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EXPERIMENTAL THERAPEUTICS

Neutralization of *Staphylococcus aureus* Protein A Prevents Exacerbated Osteoclast Activity and Bone Loss during Osteomyelitis

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ABSTRACT Osteomyelitis caused by *Staphylococcus aureus* is an important and current health care problem worldwide. Treatment of this infection frequently fails not only due to the increasing incidence of antimicrobial-resistant isolates but also because of the ability of *S. aureus* to evade the immune system, adapt to the bone microenvironment, and persist within this tissue for decades. We have previously demonstrated the role of staphylococcal protein A (SpA) in the induction of exacerbated osteoclastogenesis and increased bone matrix degradation during osteomyelitis. The aim of this study was to evaluate the potential of using anti-SpA antibodies as an adjunctive therapy to control inflammation and bone damage. By using an experimental *in vivo* model of osteomyelitis, we demonstrated that the administration of an anti-SpA antibody by the intraperitoneal route prevented excessive inflammatory responses in the bone upon challenge with *S. aureus*. *Ex vivo* assays indicated that blocking SpA reduced the priming of osteoclast precursors and their response to RANKL. Moreover, the neutralization of SpA was able to prevent the differentiation and activation of osteoclasts *in vivo*, leading to reduced expression levels of cathepsin K, reduced expression of markers associated with abnormal bone formation, and decreased trabecular bone loss during osteomyelitis. Taken together, these results demonstrate the feasibility of using anti-SpA antibodies as an antivirulence adjunctive therapy that may prevent the development of pathological conditions that not only damage the bone but also favor bacterial escape from antimicrobials and the immune system.

KEYWORDS *Staphylococcus aureus*, antivirulence therapy, osteomyelitis, protein A

Osteomyelitis remains a significant health care problem around the world (1–4). Although the advances in prophylaxis and aseptic surgical techniques have decreased the incidence of orthopedic infections, current studies indicate that infection rates for elective surgery cannot be reduced below 1% to 2% (5, 6), and the rates of recurrent or persistent infection following a two-stage revision surgery are still as high as 33% (7, 8). In addition to persistent and recurrent

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infection, sepsis is a significant concern in implant-associated osteomyelitis with approximately 10% of prosthetic joint infection cases leading to sepsis and death (9, 10).

Staphylococcus aureus is the major causative agent of osteomyelitis in adults and children. Treatment of this infection frequently fails not only due to the increasing incidence of antimicrobial-resistant isolates but also, and perhaps more importantly, due to the tremendous ability of *S. aureus* to induce an exacerbated inflammatory response, evade the immune system, adapt to the bone microenvironment, and persist within this tissue. Reservoirs of *S. aureus* within the bone include abscess communities, biofilm matrices, the osteocyte lacuno-canalicular network, bone sequestra, and host cells such as osteoblasts and osteocytes (2, 10–12).

Initially, the bacteria evade the immune system by surviving within abscesses in the bone marrow and soft tissue: which form as early as 4 days following infection (13). At this point, acute osteomyelitis can be treated by an aggressive antibiotic program and surgical debridement of soft tissue and bone marrow to remove all abscesses harboring bacteria. However, if treatment fails, the disease rapidly progresses and the presence of microorganisms in the bone induces the production of inflammatory cytokines by resident cells. Among these cytokines, tumor necrosis factor alpha (TNF-α) plays a central role in activating the endothelium and allowing the extravasation of neutrophils (14, 15). The inflammatory response to infection causes the compression of vascular channels and results in restricted blood perfusion (1, 16). This process, added to the local release of nitric oxide by the recruited neutrophils, leads to osteonecrosis and formation of sequestered avascular bone (17–19).

S. aureus can survive and form biofilms within the sequestered tissue which becomes a perfect niche where the bacteria persist well protected from antibiotics and the immune system (1, 11, 20–22). The inflammatory response induced by *S. aureus* also contributes to increased osteolysis by affecting the balance between osteoclast and osteoblast activity, generating the bone loss that characterizes osteomyelitis (10, 19).

The role of proinflammatory cytokines in the generation of bone sequestra and osteolysis is well recognized (19). However, the mechanism underlying these processes during staphylococcal infections is intricate and not completely understood. It has been proposed that it involves direct binding of cytokines to cytokine receptors expressed by osteoclast precursors and osteoblasts and indirect mechanisms through production of osteoclastogenic factors by inflammatory and resident cells. We have previously demonstrated that staphylococcal protein A (SpA), a potent inducer of inflammatory cytokines in professional and nonprofessional immune cells (23–25), evokes exacerbated osteoclastogenesis and increased bone matrix degradation (26). The aim of this study was to further elucidate the role of SpA in the induction of inflammatory mediators in the bone and to evaluate the potential of using anti-SpA antibodies to decrease inflammation, osteoclast activity, and bone damage induced by *S. aureus* during osteomyelitis.

RESULTS

Protein A contributes to the production of inflammatory mediators in the bone during *in vivo* infection. Considering the importance of inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), and IL-6 in the activation of osteoclasts and pathogenesis of bone disease (19, 27–29), we aimed to elucidate the role of protein A in the induction of these cytokines during *in vivo S. aureus* infection. The levels of TNF-α, IL-1β, and IL-6 in the bone of mice challenged with phosphate-buffered saline (PBS) (control), wild-type (WT) *S. aureus*, or the SpA$^-$ mutant were quantified and compared with the corresponding levels in the noninoculated tibia (Fig. 1A and B). Although mice challenged with the SpA$^-$ mutant had increased abundance of TNF-α and IL-1β in the infected tibia relative to the contralateral tibia, the levels of these cytokines were significantly lower than those found in the infected tibia of mice challenged with WT *S. aureus* (Fig. 1B). Regarding IL-6, a significant induction of this cytokine was observed upon *S. aureus* challenge whereas the levels found in mice inoculated with the SpA$^-$ mutant did not differ from those in the PBS-inoculated group (Fig. 1B).

We then determined the potential of neutralizing protein A *in vivo* to prevent the induction of cytokines and chemokines in the bone during *S. aureus* infection (Fig. 1A). Mice treated with the anti-SpA antibody were able to establish an inflammatory response during infection,

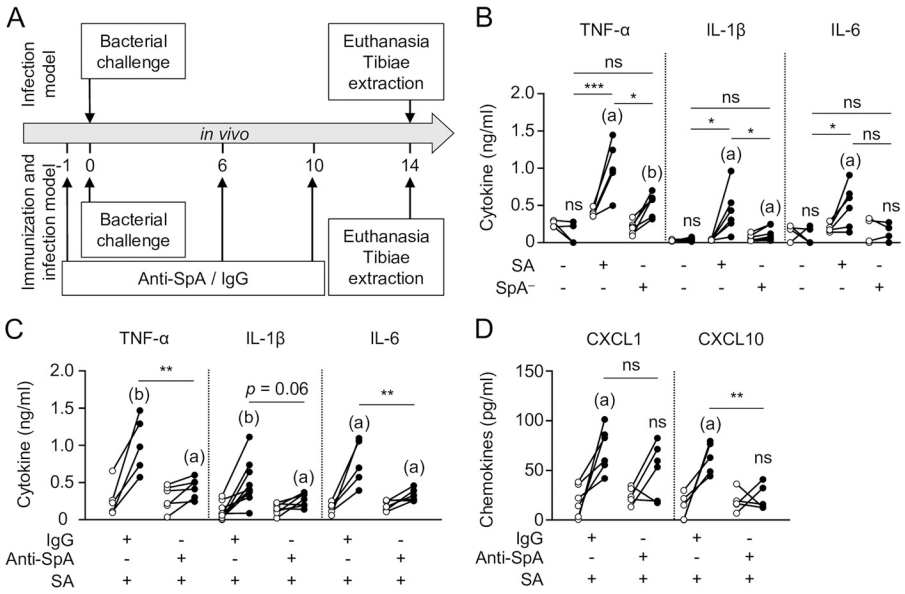

FIG 1 Role of protein A in the induction of inflammatory mediators during *S. aureus* bone infection. (A) Experimental design. (B) Groups of BALB/c mice were challenged in the left tibia with WT *S. aureus*, the isogenic SpA⁻ mutant, or PBS as control. The levels of TNF-α, IL-1β, and IL-6 in bone homogenates from the left tibiae (black circles) and from the right tibiae (open circles) were quantified 14 days after challenge. (a) $P < 0.05$; (b) $P < 0.01$; ns, nonsignificant; paired Student's *t* test comparing left and right tibiae of the same animal. *, $P < 0.05$; ***, $P < 0.001$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test comparing left tibiae among the 3 groups. (C and D) Groups of BALB/c mice were passively immunized via the i.p. route with anti-SpA antibody (75 mg/kg) or rabbit IgG (placebo) 1 day before the challenge with WT *S. aureus* and at days 6 and 10 thereafter. The levels of TNF-α, IL-1β, and IL-6 (C) as well as CXCL1 and CXCL10 (D) in bone homogenates from the left tibiae (black circles) and from the right tibiae (open circles) were quantified 14 days after challenge. (a) $P < 0.05$; (b) $P < 0.01$; ns, nonsignificant; paired Student's *t* test comparing left and right tibiae from the same animal. **, $P < 0.01$; ns, nonsignificant; Student's *t* test comparing left tibiae between the anti-SpA and the IgG groups.

as assessed by the detection of significantly increased levels of TNF-α, IL-1β, and IL-6 (Fig. 1C) in the bone, compared with the noninfected tibia. However, the levels of these cytokines in the infected tibia from immunized mice were lower than those determined in the infected tibia from mice in the placebo group (Fig. 1C), indicating that blocking protein A during *in vivo* infection can decrease the levels of inflammatory cytokines known to act as proosteoclastogenic factors in the bone. Protein A blocking did not affect the levels of CXCL1 (Fig. 1D), a chemokine critical for the attraction of neutrophils, but had a significant impact in the reduction of CXCL10 (Fig. 1D), a chemokine known to induce osteoclastogenesis (30). Passive immunization against protein A did not lead to a negative impact in bone bacterial colonization at day 14 after challenge (immunized group, $[2.2 \pm 1.2] \times 10^4$ CFU/bone; placebo group, $[1.5 \pm 1.1] \times 10^4$ CFU/bone; nonsignificant, Student's *t* test). Moreover, no bacterial dissemination was observed in either of the groups (immunized/placebo) evaluated as bacterial presence in blood and spleen.

Blocking protein A reduces priming of osteoclast precursors during *in vivo S. aureus* infection. We then decided to explore the potential of blocking protein A to decrease inflammatory signaling and osteoclast activity during *in vivo* infection. To that purpose, groups of C57BL/6 and BALB/c mice received the anti-SpA antibody or normal rabbit IgG 1 day before the subsequent challenge with *S. aureus* in the left tibia. Two days later, osteoclast precursors were obtained and stimulated with RANKL during 5 days (Fig. 2A). In the placebo group, *S. aureus* infection significantly increased the ability of osteoclast precursors to differentiate in response to RANKL independently of the genetic background analyzed (Fig. 2B and C). Conversely, osteoclast precursors from mice that were treated with the anti-SpA antibody and subsequently challenged with *S. aureus* did not show an increased capacity to differentiate into osteoclasts and the number of mature osteoclasts was equivalent to that in the noninfected group (Fig. 2B and C).

To further explore the potential mechanism involved in the increased response of osteoclast precursors from infected mice to RANKL, we determined the effect of *S. aureus* in

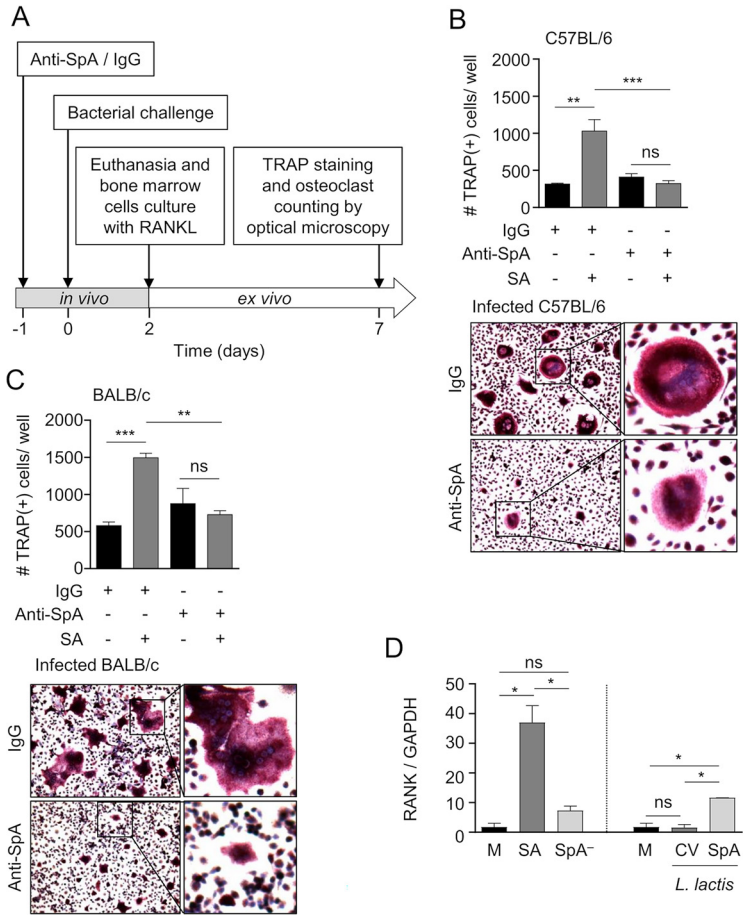

FIG 2 Effect of protein A neutralization on osteoclast precursor priming during *S. aureus* infection. (A) Experimental design. (B and C) Groups of C57BL/6 (B) and BALB/c (C) mice were passively immunized via the i.p. route with an anti-protein A antibody (75 mg/kg) or rabbit IgG (placebo) 1 day before the challenge in the left tibia with WT *S. aureus* (gray bars) or PBS (control, black bars). At 48 h postchallenge, stroma-free bone marrow cells were obtained and subsequently stimulated with RANKL (50 ng/mL) in the presence of M-CSF for 5 days. Multinucleated (having more than three nuclei) TRAP-positive cells were quantified by light microscopy. Bars represent cumulative data from 3 independent experiments with duplicates for each condition. **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test. Representative images at ×20 magnification are shown. (D) Stroma-free bone marrow cells were obtained from control BALB/c mice and cultured in the presence of M-CSF (30 ng/mL) for 72 h and subsequently stimulated with WT *S. aureus*, the isogenic SpA⁻ mutant, *L. lactis* CV, or *L. lactis* SpA (10^8 CFU/mL) in the presence of M-CSF for 9 days. The levels of RANK mRNA expression were quantified and standardized using GAPDH as control. Bars represent cumulative data from two independent experiments with duplicates for each condition. *, $P < 0.05$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test.

the expression of the RANKL receptor RANK. The levels of RANK were significantly increased in osteoclasts differentiated in response to *S. aureus* compared with medium alone (Fig. 2D). Conversely, the expression of RANK was not modified when the SpA⁻ mutant was used to stimulate the osteoclast precursors (Fig. 2D). The role of protein A in modulating RANK expression was further confirmed using *Lactococcus lactis* expressing protein A on its surface as a stimulus (Fig. 2D).

Neutralization of protein A diminished osteoclast differentiation and activity, bone resorption, and abnormal bone formation during experimental osteomyelitis. In order to evaluate the feasibility of using the anti-SpA antibody to prevent osteoclast differentiation and activation *in vivo*, we performed histomorphometric studies in immunized mice to quantify the number of osteoclasts and the osteoclast resorbing surface relative to intact trabecular bone. This analysis showed a significantly decreased number of osteoclasts per bone perimeter (N.Oc/B.pm) in tibiae from mice treated with the anti-SpA antibody relative to those determined in the placebo group (Fig. 3A, left panel, and Fig. 3B). Moreover, *in vivo* neutralization

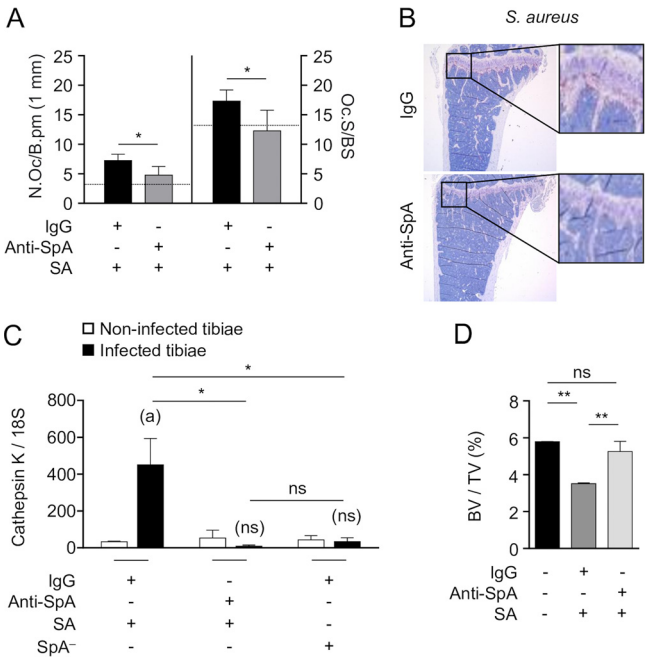

FIG 3 Impact of protein A neutralization on osteoclast differentiation and activation during *S. aureus* infection. Groups of BALB/c mice were passively immunized via the i.p. route with an anti-protein A antibody (75 mg/kg) or rabbit IgG (placebo) 1 day before the challenge with WT *S. aureus*, the isogenic SpA⁻ mutant, or PBS (as a control) and at days 6 and 10 thereafter. (A and B) The number of osteoclasts per bone perimeter (N.Oc/B.pm) and the osteoclast surface per bone surface (Oc.S/BS) were determined by histomorphometric analysis using TRAP staining on tibia tissue sections. (B) Representative images at ×10 magnification are shown. (C) The levels of cathepsin K mRNA expression at 14 days after challenge were quantified. 18S rRNA expression was used for standardization. Black bars represent left tibiae (inoculated with bacteria), and white bars represent right tibiae (noninoculated). (a) $P < 0.05$; ns, nonsignificant; Student's *t* test comparing left and right tibiae in the same group. *, $P < 0.05$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test comparing left tibiae among the three groups. (D) The trabecular bone volume per total volume (BV/TV) was evaluated 14 days after challenge via micro-computed tomography (μCT) imaging on trabecular bone in the distal tibia. (A and D) Bars represent data from 4 to 6 inoculated tibiae for each group. (A) *, $P < 0.05$, Student's *t* test. (D) **, $P < 0.01$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test.

of protein A led to decreased osteoclast surface per bone surface (Oc.S/BS) (Fig. 3A, right panel, and Fig. 3B) as well as significantly decreased expression levels of cathepsin K, a protease involved in bone matrix resorption (31) (Fig. 3C).

We have previously demonstrated that *S. aureus* protein A significantly contributes to cortical bone destruction during the course of disease in murine and rat models of osteomyelitis (26). In order to establish the potential of protein A neutralization in preventing alterations in bone architecture during *S. aureus* infection, we performed micro-computed tomography (μCT) imaging on trabecular bone in the distal tibia. The trabecular bone volume per total volume (BV/TV), a standard measure of bone volume and architecture (32), was quantified to determine the amount of trabecular bone that was lost during osteomyelitis. *S. aureus*-infected tibiae exhibited a significant loss in trabecular bone compared with the tibiae from mice inoculated with PBS (Fig. 3D). In contrast, in infected mice that had been treated with the anti-SpA antibody, the amount of trabecular bone was higher than that in nonimmunized mice and comparable to that in the control group (inoculated with PBS) (Fig. 3D). Thus, the reduced size of the osteoclasts and the diminished expression of bone-resorbing cathepsin K observed as consequence of protein A neutralization correlate with the decreased loss of trabecular bone volume.

During the course of osteomyelitis, the dysregulated activity of osteoclasts and osteoblasts leads to abnormal formation of new bone (18, 33). As part of this process, osteoblasts produce proteins including collagen I as well as osteocalcin to form the bone matrix. In the experimental infection model used, *S. aureus* promoted increased expression of osteocalcin and collagen I in a protein A-dependent manner, as evidenced by challenging mice with either the

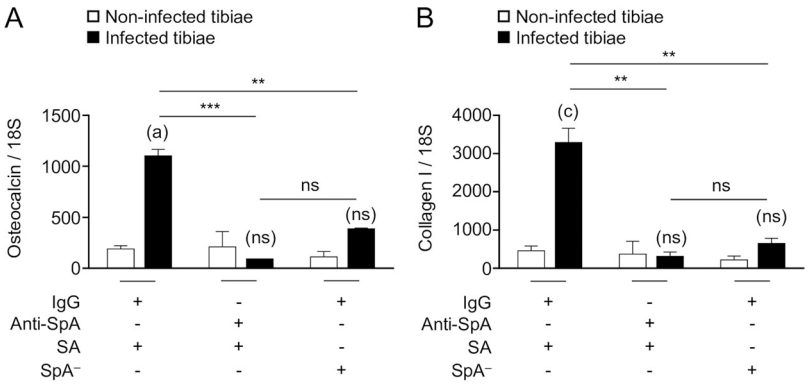

FIG 4 Impact of protein A neutralization on the expression of bone remodeling markers during *S. aureus* infection. Groups of BALB/c mice were passively immunized via the i.p. route with an anti-protein A antibody (75 mg/kg) or rabbit IgG (placebo) 1 day before the challenge with WT *S. aureus* and at days 6 and 10 thereafter. At day 14 after bacterial challenge, RNA was isolated from mouse tibia homogenates and the levels of osteocalcin (A) and collagen I (B) mRNA expression were quantified. 18S rRNA expression was used for standardization. Black bars represent left tibiae (inoculated with bacteria), and white bars represent right tibiae (noninoculated). (a) $P < 0.05$; (c) $P < 0.001$; ns, nonsignificant; Student's *t* test comparing left and right tibiae in the same group. **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; one-way ANOVA and Bonferroni's multiple-comparison test comparing left tibiae among the groups.

WT strain or the SpA$^-$ mutant (Fig. 4A and B). Neutralization of protein A by immunization evoked a significant reduction in osteocalcin and collagen I *in vivo*, and the level of expression of these molecules was comparable to that in the noninfected tibia (Fig. 4A and B).

DISCUSSION

Acute osteomyelitis is normally aggressively treated with antibiotics. However, treatment can fail even when the isolates, according to the laboratory testing, are sensitive to the antimicrobials used. The refractory behavior of *S. aureus* can be explained by its ability to hide from antibiotics and the immune system within the bone tissue. As a consequence, acute osteomyelitis often rapidly evolves into a chronic disease with poor prognosis of complete bacterial eradication and definitive cure (4, 34). Besides the importance of the inflammatory response in the defense against the invading pathogens, during bacterial osteomyelitis: inflammation significantly contributes to pathology as in many other infectious diseases (19, 35, 36). In this scenario, procedures that can modulate the excessive inflammatory response and osteolysis induced by *S. aureus* components in the bone are promising adjuvant therapies to consider.

In the present study, we provide evidence about the impact of protein A in the induction of cytokines and chemokines in the bone and demonstrate that neutralization of protein A through the systemic administration of an anti-SpA antibody significantly decreases the local production of TNF-α, IL-1β, IL-6, and CXCL10. Cytokines such as TNF-α, IL-1β, and IL-6 are essential for initiating inflammatory responses to trauma and infection (37) but are also important players in the pathogenesis of osteomyelitis. TNF-α has a well-demonstrated role in promoting the differentiation of osteoclasts (28) and at the same time inhibiting osteoblast differentiation (38, 39). IL-1β concentration is highly increased in bones during implant-associated osteomyelitis in mice (40, 41) and inhibits osteoblast differentiation (39) whereas it stimulates the production of IL-6 and RANKL, leading to increased osteoclast activity (42). Interestingly, both TNF-α and IL-1β are upregulated in patients with prosthetic joint infections (PJI) (43). Recently, it was demonstrated that IL-1β signaling contributes to bone loss but is also necessary for local control of bacterial replication (27). In the mouse model of osteomyelitis used in this study, high levels of IL-1β were induced upon bacterial challenge. Immunization with the anti-SpA antibody prevented the excessive production of IL-1β but still allowed for a significant increase of this cytokine, which could contribute to limiting the bacterial burden without the collateral damage in the bone tissue. The role of IL-6 during *in vivo* bone infection is still unclear, and two different studies using IL-6-deficient mice have shown opposite results (44, 45). Nonetheless, IL-6 is detected in serum and bone from patients

with posttraumatic osteomyelitis, and it is particularly associated with the acute phase of the disease (46, 47). *In vivo* neutralization of protein A also contributed to the reduction of CXCL10, a chemokine that has been shown to induce osteoclastogenesis (30) and is upregulated in bone samples from patients with PJI (48). Conversely, the levels of CXCL1 were not decreased in immunized mice, which could permit the recruitment of neutrophils, necessary to clear the infecting microorganism.

We have previously shown that protein A is involved in the priming of osteoclast precursors. This priming is evident as soon as 48 h after bacterial challenge, and it has a significant impact on the subsequent response of osteoclast precursors to the physiological inducer of osteoclastogenesis RANKL (26). In this regard, the present study shows that neutralization of protein A *in vivo* blocked the ability of *S. aureus* to prime osteoclast precursors. It is intriguing that although the production of RANKL by osteoblasts cultured *in vitro* in the presence of protein A has been reported previously (49), no differences in the levels of RANKL in patients with PJI were found compared with control individuals (43, 50). Considering that *S. aureus* is a pathogen that often subverts host physiological responses to its own benefit (24, 51–53), and since we have observed increased expression of RANK in osteoclast precursors stimulated with *S. aureus in vitro*, it is reasonable to hypothesize that, during bone infection, the bacteria might prime osteoclast precursors by inducing an increase in the expression of RANK to make them more susceptible to the endogenous levels of RANKL.

During chronic osteomyelitis *agr*-deficient mutants are often selected *in vivo* (54), and these mutants overexpress protein A due to the decreased levels of RNAIII (55). Staphylococcal protein A can mimic the osteoclastogenic action of TNF-α, inducing a "TNF-α-like response" upon the entry of the bacteria in the bone and before inflammatory cytokines are upregulated at the infected site (26). Therefore, blocking this bacterial molecule might be critical to impede excessive osteoclastogenesis and bone loss. Our results indicate that the neutralization of protein A prevented the differentiation and activation of osteoclasts *in vivo*. A significantly reduced number of osteoclasts was observed in the bone from immunized mice, and expression of cathepsin K, a marker of osteolysis, was significantly decreased in this group. Osteoclasts from nonimmunized mice were increased in size, indicating that a higher number of fusions as well as an active process of osteoclast differentiation was taking place during experimental infection (29). Consequently, blocking protein A led to a decreased loss of trabecular bone during experimental infection, indicating the feasibility of targeting this virulence factor to prevent bone damage during staphylococcal osteomyelitis. It is important to recognize that osteoclasts are not the only players in the pathogenesis of osteomyelitis. A critical role for osteoblasts in the deficient synthesis of new bone matrix and the generation of abnormal bone has been recognized during infection (18), and a key role for protein A in this process has been proposed (56). In this regard, our work shows that the expression of markers of reactive bone formation induced by *S. aureus*, such as osteocalcin and collagen I, was downregulated as a consequence of protein A neutralization. Alpha phenol-soluble modulins are also important staphylococcal virulence factors with a cytotoxic effect against osteoblasts (33). Recent studies have demonstrated the efficacy of diflunisal, a drug that targets the *agr* regulatory system and inhibits toxin secretion, to prevent osteoblast mortality. This approach contributed to decrease cortical bone destruction in a mouse model of osteomyelitis when delivered locally using foams or systemically using nanoparticles (57–59). Therefore, combined treatments that attempt to prevent osteoclast and osteoblast abnormal function might be considered as an adjuvant therapy during the course of osteomyelitis. This approach is in line with recent advances in the field that seem to indicate that targeting specific virulence factors associated with disease would be a promising strategy to combat staphylococcal infections in the light of the increasing appearance of antibiotic resistance (59, 60). In fact, the efficacy of using antibodies against alpha-toxin (Hla) to decrease the severity of USA300 skin infections has been recognized (61–64). More recently, a combination of two human monoclonal antibodies that together neutralize

Hla and the leukocidins LukSF-PV, HlgAB, HlgCB, LukED, and LukGH was shown to neutralize the cytotoxins of *S. aureus* in the lung and prevent damage to the mucosal barrier and innate immune cells in a rabbit model of pneumonia (65). Passive immunization with a monoclonal antibody against the glucosaminidase subunit of autolysin in combination with vancomycin reduced reinfection following revision surgery in a murine model of implant-associated osteomyelitis (66). Passive immunization against SpA has been proposed in the context of other staphylococcal diseases. The administration of a monoclonal antibody against the SpA variant SpA_{KKAA} was able to reduce abscess formation and increase phagocytosis in a renal abscess mouse infection model (67, 68). Moreover, *in vivo*-generated protein A-neutralizing antibodies contributed to *S. aureus* decolonization of the nasopharynx in mice and activated antibody responses against staphylococcal antigens that are recognizable by the immune system (69–71). In the present study, we have demonstrated the feasibility of neutralizing protein A-mediated signaling *in vivo* to avoid the induction of excessive inflammatory responses, osteoclast priming, and activation as well as bone loss during the initial phase of the osteomyelitis. In the experimental mouse model, the first dose of antibody against protein A was administered 1 day prior the bacterial challenge. Although there is always a long way from studies with laboratory animals to clinical trials, from a translational research point of view, this immunization schedule could represent the situation of a programmed implant bone surgery for which the antibody might be administered as a preventive therapy for a potential infection. Future studies using an immunization schedule in which the antibody is administered only after bacterial challenge, alone or in combination with other antivirulence agents, would allow us to expand our findings to other potential clinical settings. This approach may be considered to prevent the development of host conditions that not only damage the bone but also favor bacterial refractory behaviors against antimicrobials and the immune system.

MATERIALS AND METHODS

Animals and housing. BALB/c and C57BL/6 mice were obtained from the animal facility of the Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM, UBA-CONICET), Buenos Aires, Argentina. The procedures involving laboratory animals were approved by the Institutional Animal Care and Use Committee of the School of Medicine, University of Buenos Aires (IACUC approval number 2239/16), and followed internationally accepted guidelines. Animals were maintained in a conventional facility, with controlled temperature (22 ± 1°C), controlled humidity (55%), and a 12-h:12-h light-dark cycle, and fed *ad libitum*. Procedures were performed in an experimental room within the mouse facility. Mice were euthanized using CO_2.

Bacterial strains. *S. aureus* strain USA300 FPR3757 (WT) and the corresponding isogenic SpA-deficient mutant (SpA^-) (provided by Alice Prince, Columbia University, NY, USA) were grown in Trypticase soy broth (TSB) at 37°C with agitation until reaching an optical density at 600 nm (OD_{600}) of 0.8, washed, and suspended in PBS. The concentration was adjusted to 2×10^9 CFU/mL prior to mouse inoculation. For *in vitro* assays, *S. aureus* and the SpA^- mutant were grown in Trypticase soy agar (TSA) at 37°C. *Lactococcus lactis* MG1363 carrying the pKS80 vector containing the full-length SpA (*L. lactis* SpA) or an empty control vector (*L. lactis* CV) (provided by Tim Foster, Trinity College, Dublin, Ireland) was grown in M17 medium supplemented with 0.5% glucose and 5 μg/mL erythromycin at 30°C without agitation (25). Suspensions of *S. aureus*, the SpA^- mutant, and *L. lactis* in alpha minimal essential medium (α-MEM) (Life Technologies, Grand Island, NY) containing 1×10^9 CFU/mL were incubated at 90°C for 60 min and diluted to 1×10^8 CFU/mL prior to being used as heat-killed bacteria during *in vitro* experiments.

Mouse osteomyelitis model and immunization. Ten-week-old BALB/c and C57BL/6 mice were anesthetized with ketamine-xylazine (100/16 mg/kg of body weight), the left tibia was exposed, and a hole in the bone was made with a high-speed drill using a 1-mm-diameter bit. The tibia was inoculated with 2.5 μL of a suspension containing 1×10^6 to 2×10^6 CFU of bacteria or PBS as a control. Inocula were suspended in fibrin glue (Tissucol kit, 1 mL; Baxter Argentina-AG, Austria) (26). In certain experiments, mice were passively immunized via the intraperitoneal (i.p.) route with an anti-SpA antibody (polyclonal antibody P-3775; Sigma-Aldrich, USA) 1 day before the challenge with *S. aureus* and at days 6 and 10 thereafter. The antibody dose used was 75 mg/kg (based on preliminary studies) in 200 μL of PBS. The placebo group received rabbit IgG as a control. Mice were sacrificed and the tibiae were removed at different time points depending on the parameters to be evaluated. The anti-SpA antibody did not have an effect on bacterial growth as assessed by growing WT *S. aureus* in the presence of different concentrations of the antibody (50 to 500 μg/mL).

Osteoclast differentiation assay. α-MEM supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin was used for cell differentiation. Stroma-free bone marrow cells were obtained by flushing the tibiae with 10 mL of α-MEM and red cells were lysed with ammonium chloride (0.5 M). For priming experiments 5×10^5 cells/well (obtained from infected mice) were cultured on glass coverslips in 24-well plates (in 0.5 mL of medium) in the presence of RANKL (50 ng/mL; Miltenyi Biotec, USA) and macrophage colony-stimulating factor (M-CSF) (30 ng/mL; Miltenyi Biotec, USA) for 5 days. In separate experiments, bone marrow cells from naive

TABLE 1 Primers used for reverse transcription-quantitative PCR

Gene	Primer[a]	Annealing temp (°C)
18S rRNA	Fw: 5′-AACACGGGAAACCTCACCC-3′	60
	Rv: 5′-TCCACCAACTAAGAACGGCCA-3′	
Cathepsin K	Fw: 5′-GAGGGCCAACTCAAGAAGAA-3′	50
	Rv: 5′-GCCGTGGCGTTATACATACA-3′	
GAPDH	Fw: 5′-GAAGGTGGTGAAGCAGGCAT-3′	60
	Rv: 5′-TCGAAGGTGGAAGAGTGGGA-3′	
Osteocalcin	Fw: 5′-AAGCAGGAGGGCAATAAGGT-3′	55
	Rv: 5′-CAAGCAGGGTTAAGCTCACA-3′	
RANK	Fw: 5′-TGTGGTCTGCAGCTCTTCCA-3′	58
	Rv: 5′-CGAAGATGATGGCAGCCACTA-3′	
Type I collagen	Fw: 5′-TCTCCACTCTTCTAGTTCCT-3′	60
	Rv: 5′-TTGGGTCATTTCCACATGC-3′	

[a]Fw, forward; Rv, reverse.

mice were differentiated to osteoclast precursors by culturing them in the presence of M-CSF (30 ng/mL) for 72 h and then stimulated with *S. aureus*, the SpA$^-$ mutant, *L. lactis* CV, or *L. lactis* SpA for 9 days. Every 2 to 3 days, the culture medium and all stimuli were replaced. Cells were fixed in 2% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, USA). Multinucleated (having three or more nuclei) TRAP-positive cells were defined as osteoclasts (26).

Cytokine and chemokine detection. IL-1β, IL-6, TNF-α, CXCL1, and CXCL10 were quantified in bone homogenates 14 days after the bacterial challenge by enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs from BD Biosciences (IL-6, TNF-α, and CXCL10) or R&D Systems (IL-1β and CXCL1).

Bacterial load in the bone. Mouse tibiae were crushed and homogenized in sterile mortars. The number of CFU in the homogenates was determined by plating on TSA.

Real-time PCR. RNA was isolated from osteoclasts differentiated *in vitro* (26) or from mouse tibiae at 14 days after bacterial challenge using TRIzol reagent (Invitrogen). Bone homogenates were obtained by crushing the tissue using a glass mortar and pestle in ice (72). cDNA was made from 1 μg of RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Gene quantification was performed using the Applied Biosystems StepOne real-time PCR system and SYBR green (Applied Biosystems). The primers used for gene quantification are listed in Table 1. Forty cycles were run with denaturation at 95°C for 15 s, the corresponding annealing temperature for each gene for 30 s, and extension at 72°C for 45 s. 18S rRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for standardization. Results are depicted as the ratio of the expression of the gene of interest to that of the housekeeping gene.

μCT of trabecular bone. Tibiae were harvested 14 days postinoculation and fixed for 48 h in neutral buffered formalin at 4°C. Bones were scanned using a μCT50 scanner (Scanco Medical, Switzerland) and analyzed with micro-computed tomography (μCT) V6.3-4 software (Scanco USA, Inc., Wayne, PA). Trabecular bone measurements were obtained in the distal tibiae by advancing proximally past the growth plate 30 slices. A total of 101 slices were analyzed with an inclusive contour drawn along the endosteal surface to include trabeculae and exclude the cortical bone. Trabecular bone volume (BV) and total tissue volume (TV) were determined by segmentation of the image with a lower threshold of 329 mg hydroxyapatite (HA)/cm^3, sigma 1.3, and support 1 (27).

Bone histology and histomorphometric analysis of osteoclasts in trabecular bone. After μCT imaging, tibiae were decalcified for 3 days in 20% EDTA at 4°C. Decalcified bones were processed and embedded in paraffin before being sectioned at 4-μm thickness through the infectious nidus and bone marrow cavity using a Leica RM2255 microtome. Sectioned tibiae were stained for osteoclast detection with TRAP stain. Osteo Measure software (OsteoMetrics, Inc., Decatur, GA) was used to manually analyze TRAP-stained histologic sections at a region of interest encompassing the trabeculae proximal to the growth plate in the distal tibiae. Osteoclast number, osteoclast surface, bone perimeter, and bone surface were determined and reported per American Society for Bone and Mineral Research (ASBMR) standards (32). A Leica SCN400 slide scanner was used to scan stained tibia sections in bright field at 20×.

Statistics. Data are depicted either showing individual values or as bar graphs showing the mean and standard deviation in each group. Data were analyzed using the Student *t* test (nonpaired or paired samples) or 1-way analysis of variance (ANOVA) with Bonferroni's posttest to compare all pairs of data. GraphPad Prism software version 7.0 was used. A *P* value lower than 0.05 was considered statistically significant.

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