Staphylococcus aureus protein A binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection

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Staphylococcus aureus is the major pathogen among the staphylococci and the most common cause of bone infections. These infections are mainly characterized by bone destruction and inflammation, and are often debilitating and very difficult to treat. Previously we demonstrated that S. aureus protein A (SpA) can bind to osteoblasts, which results in inhibition of osteoblast proliferation and mineralization, apoptosis, and activation of osteoclasts. In this study we used small interfering RNA (siRNA) to demonstrate that osteoblast tumour necrosis factor receptor-1 (TNFR-1) is responsible for the recognition of and binding to SpA. TNFR-1 binding to SpA results in the activation of nuclear factor kappa B (NF\(\kappa\)B). In turn, NF\(\kappa\)B translocates to the nucleus of the osteoblast, which leads to release of interleukin 6 (IL-6). Silencing TNFR-1 in osteoblasts or disruption of the spa gene in S. aureus prevented both NF\(\kappa\)B activation and IL-6 release. As well as playing a key role in proinflammatory reactions, IL-6 is also an important osteotropic factor. Release of IL-6 from osteoblasts results in the activation of the bone-resorbing cells, the osteoclasts. Consistent with our results described above, both silencing TNFR-1 in osteoblasts and disruption of spa in S. aureus prevented osteoclast activation. These studies are the first to demonstrate the importance of the TNFR-1–SpA interaction in bone infection, and may help explain the mechanism through which osteoclasts become overactivated, leading to bone destruction. Anti-inflammatory drug therapy could be used either alone or in conjunction with antibiotics to treat osteomyelitis or for prophylaxis in high-risk patients.

INTRODUCTION

The skeletal system plays a critical role in the provision of structural support and protection of inner organs of the body (Clarke, 2008). Bone is the major tissue component of the skeletal system, which is constantly undergoing remodelling. The bone remodelling process is a tightly regulated process that is controlled by bone-forming cells, osteoblasts and bone-degrading cells, osteoclasts (Eriksen, 2010). Remodelling begins with the laying down of an organic matrix and its calcification by osteoblasts, followed by the removal of this calcified matrix by the osteoclast (Sims & Gooi, 2008). Bone remodelling is a constant process that enables the skeletal system to respond and adapt to mechanical stresses encountered in everyday life (Hill, 1998). An imbalance in bone remodelling can interfere with osteoblast function and osteoclast activity, leading to the failure of the resorbed bone to be filled by new calcified matrix. As a result, this imbalance causes increased bone fragility, which leads to a high fracture risk.

Abbreviations: FnBP, fibronectin-binding protein; IL-6, interleukin 6; NF\(\kappa\)B, nuclear factor kappa B; siRNA, small interfering RNA; TNF, tumour necrosis factor; TNFR-1, tumour necrosis factor receptor 1; TRAP, tartate-resistant acid phosphatase.
in affected individuals (Clarke, 2008). There are many factors that can trigger an imbalance, such as disorders of the endocrine system (Lombardi et al., 2011; Karsenty, 2012), but the most sustained and devastating is the presence of an infecting micro-organism in bone (Lew & Waldvogel, 2004).

Osteomyelitis is an acute or chronic infection of the bone and is characterized by suppurative inflammation, abnormal bone remodelling, together with uncontrolled bone resorption (Montanaro et al., 2011). Micro-organisms can gain entry to the bone using a number of different routes. Contiguous infection occurs following trauma, bone surgery or joint replacement. Haematogenous infection occurs as a result of a bloodstream infection or bacteraemia (Wright & Nair, 2010). Osteomyelitis can be caused by a wide range of different micro-organisms. However *Staphylococcus aureus* infection is the most common, accounting for up to 80% of clinical cases (Berendt & Byren, 2004). Treatment for osteomyelitis is often unsuccessful and requires radial debridement of the affected limb coupled with intense combination antibiotic treatment (Rao et al., 2011). Of particular concern is the rapid emergence of multiple resistant strains of *S. aureus* and the recent identification of *S. aureus* isolates that are resistant to or have reduced susceptibility to vancomycin, the last remaining antibiotic to which the bacteria was uniformly susceptible (Vander Have et al., 2009).

To date, the mechanism by which *S. aureus* causes weakening of the bones in osteomyelitis is not fully understood. However, significant advances in our understanding of the underlying mechanisms are beginning to emerge. Earlier reports demonstrate that osteoblasts are capable of internalizing *S. aureus*. Uptake is promoted by fibronectin-binding proteins (FnBPs) expressed on *S. aureus* that capture fibronectin and use it as a bridge between bacteria and the α5β1 integrin expressed on osteoblasts (Sinha et al., 1999; Ahmed et al., 2001). Integrin clustering in the osteoblast results in signalling that leads to bacterial uptake, thus rendering the bacteria safe from both immune and antibiotic attack. Once internalized, *S. aureus* cells also have the ability to induce apoptosis via the TRAIL/caspase pathway, thus contributing to bone destruction (Tucker et al., 2000). More recently, our group has demonstrated that in the absence of FnBPs, *S. aureus* is still capable of interacting with osteoblasts (Claro et al., 2011). We demonstrated that *S. aureus* is capable of triggering a series of downstream events that contribute to abnormal bone remodelling. When *S. aureus* protein A binds to osteoblasts, it leads to an increase in the expression of RANKL, an important cytokine involved in osteoclast differentiation. The binding also generates signals in the osteoblast that lead to the inhibition of mineralization and osteoblast proliferation. Finally, we demonstrated that SpA binding to osteoblasts generates a signal that induces osteoblast apoptosis. Deletion of SpA from *S. aureus* prevented these events from occurring, suggesting that the *S. aureus* SpA–osteoblast interaction is a critical event in upsetting normal bone remodelling (Claro et al., 2011; Widaa et al., 2012).

SpA is a multifunctional protein, which has been previously shown to bind a number of ligands, including tumour necrosis factor receptor 1 (TNFR-1) (Gómez et al., 2006). Osteoblasts express high levels of TNFR-1. In the current study we demonstrate that the TNFR-1 receptor plays a critical role in binding *S. aureus* SpA, and furthermore is responsible for producing interleukin 6 (IL-6) via the nuclear factor kappa B (NFκB) pathway, resulting in osteoclast activation.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. *S. aureus* strains were grown to stationary phase at 37 °C in Brain Heart Infusion broth (Oxoid). *S. aureus* strain Newman (pCU1spa+) was grown with the addition of 10 μg chloramphenicol ml⁻¹ (Sigma-Aldrich). Bacteria were harvested and washed by centrifugation at 4000 g for 5 min, resuspended in PBS, pH 7.4, and adjusted to 1 × 10⁹ cells ml⁻¹ for all studies.

**Tissue cell culture conditions.** The mouse clonal MC3T3-E1 pre-osteoblastic cell line (ATCC) was used for all experiments. This is a common cell line used routinely for investigating osteoblast function. The cells were cultured in standard T175 tissue culture flasks (Sarstedt) containing α-MEM supplemented with 10% FBS (Biosera), 2% penicillin/streptomycin solution and 1% l-glutamine (Sigma-Aldrich). The medium was replaced every 3–4 days, and after confluence, cells were harvested using trypsin-EDTA (Sigma-Aldrich) and resuspended in the standard medium. MC3T3-E1 pre-osteoblastic cells were differentiated to matrix-secreting mature osteoblasts using the standard osteogenic cocktail, by supplementation with dexamethasone (100 nM), ascorbic acid (50 μg ml⁻¹) and β-glycerolphosphate (10 mM) (Sigma-Aldrich).

The murine RAW 264.7 pre-osteoclast cell line (ATCC) was used for all osteoclast studies. RAW 264.7 cells were cultured in T175 tissue culture flasks in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (Biosera) and 1% penicillin/streptomycin solution (Sigma-Aldrich).

**Silencing of TNFR-1 on osteoblastic cells using small interfering RNA (siRNA).** siRNA silencing was used to knock down TNFR-1 using the murine Amnax Cell Line Nucleofector kit R (Lonza). Osteoblastic cells (1 × 10⁶ cells ml⁻¹) were harvested and centrifuged at 9000 g for 5 min at room temperature. After centrifugation, the supernatant was removed and each pellet was resuspended in 100 μl RT Nucleofector Solution R with 150 nM siRNA (Ambion). The suspended cells with siRNA were then electroporated using a Nucleofector I electroporator (ISIS). Immediately after electroporation, 500 μl pre-equilibrated osteoblast medium was added to the cells. Following 5 days of growth, osteoblasts were lysed and loss of expression of TNFR-1 was confirmed by Western immunoblotting.

**Western immunoblotting.** Infected, uninfected and siRNA-treated osteoblasts were lysed in RIPA buffer containing 1× protease inhibitor cocktail supplemented with phosphatase inhibitors (sodium fluoride, sodium orthovanadate and calcyclin A) on ice for 10 min. Cleared lysates were separated on a 10% SDS-PAGE gel. Proteins were electroblotted onto PVDF membranes (Roche) for 1 h. Membranes were probed with a primary anti-rabbit IgG against TNFR-1 (Santa Cruz), anti-mouse IgG against IKB (Calbiochem) or...
stopped using 1 M NaOH and the Au (Sigma-Aldrich). After 30 min incubation at 37°C, the plate was then incubated in a 250 μL hydrophobic porous sealing film. The plate was then incubated in a microplate was coated with IL-6 capture antibody and sealed with the Mouse IL-6 ELISA Ready-SET-Go kit (eBioscience). A 96-well plate was used to capture and replicate in Escherichia coli and S. aureus expressing protein A. 

Previously we demonstrated that S. aureus hydrophobic porous sealing film. The plate was then incubated in a microplate was coated with IL-6 capture antibody and sealed with the Mouse IL-6 ELISA Ready-SET-Go kit (eBioscience). A 96-well plate was used to capture and replicate in Escherichia coli and S. aureus expressing protein A. 

RESULTS

Osteoblast TNFR-1 binds to S. aureus SpA

Previously we demonstrated that S. aureus SpA binds to and triggers a series of events that contribute to bone destruction; however, the osteoblast receptor to which SpA binds has not been definitively identified. Protein A has been shown to bind a number of ligands, including TNFR-1. Osteoblasts express high levels of TNFR-1. Our previous results demonstrated that blocking osteoblast TNFR-1 with an antibody reduced osteoblast adhesion to S. aureus Newman. To confirm the interaction between osteoblast TNFR-1 and S. aureus SpA we employed siRNA techniques. Osteoblasts were preincubated with an siRNA specifically targeted at knocking down expression of TNFR-1. Western immunoblot analysis confirmed successful silencing of the TNFR-1 expression on the osteoblasts (Fig. 1a). β-Actinin was used as a loading control (Fig. 1a). siRNA-treated osteoblasts displayed significantly less binding to S. aureus than the untreated osteoblast control (Fig. 1b, P<0.01). Interestingly, siRNA-treated osteoblasts bound significantly less to the S. aureus strain defective in protein A. 

S. aureus SpA binding to TNFR-1 induces NFκB activation

As well as the TNFR-1 being recognized as a death receptor it also plays an important role in the control of gene transcription through the activation of NFκB. NFκB is a dimeric protein which is translocated from the cytoplasm to the nucleus under the control of the inhibitory protein IκB (Carmody & Chen 2007), where it activates transcriptional factors that mediate the expression of inflammatory cytokines and chemokines (Panzer et al., 2009). As a measure of NFκB activation, degradation of IκB was investigated by immunoblotting. Osteoblasts were infected over a 90 min period with S. aureus Newman. IκB degraded in a time-dependent manner over a 90 min period following infection of osteoblasts with SpA+ S. aureus Newman (Fig. 2a). Following infection with an SpA-defective mutant, no IκB degradation was detected over the 90 min period (Fig. 2b). The complemented mutant (pCU1spa+) recovered the ability to induce degradation of IκB over the 90 min period (Fig. 2c). Pre-incubating osteoblasts with an anti-TNFR-1 IgG (Fig. 2d) or using TNFR-1-silenced osteoblasts failed to induce IκB degradation in the presence of S. aureus Newman (Fig. 2d, e). These results demonstrate that in the absence of SpA on the S. aureus surface or in TNFR-1-blocked or -silenced osteoblasts, TNFR-1 binds to S. aureus SpA.

Table 1. S. aureus strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newman</td>
<td>NCTC 8178 wild-type. Isolated from a case of secondarily infected tuberculous osteomyelitis in man</td>
<td>Guthrie &amp; Lorenz (1952)</td>
</tr>
<tr>
<td>DU5876 Newman clfA</td>
<td>clfA::Tn917. Defective in clumping factor A</td>
<td>McDevitt et al. (1994)</td>
</tr>
<tr>
<td>Newman pCU1spa+</td>
<td>Cm’ Ap’ spa gene cloned into pCU1. Insertion of pCU1spa, capable of replicating in Escherichia coli and S. aureus expressing protein A</td>
<td>Claro et al. (2011)</td>
</tr>
</tbody>
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osteoblasts, the TNFR-1 pathway is not activated. As a consequence, \( \text{IkB} \) retains \( \text{NF} \kappa \text{B} \) in the cytosol, preventing the transcription of genes involved in the inflammatory response.

**S. aureus** SpA binds to osteoblast TNFR-1 and causes release of IL-6

Recent observations suggest that activated osteoblasts might serve as a source of proinflammatory cytokines. \( \text{NF} \kappa \text{B} \) activates transcriptional factors that mediate the expression of several proinflammatory cytokines. IL-6 is an example of one such proinflammatory cytokine that is found at high levels in patients with bone infection. We next investigated whether *S. aureus* SpA binding to osteoblast TNFR-1 leads to activation of \( \text{NF} \kappa \text{B} \) and thus secretion of IL-6 into the local environment. *S. aureus* strains were preincubated with untreated or siRNA-treated osteoblasts for 48 h before the supernatant was collected and assayed for IL-6. Uninfected osteoblasts released low levels of IL-6 compared with those incubated with *S. aureus* Newman, which caused an almost eightfold increase of IL-6 (Fig. 3a, \( P < 0.001 \)). In contrast, the SpA-defective mutant significantly reduced IL-6 release (Fig. 3a, \( P < 0.001 \), compared with the wild-type). The complemented mutant (pCU1spa\(^+\)) stimulated a similar level of IL-6 to that of wild-type *S. aureus* Newman (Fig. 3a, \( P \) value not significant).

To further investigate whether *S. aureus* SpA binding to osteoblast TNFR-1 caused the release of IL-6 we used osteoblasts pretreated with siRNA to knock down expression of TNFR-1. Consistent with our earlier observations, uninfected osteoblasts failed to release IL-6. In contrast to those preincubated with siRNA, untreated osteoblasts with *S. aureus* showed a significant increase in IL-6 release (Fig. 3b, \( P < 0.0001 \)). The siRNA-treated osteoblasts did not release IL-6 when challenged with *S. aureus* (Fig. 3b, \( P < 0.0001 \)). These results suggest that *S. aureus* SpA binds to osteoblast TNFR-1, causing the release of the proinflammatory cytokine IL-6.
S. aureus SpA binds osteoblast TNFR-1 in osteomyelitis

S. aureus is a commensal of humans found predominantly in the moist squamous epithelium of the anterior nares (Foster, 2009). It is also an important pathogen, causing many types of infection, including osteomyelitis. Trauma, surgery, joint replacement or bloodstream infection provides routes of entry for these bacteria into the bone tissue (Lew & Waldvogel, 2004). Osteomyelitis is characterized by progressive inflammatory destruction of the bone (Wright & Nair, 2010). The ability of S. aureus to bind to and trigger intracellular signals in the osteoblast is considered to be a primary event in the pathogenesis of osteomyelitis.

Understanding the mechanisms through which S. aureus binds to osteoblasts and generates intracellular signals leading to bone loss and bone destruction in vitro is poorly characterized. Recently, a model of the early stages of infection was proposed, where S. aureus FnBPs bind fibronectin, which in turn bridges to osteoblast $\alpha 5\beta 1$, facilitating internalization (Sinha et al., 1999; Ahmed et al., 2001). Using this strategy, S. aureus can evade immune cell recognition or antibiotic attack by hiding inside osteoblasts. SpA can promote binding to osteoblasts, which results in activation of osteoclasts and inhibition of osteoblast proliferation and mineralization. In addition, SpA binding to osteoblasts induces apoptosis via caspase 6 activation (Claro et al., 2011; Widaa et al., 2012). These effects are believed to contribute to the bone loss observed in osteomyelitis patients.

We next addressed the role of osteoblast TNFR-1. siRNA silencing of TNFR-1 on the osteoblastic cells failed to increase the TRAP level compared with uninfected osteoblasts when cultured in the presence of S. aureus Newman wild-type strain (Fig. 4, $P$ value not significant). These results further confirm that S. aureus SpA binds to osteoblast TNFR-1, inducing the release of IL-6, which leads to activation of osteoclasts.

**DISCUSSION**

S. aureus causes activation of osteoclasts

Having demonstrated the release of IL-6 from osteoblasts as a result of SpA binding to TNFR-1, we next determined whether the released IL-6 triggers osteoclastogenesis. To address this, medium was removed from both uninfected and S. aureus-infected osteoblasts and transferred to preosteoclasts for up to 21 days. Media from S. aureus-infected osteoblasts contains many secreted factors, including IL-6. TRAP is a glycosylated monomeric metalloenzyme that is highly expressed in osteoclasts, and its detection is utilized as an indicator of osteoclastogenesis. TRAP levels were quantified from osteoclastic cells after 4 days culture in media taken from uninfected and S. aureus-infected osteoblastic cells. TRAP activity from osteoclastic cells cultured in S. aureus Newman wild-type infected medium was higher than that from cells cultured in the uninfected osteoblastic cell culture medium (Fig. 4, $P<0.01$). The SpA mutant failed to induce higher TRAP levels than the uninfected cells (Fig. 4, $P$ value not significant), while the complemented mutant (pcU1spa$^+$) produced a higher level of TRAP (Fig. 4, $P<0.001$), at a similar levels to wild-type S. aureus Newman (Fig. 4, $P$ value not significant).

*Fig. 3. SpA binding to TNFR-1 induces IL-6 release on osteoblasts. (a) The amount of IL-6 released from non-infected and S. aureus-infected osteoblasts over 24 and 48 h was quantified using the mouse IL-6 ELISA. (b) The levels of IL-6 released were quantified in supernatants of both uninfected and S. aureus-infected normal and TNFR-1-silenced osteoblasts, over 48 h infection times. $*P<0.001$, $**P<0.0001$; error bars, SEM, n=3.*
Proinflammatory cytokine that is produced as a result of NFκB activation. IL-6 also plays a key role in activating the bone-resorbing cells, the osteoclasts (Ishimi et al., 1990). Upon NFκB activation, osteoblasts release IL-6, which subsequently activates osteoclasts. Neither the SpA mutant nor the TNFR-1-silenced osteoblast was able to release IL-6 and trigger activation of osteoclasts. This is a particularly important step in the development of osteomyelitis because this pathway may account for the increased bone resorption and subsequent bone loss experienced in osteomyelitis patients.

Significant emphasis has been placed on the role of TNFR-1 in cell death, and it is now generally known as a death receptor. However, more recently, TNFR-1 has also been shown to play a key role in cytokine generation and inflammation. For example, S. aureus SpA was previously shown to bind TNFR-1 in lung epithelial cells, resulting in proinflammatory signalling in the pathogenesis of staphylococcal pneumonia (Gómez et al., 2006). In the current study we demonstrate that osteoblasts express TNFR-1 and that silencing TNFR-1 on osteoblasts significantly reduces binding to S. aureus. Interestingly, binding of the SpA mutant to TNFR-1-silenced osteoblasts was also significantly reduced, suggesting that SpA may also be binding to another protein on the osteoblast surface. One distinct possibility is epidermal growth factor receptor (EGFR), as this is also expressed on osteoblasts (data not shown) and is a ligand for SpA (Gómez et al., 2007).

A key signalling component downstream of TNFR-1 is NFκB. NFκB is a dimeric protein composed of members of the Rel/NFκB family. Typically, NFκB dimers are retained in the cytoplasm by inhibitory protein IkBz (Kriete & Chen, 2007). Upon phosphorylation, IkBz becomes ubiquitinated and degraded which finally causes activation of NFκB (Carmody & Chen, 2007). A number of bacteria have been shown to activate NFκB in response to infection in various cell types, including Streptococcus pneumoniae in lung epithelial cells (Scharf et al., 2012), Streptococcus pyogenes in macrophages (Hertzen et al., 2010) and S. aureus in endothelial cells (Oviedo-Boyso et al., 2011). Here we demonstrate that S. aureus SpA binding to osteoblast TNFR-1 induces activation of NFκB. Either deleting SpA from S. aureus or reducing TNFR-1 expression by osteoblasts prevented activation of NFκB. This is an important observation, as NFκB is a critical molecular switch for several downstream events that affect host responses to infection and immune regulation. For example, once activated, free NFκB translocates into the nucleus of the cell to initiate the transcription of many NFκB-dependent genes, which include growth factors, adhesion molecules, chemokines and cytokines (Ning et al., 2010). Using a mouse model of osteomyelitis there was a significant increase in cytokines IL-1β, TNFα and IL-6 following infection with S. aureus (Yoshii et al., 2002; García-Alvarez et al., 2009). The source of these cytokines was not fully clear as S. aureus infection led to increased transcription, but not increased protein synthesis or secretion of IL-1β from murine osteoblasts (Marriott et al., 2002). Furthermore osteoblasts only release low levels of tumour necrosis factor (TNF)α (Bu et al., 2003). IL-6 is a pleiotropic cytokine that acts as a regulator of immune response and inflammation during infection or trauma (Kishimoto et al., 1992). There is growing evidence that IL-6 is an important osteotropic factor. IL-6 is produced by osteoblasts in response to S. aureus infection and has been shown to stimulate osteoclastic bone resorption (Ishimi et al., 1990). In the current study and consistent with previous reports, we demonstrated that osteoblasts secrete high levels of IL-6 following binding of S. aureus and that this contributed to osteoclast activation. This was dependent on SpA expression by S. aureus and TNFR-1 expression by the osteoblasts.

Significant progress has been made recently in outlining the mechanisms through which S. aureus induces bone destruction and bone loss. Previous reports suggest a dual mechanism to bone infection by S. aureus. In the first mechanism, S. aureus FnBP binds fibronectin and bridge the bacteria to osteoblast z5/f1. This mechanism contributes to immune evasion through internalization into the osteoblast and persistence of infection. In the second mechanism, S. aureus SpA binds osteoblast TNFR-1 and triggers a series of events that lead to inhibition of mineralization, inhibition of osteoblast proliferation and
induction of apoptosis, contributing to bone loss. The current study builds on these observations by demonstrating that SpA binding to TNFR-1 also triggers IL-6 release via activation of NFκB (Fig. 5). In turn, IL-6 activates the bone-resorbing cells, osteoclasts, which contributes to bone destruction. The significance of the current work lies in the discovery that IL-6 release from S. aureus-infected osteoblasts triggers activation of osteoclasts. In bone disease, IL-6 inhibitors have been used to treat postmenopausal osteoporosis and rheumatoid arthritis (Edwards & Williams, 2010; Jazayeri et al., 2010), indicating the benefits of preventing IL-6 responses in the bone. The involvement of osteoblast TNFR-1 in mediating the release of IL-6, which then activates the bone-resorptive cells, osteoclasts, makes the receptor a potential target for therapeutic intervention. This would prevent the destruction and loss of bone in osteomyelitis patients. Novel drug therapies could be used in conjunction with antibiotics to treat osteomyelitis or on their own as a prophylaxis for high-risk patients.

While significant advances are being made in understanding the complex interplay between S. aureus and osteoblasts, much work in this area is still needed. For example, deletion of S. aureus SpA only reduces osteoblast binding by roughly 70%, suggesting that additional interactions between S. aureus and osteoblasts occur. S. aureus expresses many potential candidates, including other LPXTG-anchored proteins, peptidoglycan and carbohydrates, which have already been shown to be involved in host recognition. In addition, S. aureus also secretes several superantigens and enterotoxins, which may also play a role in either the recognition or activation of osteoblasts, and which cannot be ruled out at this stage with respect to whether they play a role in bone infection. One major limitation of this study and of a lot of the previous investigational studies is that all experiments have been carried out in vitro using cell culture techniques. To establish whether these events occur in vivo, future studies should be carried out using animal models of osteomyelitis.

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