

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

Tânia Claro,¹ Amro Widaa,^{1,3} Cormac McDonnell,¹ Timothy J. Foster,² Fergal J. O'Brien^{3,4} and Steven W. Kerrigan^{1,5}

Correspondence
Steven W. Kerrigan
skerrigan@rcsi.ie

¹Microbial Infection Group, Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2, Ireland

²Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland

³Department of Anatomy, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2, Ireland

⁴Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland

⁵School of Pharmacy, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2, Ireland

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 (NFκB). In turn, NFκB 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 NFκB 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.

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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; NFκB, 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 radical 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 $\alpha 5\beta 1$ 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 (NF κ B) 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 confluency, 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 Amaxa Cell Line Nucleofector kit R (Lonza). Osteoblastic cells (1 × 10⁹ 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 calyculin 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 I κ B (Calbiochem) or

Table 1. *S. aureus* strains used in this study

Strain	Relevant characteristics	Reference
Newman	NCTC 8178 wild-type. Isolated from a case of secondarily infected tubercular osteomyelitis in man	Duthie & Lorenz (1952)
DU5876 Newman <i>clfA</i>	<i>clfA2::Tn917</i> . Defective in clumping factor A	McDevitt <i>et al.</i> (1994)
DU5971 Newman <i>spa</i>	<i>spa::Ka^r</i> . Defective in protein A	O'Brien <i>et al.</i> (2002)
Newman pCU1 <i>spa</i> +	Cm ^r Ap ^r <i>spa</i> gene cloned into pCU1. Insertion of pCU1 <i>spa</i> , capable of replicating in <i>Escherichia coli</i> and <i>S. aureus</i> expressing protein A	Claro <i>et al.</i> (2011)

anti-rabbit IgG against α -actinin (Santa Cruz) overnight at 4 °C with constant inversion. Unbound antibody was removed by three 10 min washes with Tris-buffered saline (TBS) buffer. Protein bands were detected using a species-specific horseradish peroxidase-conjugated secondary antibody and developed by chemiluminescence.

ELISA IL-6 production in osteoblasts following *S. aureus* binding. 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 Ready-SET-Go kit (eBioscience). A 96-well microplate was coated with IL-6 capture antibody and sealed with hydrophobic porous sealing film. The plate was then incubated overnight at 4 °C. Following this, wells were washed by addition of 250 μ l wash buffer. The plate was inverted and blotted on absorbant paper to remove any residual buffer. Each well was blocked with 200 μ l 1 \times assay diluent and the plate was incubated at room temperature (21 °C) for 1 h. Standards and samples were prepared by diluting mouse recombinant IL-6 in 1 \times assay diluent. The plate was washed again by adding 250 μ l Wash Buffer per well. Finally, 100 μ l of standard or sample was added to the 96-well plate. The plate was sealed using hydroporous sealing film and incubated at room temperature for 2 h. After washing the plate, 100 μ l of detection antibody was added to each well and incubated for 30 min at room temperature. After three washes of each well with 250 μ l Wash Buffer, 100 μ l substrate solution was added and the plate was incubated for 15 min at room temperature. Finally 50 μ l stop solution was added and the A_{450} was read using a Wallac Victor2 microplate reader system (Perkin Elmer).

Tartate-resistant acid phosphatase (TRAP) assay for osteoclastogenesis. To determine the effects of *S. aureus* on the formation and proliferation of pre-osteoclasts, RAW 264.7 cells were seeded (2×10^4 cells per well) in 12-well tissue culture plates. Conditioned 4-day medium from osteoblasts, either exposed or unexposed to *S. aureus*, was then transferred to the pre-osteoclasts, which were cultured for a further 4 days. TRAP was quantified to measure osteoclastogenesis on day 4. TRAP quantification was carried out by removing conditioned media from pre-osteoclasts and washing cells in PBS. Cells were then incubated for 1 h at 37 °C in 1 mM citrate solution. After the 1 h incubation, 20 μ l of supernatant from the wells was added in triplicate to wells of a 96-well plate containing 50 μ l 100 mM *p*-nitrophenol phosphate, 80 mM sodium tartrate, 200 mM sodium citrate and 200 mM sodium chloride (Sigma-Aldrich). After 30 min incubation at 37 °C, the reaction was stopped using 1 M NaOH and the A_{405} was measured using a microplate reader (Wallac Victor2).

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). α -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 expression of SpA (Fig. 1b, $P < 0.05$). This suggests that TNFR-1 bound to an additional adhesin (other than SpA) on *S. aureus*.

S. aureus SpA binding to TNFR-1 induces NF κ B 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 NF κ B. NF κ B is a dimeric protein which is translocated from the cytoplasm to the nucleus under the control of the inhibitory protein I κ B (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 NF κ B activation, degradation of I κ B was investigated by immunoblotting. Osteoblasts were infected over a 90 min period with *S. aureus* Newman. I κ B 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 I κ B degradation was detected over the 90 min period (Fig. 2b). The complemented mutant (pCU1*spa*⁺) recovered the ability to induce degradation of I κ B 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 I κ B 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

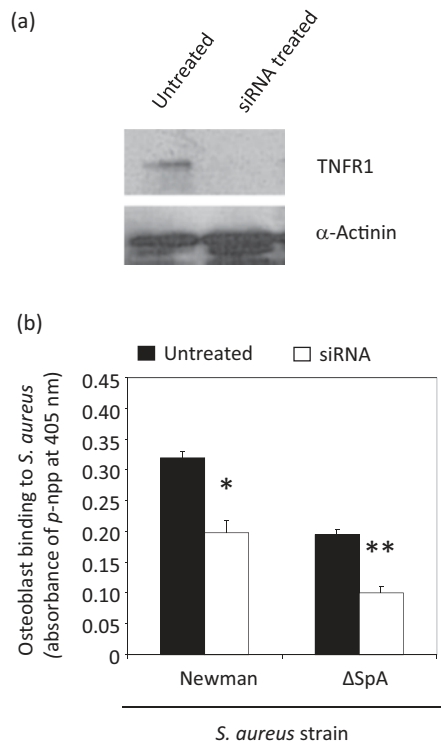


Fig. 1. TNFR-1 is the receptor for SpA on the osteoblast surface. (a) siRNA-negative and -positive osteoblast lysates were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with rabbit polyclonal anti-TNFR-1 and α -actinin as an equal loading control. Protein bands were detected using rabbit horseradish peroxidase-conjugated secondary antibody and chemiluminescence. (b) siRNA was used to silence TNFR-1 on osteoblasts. Osteoblasts were allowed to adhere to *S. aureus* Newman wild-type for 45 min at 37 °C. Binding was determined by measuring the intracellular alkaline phosphatase content at 405 nm in a microplate reader. *p*-npp, *p*-Nitrophenol phosphate. * $P < 0.01$, ** $P < 0.05$; error bars, SEM, $n = 3$.

osteoblasts, the TNFR-1 pathway is not activated. As a consequence, I κ B retains NF κ 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. NF κ 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 NF κ B and thus secretion of IL-6 into the local environment. *S. aureus* strains were preincubated with untreated or siRNA-treated

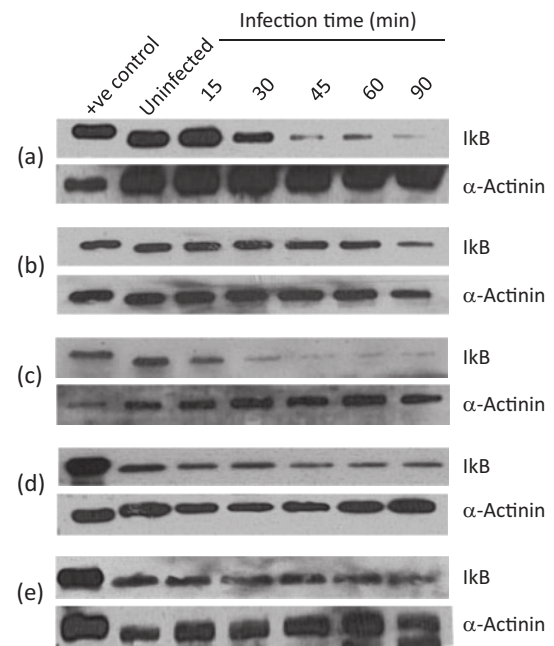


Fig. 2. SpA binding to TNFR-1 induces NF κ B activation. I κ B expression over a 90 min period in normal osteoblasts following infection with (a) *S. aureus* Newman wild-type, (b) Δ SpA, (c) pCu1SpA, (d) anti-TNFR-1 antibody and (e) TNFR-1-silenced osteoblasts. Uninfected and infected osteoblast lysates were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membranes. Membranes were probed with monoclonal I κ B antibody. Protein bands were detected using a mouse horseradish peroxidase-conjugated secondary antibody and chemiluminescence. Immunoblots are representative of three independent experiments.

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 untreated 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.

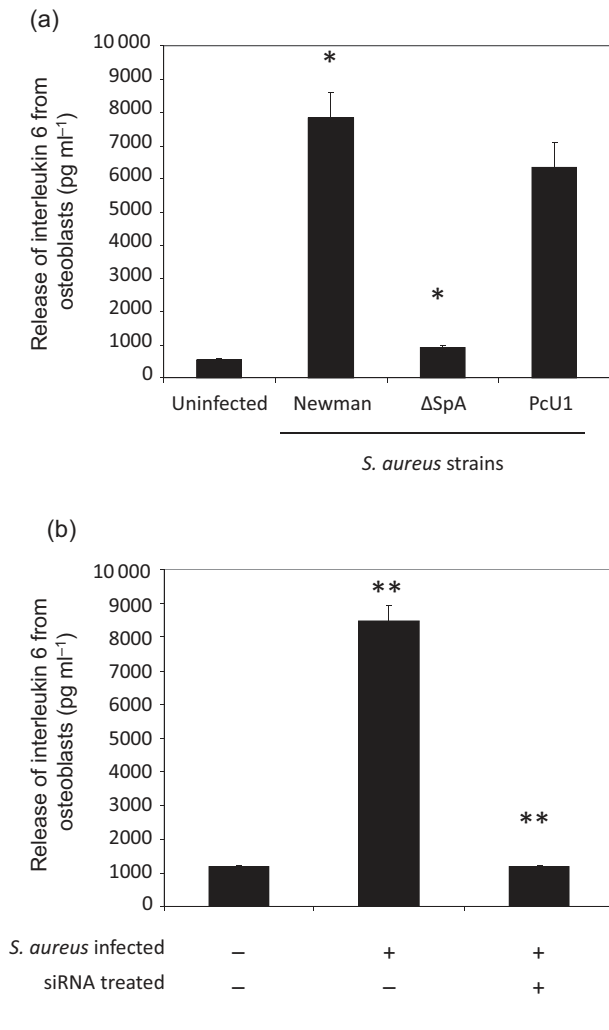


Fig. 3. SpA binding to TNFR-1 induces IL-6 release on osteoblasts. (a) The amount of IL-6 released from non-infected and *S. aureus*-infected osteoblasts over 24 and 48 h was quantified using the mouse IL-6 ELISA. (b) The levels of IL-6 released were quantified in supernatants of both uninfected and *S. aureus*-infected normal and TNFR-1-silenced osteoblasts, over 48 h infection times. * $P < 0.001$, ** $P < 0.0001$; error bars, SEM, $n = 3$.

***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 pre-osteoclasts 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).

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 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.

In the present study we demonstrate that *S. aureus* SpA binds to TNFR-1 on osteoblasts. A key target involved in the downstream signalling cascade of TNFR-1 is I κ B degradation and NF κ B activation (Guicciardi & Gores, 2009). Disruption of the *spa* gene on *S. aureus* or silencing expression of TNFR-1 on osteoblasts prevented I κ B degradation and NF κ B activation. IL-6 is an important

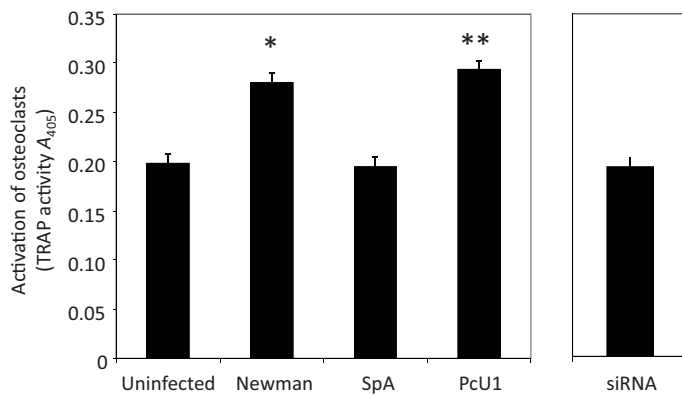


Fig. 4. *S. aureus* causes activation of osteoclasts. Pre-osteoclasts were seeded (2×10^4 cells per well) in 12-well tissue culture plates and cultured for 21 days. Conditioned media from osteoblasts (4 days), with either control buffer or formaldehyde-fixed *S. aureus* Newman, Newman Δ spa or Newman spa (pCU1spa+), was then transferred to the pre-osteoclasts. Osteoclastogenesis was determined by measuring TRAP. * $P < 0.01$, ** $P < 0.001$, $n = 3-5$. The right-hand panel shows TRAP activity after siRNA silencing of TNFR-1.

proinflammatory cytokine that is produced as a result of NF κ B activation. IL-6 also plays a key role in activating the bone-resorbing cells, the osteoclasts (Ishimi *et al.*, 1990). Upon NF κ B 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 NF κ B. NF κ B is a dimeric protein composed of members of the Rel/NF κ B family. Typically, NF κ B dimers are retained in the cytoplasm by inhibitory protein I κ B α (Kriete & Mayo, 2009). Upon phosphorylation, I κ B α becomes ubiquitinated and degraded which finally causes activation of NF κ B (Carmody & Chen, 2007). A number of bacteria have been shown to activate NF κ B in response to infection in various cell types, including *Streptococcus pneumoniae* in lung epithelial cells (Scharf *et al.*, 2012), *Streptococcus pyogenes* in macrophages (Hertzén *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 NF κ B. Either deleting SpA from *S. aureus* or reducing TNFR-1 expression by osteoblasts prevented activation of NF κ B. This is an important observation, as NF κ B is a critical molecular switch for several downstream events that affect host responses to infection and immune regulation. For example, once activated, free NF κ B translocates into the nucleus of the cell to initiate the transcription of many NF κ B-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-1 β , TNF α 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-1 β 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* FnBPs bind fibronectin and bridge the bacteria to osteoblast $\alpha 5\beta 1$. 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

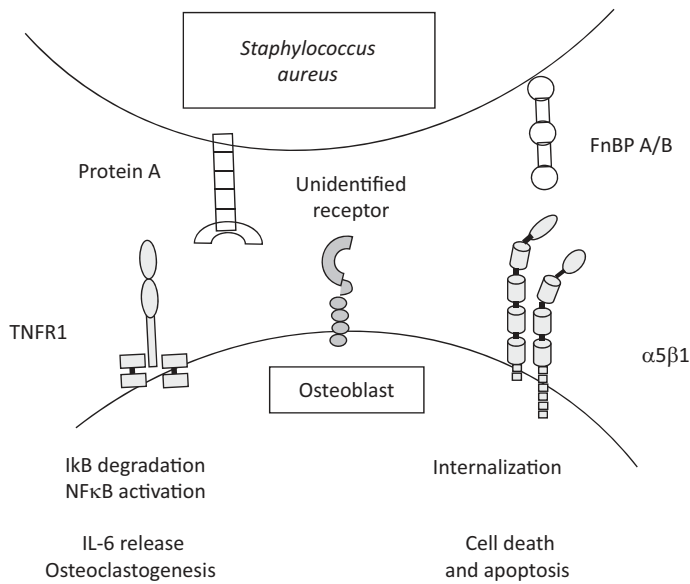


Fig. 5. Model of *S. aureus* interaction with osteoblasts. Engagement of *S. aureus* protein A with osteoblast TNFR-1 results in I κ B degradation and NF κ B activation. This results in the release of IL-6, which in turn activates the bone-resorbing cells, osteoclasts. A second mechanism exists in which *S. aureus* FnBP A/B binds fibronectin bridges to α 5 β 1 on the osteoblast and triggers internalization, resulting in cell death and apoptosis.

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 NF κ B (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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