BMC Microbiology

BioMed Central

Research article

Open Access

Staphylococcus aureus - induced tumor necrosis factor - related apoptosis - inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts

Emily H Alexander, F Andrea Rivera, Ian Marriott, Juan Anguita, Kenneth L Bost and Michael C Hudson*

Address: Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223, USA

Email: Emily H Alexander - ehalexan@zoo.uvm.edu; F Andrea Rivera - farivera@email.uncc.edu; Ian Marriott - imarriot@email.uncc.edu; Juan Anguita - janguita@email.uncc.edu; Kenneth L Bost - klbost@email.uncc.edu; Michael C Hudson* - mchudson@email.uncc.edu

* Corresponding author

Published: 2 April 2003

BMC Microbiology 2003, **3**:5

Received: 17 December 2002

Accepted: 2 April 2003

This article is available from: http://www.biomedcentral.com/1471-2180/3/5

© 2003 Alexander et al; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: *Staphylococcus aureus* infection of normal osteoblasts induces expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

Results: Normal osteoblasts were incubated in the presence of purified bacterial products over a range of concentrations. Results demonstrate that purified surface structures and a selected superantigen present in the extracellular environment are not capable of inducing TRAIL expression by osteoblasts. Osteoblasts were co-cultured with *S. aureus* at various multiplicities of infection utilizing cell culture chamber inserts. Results of those experiments suggest that direct contact between bacteria and osteoblasts is necessary for optimal TRAIL induction. Finally, *S. aureus* infection of osteoblasts in the presence of anti-TRAIL antibody demonstrates that TRAIL mediates caspase-8 activation and apoptosis of infected cells.

Conclusions: Collectively, these findings suggest a mechanism whereby *S. aureus* mediates bone destruction via induction of osteoblast apoptosis.

Background

Staphylococcus aureus is the most common cause of osteomyelitis, a disease often refractory to treatment and subject to recurrence. Traditional treatment for osteomyelitis involves surgical debridement of infected tissue, coupled with systemic antibiotic therapy. Only a few decades ago, the majority of *S. aureus* strains associated with osteomyelitis were susceptible to antibiotics including methicillin. However, the emergence of antibiotic resistance among strains of *S. aureus* has rendered methicillin and many other antibiotics less effective against this organism. Consequently, there is an urgent need for the development of novel therapies for the treatment of osteomyelitis.

S. aureus is a capable bone pathogen, in part, because it possesses several cell surface adhesion molecules that facilitate its binding to bone matrix. Binding involves a family of adhesins that interact with extracellular matrix components, and these adhesins have been termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [1]. Specific MSCRAMMs are responsible for localization of *S. aureus* to particular tissues, and include fibronectin-binding proteins (FnBPs) [2,3], fibrinogen-binding proteins [4–6], elastin-binding adhesin [7] collagen-binding adhesion [8], and a broad-specificity adhesin (MAP) that facilitates low-affinity binding of *S. aureus* to several proteins, including osteopontin,

collagen, bone sialoprotein, fibronectin, fibrinogen, and vitronectin [9]. *S. aureus* can utilize MSCRAMMs to adhere directly to cells or can attach to cells through the use of bridging ligands such as Hsp60 which has an affinity for both MSCRAMMs and the β_1-integrin host cell receptor [10,11]. Interaction of *S. aureus* with host cells via MSCRAMMs can induce signal transduction, tyrosine kinase activity, and cytoskeletal rearrangement [12].

Compared to other bacteria, *S. aureus* is generally not considered a significant intracellular pathogen. Staphylococci have typically been regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix; however, there is growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells. *S. aureus* has been shown to be ingested by non-professional phagocytes, such as mouse fibroblasts [13], mouse renal cells [14], and bovine mammary epithelial cells [15,16]. *S. aureus* also has the ability to invade mouse and human osteoblast cell lines [17,18], as well as normal mouse and human osteoblasts [19]. Finally, *S. aureus* has been shown to invade normal chick osteoblasts both in vitro and in vivo [20,21]. *S. aureus* cells were found in approximately 14% of calvarial osteoblasts after subcutaneous injection of chick embryos, and in 11% of calvarial and tibial osteoblasts following intra-allantoic injection. Similar to most in vitro studies, the majority of intracellular bacteria are eventually free in the osteoblast cytoplasm in vivo. *S. aureus* cells in calvariae and tibiae were also observed in the cytoplasm of approximately 4% of the osteocytes in mineralized bone matrix. Therefore, osteoblasts containing internalized *S. aureus* cells continue differentiating into osteocytes. In addition, *S. aureus* invasion of osteoblasts stimulates the secretion of interleukin (IL)-6, IL-12, and colony-stimulating factors [19,22,23]. These cytokines can potentially exacerbate the bone destruction already characteristic of infection by activation of osteoclasts. In addition to osteoclast activation, *S. aureus* inhibits bone-matrix synthesis, further compounding bone loss observed with osteomyelitis [24].

The ability of *S. aureus* to survive in the eukaryotic intracellular environment could explain several aspects of chronic staphylococcal diseases and long-term colonization. Internalization may provide a means of protection against host defenses and certain classes of antibiotics. Many staphylococcal infections which tend to become chronic (e.g., osteomyelitis and mastitis) are associated with multiple recurrences and do not resolve even in the presence of what seems to be an adequate humoral immune response [25–28]. For example, patients can have recurrent attacks of osteomyelitis after completion of treatment, even when causative organisms cannot be isolated [25].

Staphylococcal infections are typically associated with death of tissue, and evidence suggests intracellular bacteria are capable of inducing apoptosis. *S. aureus*-mediated apoptosis has been reported in epithelial cells [16,29,30], keratinocytes [31], endothelial cells [32,33], and osteoblasts [34]. The two global gene regulatory loci *agr* and *sar* have been demonstrated to play a role in the induction of apoptosis in epithelial cells by *S. aureus* [33]. Mutants in the *agr* or *sar* loci are internalized, but do not induce apoptosis [33]. Another study demonstrated induction of apoptosis in endothelial cells by staphylococcal alpha toxin [32].

Wesson et al. [30] demonstrated host caspases-8 and -3 to play a role in*S. aureus*-induced apoptosis, and caspase-8 is known to be associated with apoptosis triggered by engagement of death receptors [35]. Several recent studies have associated microbial induction of apoptosis with the production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Measles virus was the first infectious agent shown to induce TRAIL production [36]. Other studies have demonstrated induction of TRAIL by Theiler's murine encephalomyelitis virus (TMEV) in M1-D cells (a myelomonocytic precursor cell line) [37], reovirus infection in fibroblasts [38], HIV-1 infection in $CD4^+$ T cells [39], and adenovirus in 239T cells [40]. The demonstration of caspase-8 involvement in *S. aureus*-induced apoptosis is complemented by a recent report from our laboratory that *S. aureus* induces expression of TRAIL by infected osteoblasts [41]. We reported that *S. aureus* strongly induces TRAIL expression by both normal mouse and normal human osteoblasts, while mRNAs encoding the death-domain-containing TRAIL receptors are constitutively expressed in the same cells. *S. aureus* caused a rapid and sustained up-regulation of TRAIL mRNA and TRAIL protein expression. TRAIL is also secreted by *S. aureus*-infected osteoblasts, suggesting that TRAIL could influence cells outside the environment of a localized bacterial infection.

In the present study, we examined the ability of purified bacterial surface structures and extracellular products to induce TRAIL expression in osteoblasts. The ability of TRAIL produced by *S. aureus*-infected osteoblasts to mediate osteoblast apoptosis was also examined. Results demonstrate that purified bacterial surface structures and *S. aureus* extracellular products present outside osteoblasts are not a sufficient stimulus for TRAIL induction, and that physical contact between bacteria and osteoblasts is required for optimal induction of TRAIL expression. TRAIL produced by *S. aureus*-infected osteoblasts does induce caspase-8 activation and apoptosis. Collectively, these results reveal TRAIL induction and caspase-8 activation as a mechanism whereby *S. aureus* mediates apoptosis in osteoblasts.

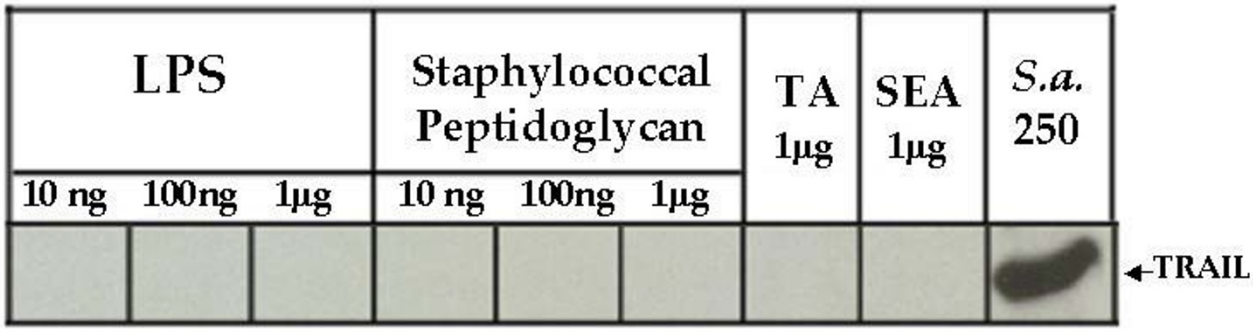

Figure 1
Western immunoblot analysis of TRAIL expression from normal human osteoblasts 24 h following incubation with LPS, peptidoglycan, teichoic acid (TA, 1 μg), staphylococcal enterotoxin A (SEA, 1 μg), or following infection with *S. aureus* at a MOI of 250:1 (S.a. 250). *S. aureus* infection was for 45 min, followed by washing and gentamicin treatment of osteoblasts for 24 h prior to protein isolation. Ten micrograms of total osteoblast protein were loaded for each lane.

Results

Purified bacterial products in the osteoblast extracellular environment do not induce TRAIL expression

In order to determine the role of S. aureus-derived peptidoglycan and teichoic acid in the expression of TRAIL by S. aureus-infected osteoblasts, cultured normal human osteoblasts were incubated with various concentrations of these purified cell wall structures (10 ng/ml, 100 ng/ml, or 1 μg/ml). Additionally, osteoblasts were incubated with various concentrations of staphylococcal enterotoxin A (SEA; 10 ng/ml, 100 ng/ml, or 1 μg/ml), used here as a representative superantigen from S. aureus, or with a purified component of gram-negative bacteria, specifically lipopolysaccharide (LPS; 10 ng/ml, 100 ng/ml, or 1 μg/ml). The concentrations of staphylococcal peptidoglycan, teichoic acid, SEA, and LPS tested did not induce TRAIL expression in osteoblasts (Fig. 1). Results for the lower concentrations of teichoic acid and SEA (10 ng/ml and 100 ng/ml) are not shown. As previously reported [41], TRAIL protein was induced in osteoblasts infected with S. aureus (Fig. 1). These results demonstrate that major surface components from these bacteria in a purified form, present in the osteoblast extracellular environment, are not sufficient to induce TRAIL expression in normal human osteoblasts.

Induction of TRAIL by S. aureus *requires physical contact between bacteria and osteoblasts*

Studies indicate that the invasion ability of bacteria are most relevant to the magnitude of the osteoblast response [42,43]. In previous experiments examining the production of TRAIL by *S. aureus*-infected osteoblasts, bacteria were in direct contact with the cells [41], such that the potential of *S. aureus* extracellular products to induce osteoblast expression and secretion of TRAIL could not be addressed. To determine if *S. aureus* extracellular products induce TRAIL expression in osteoblasts, normal mouse osteoblasts were grown in 12-well cluster plates. *S. aureus* strain UAMS-1 at a MOI of 25:1, 75:1, or 250:1 was then added separately to Transwell® cell culture chamber inserts. The inserts were placed into the 12-well plates containing osteoblasts followed by incubation for 45 minutes. The polycarbonate membrane at the base of each chamber insert contains pores 0.4 μm in size, and precludes direct interaction between the bacteria and osteoblasts. However, the inserts allow for the exchange of extracellular products. Direct physical contact between *S. aureus* and osteoblasts at a MOI of 250:1 induced osteoblast TRAIL protein expression (Fig. 2). TRAIL protein was not expressed in osteoblast cultures co-incubated with *S. aureus* at various MOIs where physical contact between the bacteria and osteoblasts was prevented (Fig. 2). These results demonstrate that physical contact between the host and pathogen is required for optimal TRAIL induction. The importance of bacterial internalization, however, could not be addressed in these experiments.

TRAIL induces apoptosis in osteoblasts

TRAIL may mediate a number of physiological responses; therefore, in order to determine if TRAIL expressed by *S. aureus*-infected osteoblasts mediates osteoblast apoptosis, cultured normal mouse osteoblasts were infected with *S. aureus* strain UAMS-1 at various MOIs in the presence and absence of an anti-human TRAIL polyclonal antibody. Osteoblasts were stained with annexin V and propidium iodide (PI). Redistribution of phosphotidylserine from

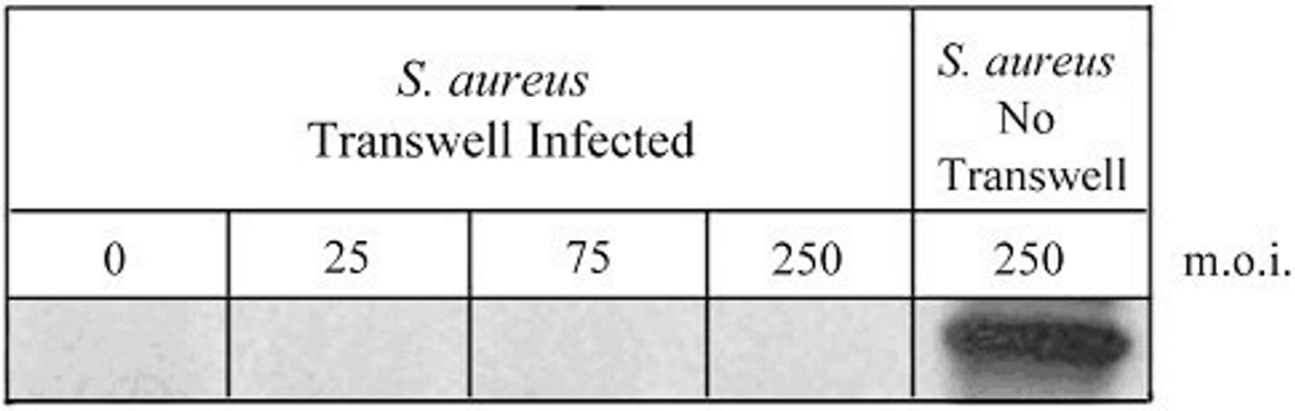

Figure 2
Western immunoblot analysis of TRAIL expression from normal mouse osteoblasts co-cultured with *S. aureus*. Normal mouse osteoblasts were grown in the lower chamber of Transwell plates. *S. aureus* at a MOI of 25:1, 75:1, or 250:1 was added separately to Transwell® cell culture chamber inserts. As a control, osteoblasts were directly infected with *S. aureus* at a MOI of 250:1. TRAIL expression was monitored at 45 minutes following the addition of bacteria. Twelve micrograms of total osteoblast protein was loaded for each lane.

the inner leaflet to the outer leaflet of the plasma membrane occurs early and is a hallmark of the apoptotic process. Annexin V has a high affinity for phosphatidylserine and was used to detect apoptotic cells. Propidium iodide can be used to distinguish early apoptotic cells from late apoptotic cells. Early apoptotic cells stain positive for annexin V and negative for PI, where as late apoptotic cells stain positive for both annexin V and PI [44]. After staining, osteoblasts were analyzed using FACS (Fig. 3). Approximately 14.89% of the cells from uninfected osteoblast cultures were annexin V positive. The percentage of annexin V-positive cells increased to 32.17% following infection with *S. aureus* at a MOI of 75:1, corresponding to a greater than 2-fold increase over that of uninfected control osteoblast cultures. A 25% reduction in annexin V-positive cells was observed in the presence of 1 μg/ml of anti-human TRAIL antibody. A 2 μg/ml concentration of anti-TRAIL antibody further reduced the population of annexin V-positive cells to 25.42% of total osteoblasts, corresponding to an approximately 40% decrease in the level of annexin V-positive cells in *S. aureus*-infected cultures. A dose-dependent decrease in annexin V-positive cells was observed by the addition of increasing amounts of the anti-human TRAIL antibody. These results strongly suggest TRAIL production induces osteoblast apoptosis in *S. aureus*-infected osteoblast cultures. The role of TRAIL in *S. aureus*-induced apoptosis is likely more significant than what is revealed by these experiments, since an anti-human TRAIL antibody was used to neutralize mouse TRAIL protein. Utilization of an anti-mouse TRAIL antibody would be predicted to further reduce the level of *S. aureus*-induced apoptosis in infected mouse osteoblast cultures.

S. aureus *induces caspase-8 activation in osteoblasts*

S. aureus-mediated apoptosis has been reported for a variety of cells including osteoblasts. A recent report demonstrated that *S. aureus*-induced apoptosis of epithelial cells involves activation of caspases-8 and-3 [30]. To determine whether caspase-8 is activated in *S. aureus*-infected osteoblasts, cultured normal mouse osteoblasts were infected with *S. aureus* strain UAMS-1 at various MOIs. As a positive control, osteoblasts were stimulated with cycloheximide (CHX), a compound known to induce caspase-8 activation [45]. At various times post-infection, endogenous caspase-8 activity was quantified.

Activated caspase-8 was detected in uninfected control osteoblast cultures (Fig. 4) and is attributable to apoptosis and cell turnover in the in vitro osteoblast tissue culture system. Osteoblasts infected with *S. aureus* at a MOI of 75:1 demonstrated a 2-fold increase in activated caspase-8 45 min post-infection, which was significantly higher than the level of activated caspase-8 in the uninfected control ($p < 0.05$) (Fig. 4). This early activation of caspase-8 in *S. aureus*-infected osteoblasts coincides with previous observations in other systems. For example, caspase-8 is activated in HeLa cells within 1 h following the administration of TRAIL [46]. The level of caspase-8 activation in osteoblasts exposed to CHX, a known activator of caspase-

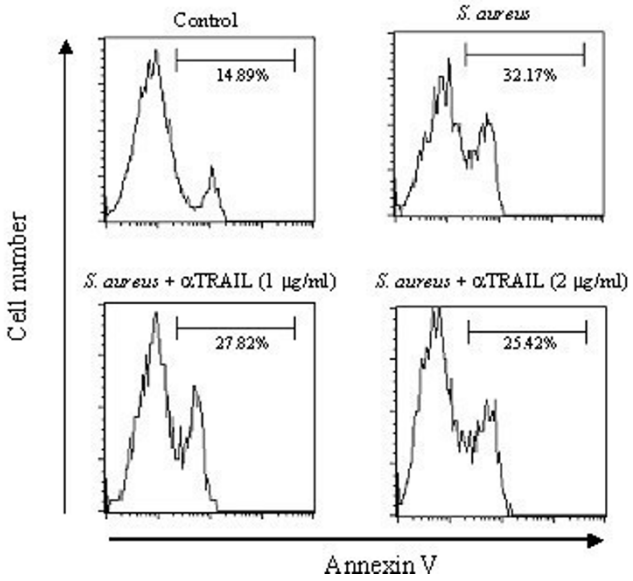

Figure 3
FACS analysis of normal mouse osteoblasts infected with *S. aureus* at a MOI of 75:1 in the presence or absence of anti-human TRAIL antibody (1.0 or 2.0 μg/ml). Following a 45-minute incubation, osteoblasts were washed 3 times with HBSS and incubated in medium containing 25 μg of gentamicin per ml to kill extracellular bacteria. Cells were stained with annexin V and propidium iodide 24 hours post-infection and analyzed using FACS. Results are presented from one osteoblast culture, and are representative of results from three different osteoblast preparations.

8 [45], was significantly higher than the level of activity in uninfected cells and was equivalent to the level in *S. aureus*-infected osteoblasts (Fig. 4). *S. aureus*-induced caspase-8 activation was sustained for 2 h following infection (data not shown). In fact, the levels of activated caspase-8 were significantly higher at 2 h than at 45 min post-infection in *S. aureus*-infected osteoblasts.

Caspase-8 has been identified as an initiator caspase activated in death receptor-mediated apoptosis [47]. Therefore, we investigated whether blockade of TRAIL expressed by *S. aureus*-infected osteoblasts had any effect on the levels of caspase-8 enzymatic activity. Mouse osteoblasts were infected with *S. aureus* in the presence of increasing concentrations of anti-human-TRAIL antibody. Figure 4 demonstrates that anti-TRAIL antibody interferes with caspase-8 activation. A clear dose-response was observed in the levels of caspase-8 enzymatic activity. As the concentrations of the anti-TRAIL antibody increase, the levels of caspase-8 specific activity decrease. The highest concentration of anti-TRAIL antibody utilized significantly reduced the level of caspase-8 enzymatic activity in *S. aureus*-infected osteoblast cultures at 45 min and 2 h post-infection ($p < 0.05$) (Fig. 4 and data not shown). These results suggest that caspase-8 activation is mediated by TRAIL, and that induction of apoptosis by TRAIL utilizes an intracellular mechanism involving caspase-8 activation.

Discussion

TRAIL is a member of the TNF gene superfamily of receptors and ligands and was discovered based on sequence homology to other members of the superfamily using an EST database [48]. Among other family members, TRAIL is most similar to Fas ligand, sharing 28% homology. TRAIL can be expressed as a membrane-bound protein or solubilized by metalloendoproteinase cleavage. The bioactive form of TRAIL is as a homotrimer [49]. There are five known human TRAIL receptors, including R1, R2, R3, R4, and osteoprotegerin (OPG); however, R1 and R2 are the only TRAIL receptors known to contain complete death domains. The signaling pathways of TRAIL and its receptors are not fully known [50]; however, recent studies suggest TRAIL activates pathways similar to those activated by TNF and its receptors [49]. Binding of TRAIL to death-domain containing receptors can cause apoptosis via activation of caspase-8 in Jurkat and HeLa cells [46,49].

We have previously demonstrated that *S. aureus* induces TRAIL expression in normal human and mouse osteoblasts [41]. Messenger RNA encoding TRAIL is rapidly and dramatically up-regulated following exposure of osteoblasts to *S. aureus*. TRAIL mRNA expression is absent in uninfected cultures and shows a dose-dependent response to *S. aureus*. In contrast to the inducible nature of TRAIL mRNA, the messages encoding death-domain-containing TRAIL receptors are constitutively expressed in human and mouse osteoblasts [41]. Additionally, TRAIL protein is expressed and secreted following infection of osteoblasts with *S. aureus*. In contrast, uninfected osteoblasts do not express significant amounts of TRAIL protein.

The current study examined possible mechanisms whereby *S. aureus* induces TRAIL expression in osteoblasts. Osteoblasts were incubated with various concentrations of major surface components of *S. aureus*, peptidoglycan and teichoic acid, as well as LPS of gram-negative bacteria to determine if these were potential inducers of TRAIL expression. These bacterial components were chosen based on their ability to induce inflammatory responses. The two major components of the staphylococcal cell wall, peptidoglycan and lipoteichoic acid have been reported to produce inflammation [51,52] and specifically to induce arthritis [53]. Bacterial peptidoglycan is also known to stimulate the bone-resorbing activity of macrophages [54]. Furthermore, staphylococci produce an array of

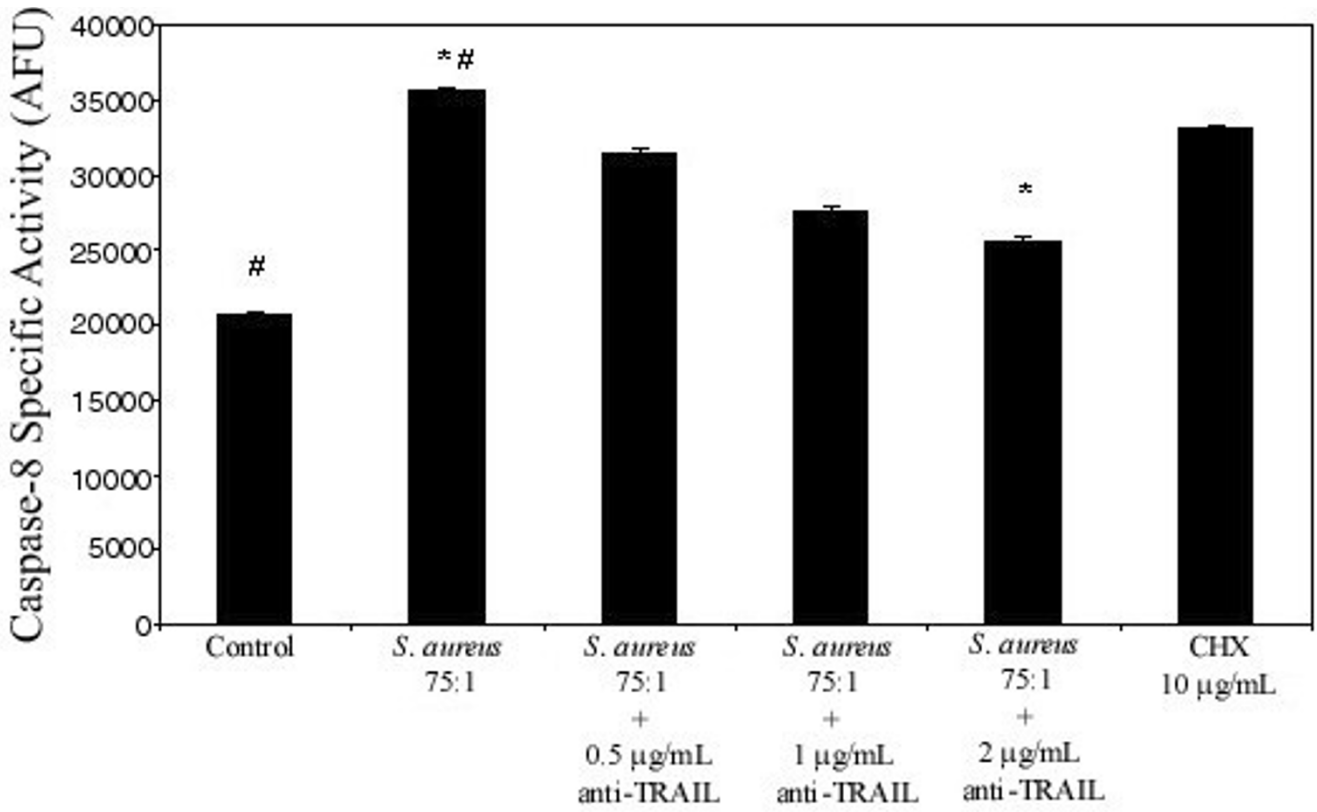

Figure 4
Caspase-8 is activated in normal mouse osteoblasts infected with *S. aureus* at a MOI of 75:1, or following incubation with 10 μg/ml cycloheximide (CHX). Caspase-8 enzymatic activity was analyzed 45 min following infection. *S. aureus*-infected osteoblasts were incubated for 45 min in the presence of increasing concentrations of anti-human TRAIL antibody (0.5 μg/ml, 1.0 μg/ml, and 2.0 μg/ml). Values are presented as the mean +/- standard deviation from three independent osteoblast cultures. Data were analyzed utilizing the Student's t-test, *,# $P < 0.05$.

secreted products capable of inducing or sustaining inflammation. For example, the staphylococcal superantigens induce prolonged inflammatory responses associated with arthritis and destruction of bone connective tissue [55].

Our results demonstrate that peptidoglycan, teichoic acid, LPS, and the staphylococcal superantigen staphylococcal enterotoxin A (SEA), when present in the osteoblast extracellular environment, do not induce TRAIL expression (Fig. 1). These findings do not eliminate the possibility that these bacterial structures contribute to TRAIL induction. They are nevertheless insufficient by themselves to elicit such a response in osteoblasts exposed extracellularly to the molecules at the concentrations analyzed. The concentrations of bacterial products chosen were based on previous reports suggesting these amounts are appropriate and sufficient to elicit cytokine responses from human cells [56,57]. It is possible that one or more of the purified products analyzed contribute to TRAIL induction, but only if expressed inside osteoblasts.

While intracellular *S. aureus* is known to induce TRAIL expression in osteoblasts [41], the ability of extracellular staphylococcal products to induce TRAIL expression has not been previously investigated. Results from the Transwell® co-culture experiments (Fig. 2) demonstrate that extracellular bacterial products do not induce TRAIL expression. *S. aureus* at a MOI of 25:1, 75:1, or 250:1 was unable to induce TRAIL expression when direct contact between bacteria and osteoblasts was precluded. Even at the highest MOI used, no TRAIL was detected in response to the co-incubation with*S. aureus*. The bacteria must be in physical contact, and potentially internalized by the osteoblasts, before TRAIL expression is induced. These findings could indicate that attachment of *S. aureus* to osteoblasts

initiates a cascade ultimately inducing TRAIL expression. Alternatively, intracellular bacteria may be the only *S. aureus* cells capable of inducing TRAIL expression by osteoblasts.

TRAIL clearly mediates cell death in other tissues, but also has the potential to mediate a variety of other physiological responses in bone. Osteoblasts are known to secrete tumor necrosis factor-related activation-induced cytokine (TRANCE) (RANK-L), which acts on the TRANCE receptor (TRANCE-R) (RANK) found on osteoclast precursors. Binding of TRANCE to TRANCE-R induces osteoclastogenesis and therefore bone resorption. Osteoblasts are also known to secrete osteoprotegerin (OPG), which binds TRANCE, thus inhibiting osteoclastogenesis. Because OPG is a soluble receptor for TRAIL [58], *S. aureus*-induced TRAIL secretion could have a profound effect on both osteoblasts and osteoclasts. Therefore, the ability of TRAIL expressed by *S. aureus*-infected osteoblasts to mediate osteoblast apoptosis was examined.

S. aureus induces osteoblast apoptosis [34]. When osteoblasts were exposed to *S. aureus* at a MOI of 75:1, the percentages of annexin V-positive cells increased from 14.89% to 32.17% of cells in culture (Fig. 3). Numbers of annexin V-positive cells were reduced 40% by the addition of anti-TRAIL antibody (2 μg/ml) to *S. aureus*-infected osteoblast cultures. While the antibodies did not completely inhibit *S. aureus*-induced apoptosis, these results demonstrate that TRAIL contributes to apoptosis in *S. aureus*-infected cultures. TRAIL-mediated apoptosis of *S. aureus*-infected osteoblasts suggests a mechanism whereby *S. aureus* induces the bone loss and destruction associated with osteomyelitis. The inability of the anti-TRAIL antibodies to completely block apoptosis induced by *S. aureus* may be attributable to the specificity of the antibody used for these experiments or the dosage used, since an anti-human TRAIL antibody was used to neutralize mouse TRAIL protein. The role of TRAIL in *S. aureus*-induced apoptosis is therefore likely to be more significant than what is revealed by these experiments. Utilization of an anti-mouse TRAIL antibody would be predicted to be more effective in reducing the level of *S. aureus*-induced apoptosis in infected mouse osteoblast cultures. In addition, *S. aureus* may also induce apoptosis by a mechanism distinct from TRAIL. It is known, for example, that *S. aureus* α-toxin induces apoptosis in Jurkat cells via the Bcl-2-controlled mitochondrial death pathway [32]; therefore, neutralization of TRAIL would not be expected to interfere with this mechanism of apoptosis induction.

TRAIL-induced apoptosis involves activation of caspase-8 in other systems. Earlier studies have shown that blockade of caspase-8 activation in HeLa cells and deletion of caspase-8 in Jurkat cells prevented activation of downstream signaling, resulting in a failure of the cells to undergo apoptosis in response to TRAIL [46]. Similarly, another group has demonstrated that apoptosis induced by *S. aureus* in bovine mammary epithelial (MAC-T) cells utilizes a mechanism involving caspases-8 and -3, which are two key components of the proteolytic cascade that leads to apoptosis [30]. Results from the current study demonstrate a two-fold increase in caspase-8 activation upon infection with *S. aureus* (Fig. 4). Active caspase-8 was detected in uninfected control osteoblast cultures (Fig. 4), and is attributable to apoptosis and cell turnover in the in vitro osteoblast tissue culture systems. The level of activated caspase-8 in *S. aureus*-infected osteoblasts was significantly higher than the level of activated caspase-8 in uninfected osteoblast control cultures ($p < 0.05$). Caspase-8 activation in osteoblasts exposed to CHX was also higher than the level of activity in uninfected cells, but equivalent to the level in infected osteoblasts (Fig. 4). These results demonstrate that *S. aureus* is a potent inducer of caspase-8 activation in osteoblasts, equivalent to a known inducer of caspase-8 [45]. Overall, the levels of caspase-8 enzymatic activity at 2 h were significantly higher compared to the ones obtained 45 min post-infection (data not shown). Neutralization of TRAIL by the addition of the anti-TRAIL antibody interfered with caspase-8 activation, demonstrating a dose-dependent response. As the concentrations of the anti-TRAIL antibody increased, the levels of caspase-8 activation decreased. The highest concentration of antibodies used significantly reduced the level of caspase-8 activation in *S. aureus*-infected osteoblast cultures ($p < 0.05$). These findings demonstrate that activation of caspase-8 in *S. aureus*-infected osteoblasts is at least in part TRAIL-dependent.

Conclusions

Collectively, these findings reveal that *S. aureus* induces apoptosis in osteoblasts via a mechanism involving TRAIL induction and caspase-8 activation. Induction of TRAIL by *S. aureus* requires the bacteria to be in contact with and potentially internalized by the osteoblasts. Although the exact mechanism whereby *S. aureus* induces TRAIL expression remains to be determined, this study suggests that major bacterial surface structures present in the osteoblast extracellular environment do not induce TRAIL. Since TRAIL mediates a number of physiological responses, the impact of *S. aureus*-induced TRAIL expression on normal bone physiology should be explored. The role of *S. aureus*-induced TRAIL in apoptosis and osteoclastogenesis in vivo is currently being examined.

Methods

Normal mouse osteoblast cell culture

Normal osteoblast cell cultures were prepared from mouse neonates according to a method previously described for chicken embryos [59]. Bone-forming cells were

isolated from mouse neonate calvariae by sequential collagenase and protease digestions. The periostea were removed, the frontal bones were harvested free of the suture regions, and the bones were incubated for 10 min at 37°C in 10 ml of digestion medium, containing collagenase (375 U/ml, type VII; Sigma Chemical Company, St. Louis, Mo.) and protease (7.5 U/ml; Sigma). The digestion medium and released cells were removed and discarded. Ten milliliters of fresh digestion medium was added, and incubation was continued for 20 min. Cells were harvested by centrifugation and rinsed 3 times in 25 mM HEPES-buffered Hanks' balanced salt solution (pH 7.4; HBSS). The digestion step was repeated twice, and the 3 cell isolates were pooled in mouse osteoblast growth medium (OBGM), consisting of Dulbecco's modified Eagle's medium containing 25 mM HEPES, 10% fetal bovine serum (Sigma), 2 g of sodium bicarbonate per liter, 75 μg of glycine/ml, 100 μg of ascorbic acid/ml, 40 ng of vitamin B_{12}/ml, 2 μg of *p*-aminobenzoic acid/ml, 200 ng of biotin/ml, and penicillin (100 U/ml)-streptomycin (100 μg/ml)-amphotericin B (25 μg/ml) (pH 7.4) [60]. Cells were seeded in six-well cluster plates and incubated at 37°C in a 5% CO_2 atmosphere until they reached confluence (6 to 7 days).

Characterization of normal mouse osteoblasts

Mouse osteoblasts were grown on glass coverslips in 24-well plates until they were confluent; they were then fixed and permeabilized using CytoFix/CytoPerm according to the methods recommended by the manufacturer (PharMingen, San Diego, Calif.). Rabbit antibodies specific for osteocalcin (1:100 dilution; Peninsula Laboratories, Belmont, Calif.), type I collagen (1:40 dilution; Chemicon, Temecula, Calif.), 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 immunoglobulin G antibody (1:50 dilution; Sigma) was added with incubation for 45 min at 4°C. After the samples were washed, at least 500 cells were scored for positive fluorescence using an Olympus BX60 fluorescence microscope. Osteocalcin, type I collagen, and alkaline phosphatase were selected for analysis, since the expression of these proteins has been used to define osteoblasts as such [61–63].

Normal human osteoblast cultures

Normal human osteoblasts (Clonetics, San Diego, Calif.) were propagated according to the guidelines provided by the vendor. Cells were seeded in 25-cm^2 flasks and incubated at 37°C in a 5% CO_2 atmosphere in medium supplied by the manufacturer; this medium contains 10% fetal bovine serum, ascorbic acid, and gentamicin. After the cells reached approximately 80% confluence (5 to 9 days), they were removed from flasks with 0.025% trypsin-0.01% EDTA, washed in medium, and seeded into six-well plates. Cells were used as described below once they reached approximately 80% confluence (6 to 7 days). These commercially available cells have been extensively characterized as osteoblasts [61,64].

Bacterial strain and growth conditions

S. aureus strain UAMS-1 (ATCC 49230) (osteomyelitis clinical isolate) was grown overnight in 5 ml of tryptic soy broth at 37°C with aeration. Bacteria were harvested by centrifugation for 10 min at 4,300 × *g* at 4°C and washed in 5 ml of HBSS. Bacteria were then resuspended in mouse OBGM.

Infection and invasion assay

Following resuspension of bacteria in OBGM, bacterial cell density was determined via spectrophotometric analysis. Cells were then diluted in OBGM to obtain the desired multiplicity of infection (MOI). Osteoblasts were infected with *S. aureus* at a MOI of 25:1, 75:1, or 250:1 (*S. aureus* organisms to osteoblasts). The highest MOIs used resulted in approximately one internalized bacterium per osteoblast in culture (data not shown). Following a 45 min infection, osteoblasts were washed three times with HBSS and incubated in medium containing 25 μg of gentamicin per ml to kill extracellular bacteria. At various times following bacterial invasion, osteoblasts were lysed and total osteoblast protein isolated for further analysis.

Osteoblast lysis and protein isolation

Osteoblasts were washed twice with phosphate-buffered saline (PBS) pH 7.4. One ml of PBS was then added to each tissue culture well and osteoblasts were harvested by scraping. Osteoblasts in PBS were transferred to microcentrifuge tubes and collected by centrifugation at 13,600 × *g* for 3 min at 4°C using a Fisher Scientific Micro-Centrifuge. Pellets were washed with 1 ml of PBS and then resuspended in 100 μl of whole extract lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 25 mM β-glycerolphosphate pH 7.4, 2 mM PPiNa, 2 mM EDTA pH 7.4, 1% Triton X-100, and 10% glycerol). Following the addition of lysis buffer, samples were incubated on ice for 15 min and then centrifuged at 13,600 × *g* for 5 min at 4°C. Cell lysates were transferred to fresh microcentrifuge tubes. Protein concentrations of the cell lysates were quantified as described [65] and the samples then stored at -80°C until analyzed by western immunoblot techniques.

Exposure of osteoblasts to purified bacterial products

Cultured normal human osteoblasts were seeded into 6-well cluster plates in medium provided by the manufacturer and incubated at 37°C in a 5% CO_2 atmosphere. After the cells reached approximately 80% confluence (5 to 9 days), they were treated with purified lipopolysaccharide (LPS), staphylococcal peptidoglycan, teichoic acid, or

staphylococcal enterotoxin A (Sigma Chemical Company, St. Louis, Mo.) over a range of concentrations. Following a 24 h exposure, osteoblasts were lysed and total osteoblast proteins isolated and analyzed using western immunoblot techniques.

Transwell co-cultures of S. aureus *and normal mouse osteoblasts*

Normal mouse osteoblasts were grown in 12-well cluster plates containing 1.5 mL of growth medium per well. *S. aureus* at an MOI of 25:1, 75:1, or 250:1 was added separately to Transwell® cell culture chamber inserts in 0.5 ml of growth medium. Inserts were placed into the 12-well plates containing osteoblasts for 45 minutes. The polycarbonate membrane at the base of each chamber insert contains pores 0.45 μm in size and precludes direct interaction between the bacteria and osteoblasts. However, the pore size allows for the exchange of extracellular products. Following the 45-minute incubation, chamber inserts were removed, osteoblasts lysed and proteins analyzed using western immunoblot techniques.

FACS analysis of apoptosis

The Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OG) was used to detect osteoblast apoptosis. Briefly, normal mouse osteoblasts were infected with *S. aureus* strain UAMS-1 at various MOI in the presence and absence of an anti-TRAIL polyclonal antibody (rabbit anti-human TRAIL/Apo2L, Cell Sciences, Inc., Norwood, MA). Following a 45-minute incubation, osteoblasts were washed 3 times with HBSS and incubated in medium containing 25 μg of gentamicin per ml to kill extracellular bacteria. Anti-TRAIL antibody was added to the cultures again to neutralize any TRAIL produced by the osteoblasts in response to the infection. At various times post-infection, osteoblasts were incubated with 0.025% trypsin-0.01% EDTA for 10 min at 37°C, collected by centrifugation, washed with PBS, and stained with Alexa Fluor 488 annexin V and propidium iodide according to published guidelines. Cells were analyzed using a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA).

Detection of Caspase-8 activity

Caspase-8 enzymatic activity was detected using a caspase-8 fluorometric kit (R&D Systems, Minneapolis, MN) according to published guidelines. Briefly, cultured normal mouse osteoblasts were infected with *S. aureus* in the presence or absence of anti-TRAIL polyclonal antibody as described above or stimulated with cychloheximide (10 μg/ml). At various time points, osteoblasts were lysed with lysis buffer and total protein concentration determined as described [65]. Cell lysates were incubated with the caspase-8 fluorogenic substrate (IETD-AFC) at 37°C for 2 h. Sample fluorescence was detected using a CytoFluor multi-well plate reader (Applied Biosystems, Foster City, CA), where the amount of fluorescence directly corresponds to the amount of caspase-8 activity.

SDS-PAGE and western immunoblot analysis

Equivalent amounts of protein from the cell lysate fractions described above were mixed with concentrated 2X sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (0.125 M Tris-HCL [pH 6.8], 4% [wt/vol] SDS, 10% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue), boiled for 5 min, and cleared by centrifugation at 13,600 × *g* for 5 min. Osteoblast proteins were separated by SDS-PAGE as described by Laemmli [66]. The proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Fisher Biotech), using the Mini Trans-Blot apparatus (BioRad, Hercules, CA) at 300 mA for 1 h at 4°C according to manufacturer's recommendations. Membranes were blocked with Buffer A (Tris-buffered saline (TBS) (Bio-Rad) containing 5.0% skim milk and 0.5% Tween 20 (Sigma)) for 1 h with gentle shaking, then washed with Buffer B (TBS containing 0.1% Tween 20) 3 times for 5 min each. A two-step detection method was used to identify TRAIL. Blots were first incubated overnight with gentle shaking at 4°C with rabbit anti-human TRAIL polyclonal antibodies (Cell Sciences, Inc., Norwood, MA) at a concentration of 0.2 μg/ml in Buffer A. Membranes were then washed the following day 3 times for 5 min in Buffer B with gentle shaking, then incubated for 1 h with gentle shaking at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (Amersham Biosciences Corp., Piscataway, NJ) diluted 1:5000 in Buffer A. Reactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) following exposure to X-ray film and subsequent film development.

Authors Contributions

EHA carried out the studies to examine the influence of purified bacterial products and cell culture inserts, and measures of apoptosis

FAR performed the caspase studies

IM performed FACS analysis

JA performed FACS analysis

KLB conceived of the design of the study

MCH conceived of the design of the study, and coordinated the experiments

All authors read and approved the final manuscript

Acknowledgements

This work was supported by grant F900 from the Aircast Foundation, Inc. (M.C.H.), and by grants AR 47585 and AI 48842 from the National Institutes of Health (I. M.)

References

1. Patti JM, Allen BL, McGavin MJ and Hook M **MSCRAMM-mediated adherence of microorganisms to host tissues** *Annu Rev Microbiol* 1994, **48:**585-617
2. Hynes RO **Fibronectins** *Springer, New York Berlin Heidelberg* 1990,
3. Jonsson K, Signas C, Muller HP and Lindberg M **Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene** *Eur J Biochem* 1991, **202:**1041-1048
4. Boden MK and Flock JI **Cloning and characterization of a gene for a 19 kDa fibrinogen-binding protein from *Staphylococcus aureus*** *Mol Microbiol* 1994, **12(4):**599-606
5. Cheung AI, Projan SJ, Edelstein RE and Fischetti VA **Cloning, expression, and nucleotide sequence of a *Staphylococcus aureus* gene (fbpA) encoding a fibrinogen-binding protein** *Infect Immun* 1995, **63(5):**1914-1920
6. McDevitt D, Francois P, Vaudaux P and Foster TJ **Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*** *Mol Microbiol* 1994, **11(2):**237-248
7. Park PW, Rosenbloom J, Abrams WR and Mecham RP **Molecular cloning and expression of the gene for elastin-binding protein (ebpS) in *Staphylococcus aureus*** *J Biol Chem* 1996, **271(26):**15803-15809
8. Patti JM, Jonsson H, Guss B, Switalski LM, Wiberg K, Lindberg M and Hook M **Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin** *J Biol Chem* 1992, **267(7):**4766-4772
9. McGavin MH, Krajewska-Pietrasik D, Ryden C and Hook M **Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity** *Infect Immun* 1993, **61(6):**2479-2485
10. Dziewanowska K, Carson AR, Patti JM, Deobald CF, Bayles KW and Bohach GA **Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells** *Infect Immun* 2000, **68(11):**6321-6328
11. Shakoori AR, Oberdorf AM, Owen TA, Weber LA, Hickey E, Stein JL, Lian JB and Stein GS **Expression of heat shock genes during differentiation of mammalian osteoblasts and promyelocytic leukemia cells** *J Cell Biochem* 1992, **48(3):**277-287
12. Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR and Bohach GA **Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells** *Infect Immun* 1999, **67(9):**4673-4678
13. Usui A, Murai M, Seki K, Sakurada J and Masuda S **Conspicuous ingestion of *Staphylococcus aureus* organisms by murine fibroblasts in vitro** *Microbiol Immunol* 1992, **36(5):**545-550
14. Murai M, Usui A, Seki K, Sakurada J and Masuda S **Intracellular localization of *Staphylococcus aureus* within primary cultured mouse kidney cells** *Microbiol Immunol* 1992, **36(5):**431-443
15. Almeida RA, Matthews KR, Cifrian E, Guidry AJ and Oliver SP ***Staphylococcus aureus* invasion of bovine mammary epithelial cells** *J Dairy Sci* 1996, **79(6):**1021-1026
16. Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA and Trumble WR **Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells** *Infect Immun* 1998, **66(1):**336-342
17. Ellington JK, Reilly SS, Ramp WK, Smeltzer MS, Kellam JF and Hudson MC **Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts** *Microb Pathog* 1999, **26(6):**317-323
18. Jevon M, Guo C, Ma B, Mordan N, Nair SP, Harris M, Henderson B, Bentley G and Meghji S **Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts** *Infect Immun* 1999, **67(5):**2677-2681
19. Bost KL, Ramp WK, Nicholson NC, Bento JL, Marriott I and Hudson MC ***Staphylococcus aureus* infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production** *J Infect Dis* 1999, **180(6):**1912-1920
20. Hudson MC, Ramp WK, Nicholson NC, Williams AS and Nousiainen MT **Internalization of *Staphylococcus aureus* by cultured osteoblasts** *Microb Pathog* 1995, **19(6):**409-419
21. Reilly SS, Hudson MC, Kellam JF and Ramp WK **In vivo internalization of *Staphylococcus aureus* by embryonic chick osteoblasts** *Bone* 2000, **26(1):**63-70
22. Bost KL, Bento JL, Ellington JK, Marriott I and Hudson MC **Induction of colony-stimulating factor expression following Staphylococcus or Salmonella interaction with mouse or human osteoblasts** *Infect Immun* 2000, **68(9):**5075-5083
23. Yao L, Bengualid V, Lowy FD, Gibbons JJ, Hatcher VB and Berman JW **Internalization of *Staphylococcus aureus* by endothelial cells induces cytokine gene expression** *Infect Immun* 1995, **63(5):**1835-1839
24. Lerner UH, Modeer T, Krekmanova L, Claesson R and Rasmussen L **Gingival crevicular fluid from patients with periodontitis contains bone resorbing activity** *Eur J Oral Sci* 1998, **106(3):**778-787
25. Craigen MA, Watters J and Hackett JS **The changing epidemiology of osteomyelitis in children** *J Bone Joint Surg Br* 1992, **74(4):**541-545
26. Greer RBI and Rosenberg AE **Case records of the Massachusetts General Hospital** *Case 6 N Engl J Med* 1993, **328:**422-428
27. Korovessis P, Fortis AP, Spastris P and Droutsas P **Acute osteomyelitis of the patella 50 years after a knee fusion for septic arthritis. A case report** *Clin Orthop* 1991, **272:**205-207
28. Lowy FD ***Staphylococcus aureus* infections** *N Engl J Med* 1998, **339(8):**520-532
29. Kahl BC, Goulian M, van Wamel W, Herrmann M, Simon SM, Kaplan G, Peters G and Cheung AL ***Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line** *Infect Immun* 2000, **68(9):**5385-5392
30. Wesson CA, Deringer J, Liou LE, Bayles KW, Bohach GA and Trumble WR **Apoptosis induced by *Staphylococcus aureus* in epithelial cells utilizes a mechanism involving caspases 8 and 3** *Infect Immun* 2000, **68(5):**2998-3001
31. Nuzzo I, Sanges MR, Folgore A and Carratelli CR **Apoptosis of human keratinocytes after bacterial invasion** *FEMS Immunol Med Microbiol* 2000, **27(3):**235-240
32. Menzies BE and Kourteva I ***Staphylococcus aureus* alpha-toxin induces apoptosis in endothelial cells** *FEMS Immunol Med Microbiol* 2000, **29(1):**39-45
33. Wesson CA, Liou LE, Todd KM, Bohach GA, Trumble WR and Bayles KW ***Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis** *Infect Immun* 1998, **66(11):**5238-5243
34. Tucker KA, Reilly SS, Leslie CS and Hudson MC **Intracellular *Staphylococcus aureus* induces apoptosis in mouse osteoblasts** *FEMS Microbiol Lett* 2000, **186(2):**151-156
35. Thornberry NA and Lazebnik Y **Caspases: enemies within** *Science* 1998, **281:**1312-1316
36. Vidalain PO, Azocar O, Lamouille B, Astier A, Rabourdin-Combe C and Servet-Delprat C **Measles virus induces functional TRAIL production by human dendritic cells** *J Virol* 2000, **74(1):**556-559
37. Jelachich ML and Lipton HL **Theiler's murine encephalomyelitis virus induces apoptosis in gamma interferon-activated M1 differentiated myelomonocytic cells through a mechanism involving tumor necrosis factor alpha (TNF-alpha) and TNF-alpha-related apoptosis-inducing ligand** *J Virol* 2001, **75(13):**5930-5938
38. Clarke P, Meintzer SM, Gibson S, Widmann C, Garrington TP, Johnson GL and Tyler KL **Reovirus-induced apoptosis is mediated by TRAIL** *J Virol* 2000, **74(17):**8135-8139
39. Miura Y, Misawa N, Maeda N, Inagaki Y, Tanaka Y, Ito M, Kayagaki N, Yamamoto N, Yagita H, Mizusawa H and Koyanagi Y **Critical contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to apoptosis of human CD4+ T cells in HIV-1-infected hu-PBL-NOD-SCID mice** *J Exp Med* 2001, **193(5):**651-660
40. Benedict CA, Norris PS, Prigozy TI, Bodmer JL, Mahr JA, Garnett CT, Martinon F, Tschopp J, Gooding LR and Ware CF **Three adenovirus E3 proteins cooperate to evade apoptosis by tumor necrosis factor-related apoptosis-inducing ligand receptor-1 and -2** *J Biol Chem* 2001, **276(5):**3270-3278
41. Alexander EH, Bento JL, Hughes FM Jr, Marriott I, Hudson MC and Bost KL ***Staphylococcus aureus* and *Salmonella enterica* serovar**

Dublin induce tumor necrosis factor-related apoptosis-inducing ligand expression by normal mouse and human osteoblasts *Infect Immun* 2001, **69(3):**1581-1586
42. Bost KL, Bento JL, Petty CC, Schrum LW, Hudson MC and Marriott I **Monocyte chemoattractant protein-1 expression by osteoblasts following infection with *Staphylococcus aureus* or Salmonella** *J Interferon Cytokine Res* 2001, **21(5):**297-304
43. Ellington JK, Elhofy A, Bost KL and Hudson MC **Involvement of mitogen-activated protein kinase pathways in *Staphylococcus aureus* invasion of normal osteoblasts** *Infect Immun* 2001, **69(9):**5235-5242
44. Bonzon C and Fan H **Moloney murine leukemia virus-induced preleukemic thymic atrophy and enhanced thymocyte apoptosis correlate with disease pathogenicity** *J Virol* 1999, **73(3):**2434-2441
45. Tang D, Lahti JM, Grenet J and Kidd VJ **Cycloheximide-induced T-cell death is mediated by a Fas-associated death domain-dependent mechanism** *J Biol Chem* 1999, **274(11):**7245-7252
46. Seol DW, Li J, Seol MH, Park SY, Talanian RV and Billiar TR **Signaling events triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): caspase-8 is required for TRAIL-induced apoptosis** *Cancer Res* 2001, **61(3):**1138-1143
47. Cohen GM **Caspases: the executioners of apoptosis** *Biochem J* 1997, **326(Pt 1):**1-16
48. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A **Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family** *J Biol Chem* 1996, **271(22):**12687-12690
49. Ashkenazi A and Dixit VM **Death receptors: signaling and modulation** *Science* 1998, **281(5381):**1305-1308
50. Hu WH, Johnson H and Shu HB **Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways** *J Biol Chem* 1999, **274(43):**30603-30610
51. Wakabayashi G, Gelfand JA, Jung WK, Connolly RJ, Burke JF and Dinarello CA ***Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. Comparison to *Escherichia coli*** *J Clin Invest* 1991, **87(6):**1925-1935
52. Wang ZM, Liu C and Dziarski R **Chemokines are the main proinflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglycan, and endotoxin** *J Biol Chem* 2000, **275(27):**20260-20267
53. Schwab JH, Brown RR, Anderle SK and Schlievert PM **Superantigen can reactivate bacterial cell wall-induced arthritis** *J Immunol* 1993, **150(9):**4151-4159
54. Bristol-Rothstein LA and Schwab JH **Bone-resorbing activity is expressed by rat macrophages in response to arthropathic streptococcal cell wall polymers** *Inflammation* 1992, **16(5):**485-496
55. Nagai H, Takaoka Y, Kamada H and Mori H **The model of arthritis induced by superantigen in mice** *Life Sci* 1994, **55(12):**L233-237
56. Gasper NA, Petty CC, Schrum LW, Marriott I and Bost KL **Bacterium-Induced CXCL10 Secretion by Osteoblasts Can Be Mediated in Part through Toll-Like Receptor 4** *Infect Immun* 2002, **70(8):**4075-4082
57. Yang S, Tamai R, Akashi S, Takeuchi O, Akira S, Sugawara S and Takada H **Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture** *Infect Immun* 2001, **69(4):**2045-2053
58. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC and Young PR **Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL** *J Biol Chem* 1998, **273(23):**14363-14367
59. Ramp WK, Lenz LG and Kaysinger KK **Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells** *Bone Miner* 1994, **24(1):**59-73
60. Ramp WK, Dillaman RM, Lenz LG, Gay DM, Roer RD and Ballard TA **A serum substitute promotes osteoblast-like phenotypic expression in cultured cells from chick calvariae** *Bone Miner* 1991, **15(1):**1-17
61. Gallagher JA, Gundle R and Beresford JN **Isolation and culture of bone forming cells (osteoblasts) from human bone** *Humana Press, Totowa, NJ* 1996,
62. Marie PJ, Lomri A, Sabbagh A and Basle M **Culture and behavior of osteoblastic cells isolated from normal trabecular bone surfaces** *In Vitro Cell Dev Biol* 1989, **25(4):**373-380
63. Rodan GA, Heath JK, Yoon K, Noda M and Rodan SB **Diversity of the osteoblastic phenotype** *Ciba Found Symp* 1988, **136:**78-91
64. Gundle R and Beresford JN **The isolation and culture of cells from explants of human trabecular bone** *Calcif Tissue Int* 1995, **56(Suppl 1):**S8-10
65. Bradford MM **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding** *Anal Biochem* 1976, **72:**248-254
66. Laemmli UK **Cleavage of structural proteins during the assembly of the head of bacteriophage** *Nature* 1970, **227(259):**680-685

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

BioMedcentral