Dis* 2001, 7:327–332