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PII: S0925-4439(16)30184-3
DOI: doi: 10.1016/j.bbadis.2016.07.016
Reference: BBADIS 64520

To appear in: *BBA - Molecular Basis of Disease*

Received date: 30 April 2016
Revised date: 4 July 2016
Accepted date: 26 July 2016

Please cite this article as: Andrea Mendoza Bertelli, María Victoria Delpino, Santiago Lattar, Constanza Giai, Mariángeles Noto Llana, Norberto Sanjuan, James E. Cassat, Daniel Sordelli, Marisa I. Gomez, *Staphylococcus aureus* protein A enhances osteoclastogenesis via TNFR1 and EGFR signaling, *BBA - Molecular Basis of Disease* (2016), doi: 10.1016/j.bbadis.2016.07.016

Staphylococcus aureus protein A enhances osteoclastogenesis via TNFR1 and EGFR signaling.

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ABSTRACT

Staphylococcus aureus is a major causative agent of osteomyelitis in adults and children. The increasing incidence of antimicrobial resistant isolates and the morbidity of this type of infection denote that alternative therapeutic approaches are required. *S. aureus* protein A interacts with TNFR1 and EGFR expressed at the surface of host cells. Given the importance of TNF-α and EGFR/RANKL crosstalk in enhancing osteoclast differentiation, the aim of this study was to determine the role of protein A in the induction of osteoclastogenesis and bone resorption during staphylococcal osteomyelitis. We determined that protein A plays a critical role in osteoclast differentiation and activation by initiating TNFR1 and EGFR mediated signaling. Moreover, we demonstrated that protein A significantly contributes to increased osteoclast differentiation and activation as well as cortical bone destruction during the course of disease using experimental models of osteomyelitis. Our findings strongly suggest targeting protein A and TNFR1 as an adjunctive strategy to control bone damage during the initial course of *S. aureus* osteomyelitis.

Key Words: *Staphylococcus aureus*, protein A, osteoclastogenesis, TNFR1, EGFR

1. Introduction

Staphylococcus aureus osteomyelitis is the greatest concern in patient care among orthopedic surgeons as this pathogen causes from 50 to 70% of adult osteomyelitis [1–4]. The increasing incidence of antimicrobial resistant isolates and the morbidity of this type of infection denote that alternative therapeutic approaches are required [5–8]. In addition, the ability of *S. aureus* to be internalized by osteoblasts [9] and to form biofilms [10] may lead to persistence and refractory behavior to antibiotic treatment regardless of antibiotic susceptibility *in vitro* [2]. The skeleton is a dynamic organ system which is constantly being rejuvenated and remodeled [11]. Osteoblasts are responsible for the deposition, calcification and mineralization of bone matrix whereas osteoclasts conduct bone resorption by acidification and release of lysosomal enzymes. The entry of *S. aureus* to the healthy bone leads to the development of osteomyelitis, which results in disruption of the fine balance between osteoblast and osteoclast activity [2,12,13].

Many *S. aureus* virulence factors have been suggested to play a role in the pathogenesis of bone infections [2]. We have previously identified protein A as a major inducer of inflammatory responses due to its ability to activate host cell signaling cascades by interacting with the TNF receptor 1 (TNFR1) and the EGF receptor (EGFR) [14–17]. Several studies have recently demonstrated the role of protein A-TNFR1 interaction in the production of soluble mediators by pre-osteoblasts [18] as well as the induction of osteoblast apoptosis, decreased proliferation and mineralization [19] and increased Receptor Activator for Nuclear Factor κ B Ligand (RANKL) secretion [20], suggesting a role for this protein in negative modulation of bone deposition. The action

of protein A on the major skeletal cell type involved in bone resorption, the osteoclast, however, has not been investigated intensively [20]. Considering that TNF-α signaling and EGFR/RANKL crosstalk contribute to osteoclast differentiation [21,22] we postulate that protein A may induce increased osteoclastogenesis and positively modulate bone resorption during *S. aureus* infection.

2. Materials and Methods

2.1. Ethics statement

Blood samples were obtained from healthy blood donors according to the Declaration of Helsinki. All animal procedures were performed according to the NIH rules and standards for the use of laboratory animals [23] and were approved by the "Institutional Committee for Care and Use of Laboratory Animals" (CICUAL) of the School of Medicine, University of Buenos Aires.

2.2. Animals

BALB/c, C57BL/6 and TNFR1 deficient (*tnfr1*$^{-/-}$) mice as well as Wistar rats were obtained from the School of Medicine and the School of Pharmacy and Biochemistry at the University of Buenos Aires, respectively. Animals were housed in groups of five, under controlled temperature (22°C) and artificial light under a 12-h cycle period, were provided with food and water ad libitum and euthanized using CO_2.

2.3. Recombinant proteins and bacterial strains

Full-length protein A (SpA), protein A domain D or the L17A mutant were expressed as GST-fusion proteins [16]. Potentially remaining lipopolysaccharide traces were removed using Detoxi-Gel Endotoxin Removing Gel columns (Pierce, Holmdel, NJ). The absence of lipopolysaccharide was confirmed using a polymyxin B inhibitory assay [24]. *S. aureus* (strains USA300 FPR3757 and Newman) and the corresponding isogenic SpA- mutants (provided by Dr. Alice Prince, Columbia University, NY, USA) were grown in trypticase soy agar at 37°C. *Lactococcus lactis* MG1363 carrying the pKS80 vector containing the full length SpA or an empty control vector (provided by Dr. Tim Foster, Trinity College, Dublin, Ireland) were grown in M17 medium supplemented with 0.5% glucose and 5 µg/ml erythromycin at 30ºC without agitation [15]. Cells were harvested by centrifugation, the pellet was suspended in α-MEM medium (Life Technologies, Grand Island NY) and suspensions containing 1 x 10^9 CFU/ml were incubated at 60°C for 60 min and diluted to 1 x 10^8 CFU/ml prior to be used as heat-killed bacteria during *in vitro* experiments. This procedure did not alter surface exposed protein A interaction with TNFR1 [16,20].

2.4. Osteoclast differentiation assay

Alpha-MEM medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS (Gibco, Life Technologies), 100 U penicillin/ml, and 100 µg streptomycin/ml was used. Stroma-free bone marrow cells from BALB/c, C57BL/6 or *tnfr1*$^{-/-}$ mice were obtained by flushing the femur (*in vitro* assays) or tibia (post-inoculation assays) with 10 ml of α-MEM and red cells were lysed with ammonium chloride (0.5 M). Peripheral blood

mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation. Monocytes were purified of PBMCs using magnetic beads labeled with anti-CD14 (Miltenyi Biotec). Purity of approximately 95% was verified by flow cytometry and cell viability >95% was determined by trypan blue exclusion. Cells were cultured in the presence of 30 ng/ml human M-CSF (R&D Systems, Minneapolis, MN, USA) for 72 h on glass coverslips in 24-well plates [$5x10^5$ cells/well (bone marrow cells) or $2.5x10^5$ cells/well (monocytes) in 0.5ml of media] to differentiate into osteoclast precursors [25] which were stimulated in the presence of M-CSF (30 ng/ml). Every 2-3 days, the culture media and all stimuli were replaced. Cells were fixed in 4% paraformaldehyde and stained for Tartrate-Resistant Acid Phosphatase (TRAP) (Sigma-Aldrich, St. Louis, MO, USA). Multinucleated (more than three nuclei), TRAP-positive cells were defined as osteoclasts. MMP-9 activity in supernatants was assayed as described previously [26] and the levels of mouse and human TNF-α as well as mouse MMP-9 were quantified using commercially available ELISA kits [R&D Systems (mouse TNF-α and MMP-9), BD Biosciences (human TNF-α)].

2.5. Pit formation assay

Bone marrow cells from C57BL/6 or *tnfr1* $^{-/-}$mice ($2x10^5$ cells/well in 0.2 ml of media) were plated on dentine disks (BD BioCoat Osteologic, BD Biosciences, San Diego, CA, USA), differentiated into osteoclast precursors as described above and subsequently cultured in complete medium containing $1x10^8$ CFU/ml of heat-killed bacteria in the presence of M-CSF (30 ng/ml) for 9 days. Media and all reagents were replaced daily. Dentine discs were washed with 1M NH_4OH to remove adherent cells,

rinsed with water and resorption lacunae were visualized by light microscopy. Digital images of representative fields for each well were taken. The assay was run in duplicated wells.

2.6. Real-time Polymerase Chain Reaction

RNA was isolated using TRIzol Reagent (Invitrogen). Complementary DNA was made from 1 µg of RNA using M-MLV Reverse Transcriptase (Promega). The following primers were used for amplification: mouse RANK: 5'-TGTGGTCTGCAGCTCTTCCA-3' and 5'-CGAAGATGATGGCAGCCACTA-3', annealing: 58ºC; mouse Cathepsin K: 5'-GAGGGCCAACTCAAGAAGAA-3' and 5'-GCCGTGGCGTTATACATACA-3', annealing: 50ºC; mouse GAPDH: 5′-GAAGGTGGTGAAGCAGGCAT-3′ and 5′-TCGAAGGTGGAAGAGTGGGA-3′, annealing: 60ºC. Forty cycles were run with denaturation at 95°C for 15 seconds, the corresponding annealing temperature for each gene for 30 seconds and extension at 60°C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (mGAPDH) was used as control for standardization.

2.7. Mouse and rat osteomyelitis models

BALB/c mice or Wistar rats (10 weeks old) were anesthetized with ketamine/xylazine, 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 5 µl (rat) or 2.5 µl (mouse) of a suspension containing 1-2 $x10^6$ CFU of *S. aureus* FPR3757, the isogenic SpA- mutant or PBS as control. The different inocula were suspended in fibrin glue (Tissucol kit 1 ml; Baxter Argentina-AG, Vienna, Austria) [27]. At 48 hours after

inoculation bone marrow cells from infected tibias were obtained to be used in osteoclastogenesis assays. In parallel experiments, 14 days after inoculation the tibias were subjected to micro-CT analysis. Left and right tibias from rats were excised and evaluated morphometrically, and the osteomyelitic index (OI) was determined as described previously [28]. Briefly, the following measurements were made using calipers: (i) the distance between the inoculation point and the distal end of the left tibia (DT); (ii) the left tibia section diameter at the inoculation site (Di) and the perpendicular diameter at the same site (Dp); (iii) Di and Dp were also measured in the uninfected right tibia of the same rat at the DT determined in the diseased left tibia (control). The osteomyelitic index (OI) was determined as follows: OI = (Dp +Di) infected - (Dp +Di) control. To assess the bacterial load, the infected bones were crushed, homogenized in sterile mortars and quantitatively cultured on TSA plates.

2.8. Histopathological and osteoclast evaluation during in vivo infection

Tibias were fixed overnight in 4% paraformaldehyde, followed by decalcification in 10% EDTA in 1 mM TrisHCl (pH 7.4) for 2 weeks at 4°C, dehydrated in ethanol at 96 and 100%, clarified in xylene, and routinely embedded in paraffin. Haematoxylin-eosin and TRAP staining were performed on tissue sections.

2.9. Microcomputed Tomography (micro-CT)

Analysis of cortical bone destruction was determined by micro-CT imaging with a μCT50 (Scanco Medical) and the manufacturer's analytical software as previously described [29]. Briefly, axial images of each tibia were acquired with 5.0 mmvoxels at 70

kV, 200 mA, 2,000 projections per rotation, and an integration time of 350 ms in a 10.24 mm field of view. Each imaging scan comprised 1,635 slices (8.125 mm) of the length of the tibia, centered on the inoculation site as visualized in the scout-view radiographs. For analysis of cortical bone destruction, a volume of interest (VOI) including only the original cortical bone and any destruction was selected by drawing of inclusive contours on the periosteal surface and excluding contours on the endosteal surface. Volume of cortical bone destruction was determined by segmentation of the image with a lower threshold of 0 and an upper threshold of 595 mg HA/ccm, sigma 1.3, and support 1, to exclude bone in the analysis. The direct voxel counting method was used for all reported calculations in each analysis.

2.10. Statistics

Data from samples with normal distribution are depicted as bar graph showing the mean and standard deviation and were analyzed using the Student *t* test or 1-way ANOVA with the Bonferroni post-test to compare all pairs of data. For data from samples that do not follow normal distribution the median values are depicted and data were analyzed with the non-parametric Mann-Whitney test from the Graph Pad Prism Software version 4.0. A *p* value lower than 0.05 was considered statistically significant.

3. Results

3.1. S. aureus protein A induces osteoclastogenesis

First, we established the potential role of *S. aureus* protein A in driving osteoclast differentiation. A significant increase in the number of osteoclasts was observed in response to protein A compared with cells stimulated with GST or media alone as control (Fig. 1A). In order to evaluate the osteoclastogenic potential of protein A in the context of the bacterial cell wall, we used the *Lactococcus lactis* heterologous expression system. A significant increase in the number of osteoclasts was induced by *L. lactis* SpA (Fig. 1B) and this response was significantly inhibited by an anti-protein A neutralizing antibody (Fig. 1B). Considering that bacteria-host interactions may vary among different hosts the role of SpA in the induction of osteoclastogenesis was further confirmed using osteoclast precursors from C57BL/6 mice (Fig. 1B).

We then determined the contribution of protein A-mediated osteoclastogenesis to overall *S. aureus*-induced osteoclast differentiation. A significant number of osteoclasts were observed after stimulation of osteoclast precursors with *S. aureus* (Fig. 1C). A significant role for protein A in this process was demonstrated by using an isogenic mutant lacking protein A expression (SpA-) (Fig. 1C). Moreover, osteoclast differentiation induced by *S. aureus* was significantly decreased in the presence of an anti-protein A neutralizing antibody (Fig. 1C).

Considering that *Staphylococcus aureus* is a major cause of osteomyelitis in humans, experiments using human monocytes as osteoclast precursors were also conducted. A significant increase in the number of osteoclasts was observed in

response to *S. aureus* (Fig. 1D), and protein A significantly contributed to osteoclast differentiation as demonstrated using the isogenic SpA- mutant (Fig. 1D). These observations were further confirmed by stimulating the cells with *L. lactis* SpA (Fig. 1D).

Since TNF-α may significantly contribute to osteoclast differentiation [13,21], we determined the induction of this inflammatory cytokine in precursors from BALB/c mice. *S. aureus* induced an early increase in TNF-α and the levels of this cytokine were significantly lower in response to the SpA- mutant as well as in cells stimulated with *S. aureus* in the presence of the anti-protein A neutralizing antibody (Fig. 2A). Moreover, *L. lactis* SpA induced significant levels of TNF-α (Fig. 2B). Similar results were observed using C57BL/6 mice (Fig. 2C and D). TNF-α was also induced in human monocytes in response to *S. aureus* (Fig. 2E) or *L. lactis* SpA (Fig. 2F) and the levels of this cytokine were significantly lower in response to the SpA- mutant (Fig. 2E).

3.2. Osteoclastogenesis induced by protein A is mediated by TNFR1 and EGFR

In order to establish the signaling pathways involved in protein A–induced osteoclastogenesis the role of TNFR1 and EGFR, both previously shown to be involved in protein A signaling in several cell types [14,17,19,20] was investigated. A significant decrease in the number of osteoclasts derived from precursor cells in response to *L. Lactis* SpA and protein A was observed using progenitors from *tnfr1*$^{-/-}$ mice as compared to C57BL/6 mice (Fig. 3A). TNF-α and RANKL were used as positive controls. The absence of baseline deficits in osteoclast precursors from *tnfr1*$^{-/-}$ mice was demonstrated using RANKL as stimuli (Fig. 3A). We then evaluated the contribution of EGFR by using a mutated protein A (SpAL17A) that is not able to signal through this receptor [17]. A

significant decrease in osteoclastogenesis was observed in cells from C57BL/6 mice stimulated with the SpAL17A protein compared with that observed in response to the wild type SpA-D (Fig. 3B). Moreover, when osteoclast precursors from *tnfr1*$^{-/-}$ mice were stimulated with SpAL17A, the osteoclastogenesis was equivalent to that observed in control cells (Fig. 3B). Taken together these results demonstrate that protein A induces osteoclastogenesis by activating TNF and EGF receptors in osteoclast precursors.

As we previously showed that protein A induces early TNF-α production (Fig. 2), we then evaluated whether protein A was able to induce osteoclastogenesis through direct TNFR1 activation or the signaling was secondary to TNF-α production. A significant decrease in osteoclastogenesis was observed when cells from C57BL/6 mice were stimulated with SpA-D in the presence of an anti-TNF-α neutralizing antibody (Fig. 3C). Moreover, when the cells were stimulated with the SpAL17A protein, which only signals through TNFR1, in the presence of an anti-TNF antibody the osteoclastogenesis was completely abrogated (Fig. 3C) indicating the importance of TNF-α in protein A-induced osteoclastogenesis.

We then evaluated the role of TNF-α in the osteoclastogenesis induced by *S. aureus*. A significant reduction in the number of osteoclasts was observed when cells from *tnfr1*$^{-/-}$ mice were stimulated with *S. aureus* (Fig. 3D). The number of osteoclasts differentiated from *tnfr1*$^{-/-}$precursors in response to the protein A deficient mutant was decreased compared with the differentiation observed in osteoclast precursors from C57Bl/6 mice (Fig. 3D), suggesting that other molecules in addition to SpA induce TNF-α responses critical for osteoclastogenesis. This was further confirmed in osteoclast

precursors from C57Bl/6 mice stimulated with the SpA- mutant in the presence of an anti-TNF-α antibody (Fig. 3D).

3.3. Protein A and TNFR1 signaling are critical for osteoclast activity

The most important proteinases involved in organic bone matrix degradation are cathepsin K and MMP-9 [30,31]. Therefore, we quantified the levels of cathepsin K expression and found that it was significantly increased in osteoclasts differentiated in response to *S. aureus* or *L.lactis* SpA (Fig. 4A). Conversely, the SpA- mutant induced significantly lower levels of cathepsin K expression (Fig. 4A). Induction of cathepsin K was not observed in osteoclast precursors from *tnfr1*$^{-/-}$ mice (Fig. 4A). In addition, increased levels of MMP-9 were observed in the supernatants of cells stimulated with *S. aureus* compared with those found in the supernatants of cells stimulated with the SpA- mutant (Fig. 4B). The differences in MMP-9 expression correlated with increased gelatinase activity induced by *S. aureus,* which according to the molecular weight of the band corresponded to MMP-9, compared with that induced by the SpA- mutant (Fig. 4C). MMP-9 expression and activity was decreased in supernatants from cells stimulated with *S. aureus* in the presence of an anti-protein A neutralizing antibody (Fig. 4B and C).

The ability of osteoclasts differentiated in response to *S. aureus* to resorb dentine was determined as an indicator of its functionality. Osteoclast precursors from C57BL/6 mice stimulated with *S. aureus* or *L. lactis* SpA were able to resorb dentine whereas cells stimulated with the SpA- mutant or *L. lactis* CV did not form resorption pits (Fig. 4C). These results indicate that whereas other staphylococcal antigens besides protein

A may induce osteoclast differentiation as evidenced using the SpA- mutant (Fig.1 and Fig. 3), protein A is critical for the induction of osteoclast activity (Fig. 4C). In addition, TNFR1 signaling also proved critical for osteoclast activity as demonstrated by the lack of dentine resorption pits when cells from *tnfr1*$^{-/-}$ mice were used as osteoclast precursors (Fig. 4C).

3.4. S. aureus protein A contributes to bone damage during experimental osteomyelitis.

The role of protein A in bone damage during staphylococcal infection was evaluated using previously described animal models [28,29]. In order to assess the early impact of protein A expression on osteoclast differentiation during *in vivo* infection, we first determined the ability of *S. aureus* and the SpA- mutant to prime osteoclast precursors. Bone marrow cells from mice previously inoculated with *S. aureus* showed significantly increased ability to differentiate into mature osteoclasts in response to RANKL than those obtained from mice inoculated with the SpA- mutant (Fig. 5A). Moreover, the osteoclastogenic potential of cells from mice inoculated with the SpA- mutant did not differ from cells obtained from control mice inoculated with PBS highlighting the importance of protein A in the early priming of osteoclast precursors (Fig. 5A). The decreased priming in cells from SpA- inoculated mice was not due to differences in the amount of bacteria present in the bone as assessed by bacterial count in bone homogenates (Fig. 5B). In order to determine the consequences of the increased osteoclastogenic activity during *S. aureus* infection, cortical bone destruction was quantified by micro-CT 14 days after inoculation as previously described [29]. A significant increase in bone destruction was observed in the tibias from mice inoculated

with *S. aureus* compared with that quantified in mice inoculated with PBS (Fig. 5C and D; Movie S1 and S2). Conversely, cortical bone loss in the tibias from mice inoculated with the SpA- mutant was nearly equivalent to that observed in mice inoculated with PBS as control (Fig. 5C and D; Movie S1 and S3). At 14 days after challenge, no significant differences in the bacterial load of the bones were observed between the *S. aureus* and the SpA- inoculated mice (Fig. 5E).

Using the rat osteomyelitis model [28] MMP-9 activity in bone homogenates was evaluated as an indicator of the presence of functional osteoclasts. Increased gelatinase activity was detected in bone from rats inoculated with *S. aureus* whereas no increase in MMP-9 activity was observed in bone from rats inoculated with the SpA- mutant (Fig. 6A). A significant macroscopic enlargement of the bone at the inoculation site of the tibia, represented as the osteomyelitic index, was observed at 15 weeks after challenge and the osteomyelitic index was significantly lower in tibia from rats inoculated with the SpA- mutant (Fig. 6B). Histopathological analysis of tibia sections from infected rats at 96 hours post-challenge revealed only congestion and a mild neutrophil exudate at the site of inoculation in all groups (data not shown). After 15 weeks, rats inoculated with the SpA- mutant showed no histological changes indicative of acute inflammation (Fig. 6C), whereas those inoculated with *S. aureus* developed lesions characterized by edema, congestion and intense neutrophil infiltrates in the bone marrow as well as fragments of detached, dead bone (sequestra) surrounded by pockets of pus (Fig. 6C). Increased osteoclastogenesis during *in vivo S. aureus* infection was also evidenced by the presence of TRAP positive multinucleated cells in tibia sections (Fig. 6C). Conversely, multinucleated TRAP positive cells were not observed in tibia sections from rats

inoculated with the SpA- mutant (Fig. 6C) further confirming that the presence of protein A is critical for bacterial-induced osteoclastogenesis *in vivo*. At this time point, 15 weeks after the onset of infection, a significant decrease in bacterial counts in the bones of rats inoculated with the SpA- mutant was observed (Fig. 6D). The differential clearance could be partially explained by the reduced amount of potential niches for bacterial persistence in the bones inoculated with the mutant compared with those inoculated with *S. aureus* in which sequestra are observed. In addition, the known ability of protein A to inhibit opsonophagocytosis could contribute to enhanced bacterial clearance at a time point in which specific antibodies are likely to be present.

4. Discussion

Although the importance of *S. aureus* as a causative agent of osteomyelitis is widely recognized, the molecular events that take place during the interaction of this pathogen with host cells within the bone microenvironment are certainly not completely understood. It has been established that the fine regulation of the osteoblast and osteoclast crosstalk is critical for bone physiology, and pro-inflammatory cytokines disrupt the balance between the activities of these two cell types [13]. In order to understand the molecular basis of bone damage during osteomyelitis, a great deal of attention has been given to the interaction of *S. aureus* with osteoblasts and it was suggested that osteoblasts would interpret the majority of the extracellular signals and subsequently modulate osteoclast differentiation and function via RANKL (2, 19, 20, 27, 28). Current experimental evidence also demonstrates that osteoclast precursors are able to sense different stimula [25,34–36]. Recent studies using live bacteria

demonstrated that *S. aureus* modulates cytokine production in osteoclast precursors and increases the resorption activity of mature osteoclasts in the absence of osteoblasts [37]. Considering the ability of *S. aureus* protein A to signal through TNFR1 and EGFR, in this study we investigated the role of this staphylococcal surface protein in modulating osteoclastogenesis. We demonstrated that protein A plays a critical role in osteoclast differentiation and activation *in vitro* using murine bone marrow cells and human monocytes primed with M-CSF as osteoclast precursors. Moreover, protein A significantly contributed to increased osteoclast differentiation and activation as well as cortical bone destruction during the course of disease in two experimental models of osteomyelitis.

Using *tnfr1*$^{-/-}$ deficient mice and a mutated SpA that cannot signal through EGFR, we demonstrated that the ability of protein A to induce osteoclastogenesis was mediated by TNFR1 and EGFR signaling. The role of TNF-α in the induction of osteoclastogenesis has been previously demonstrated [22]. *S. aureus* and protein A induced the early production of this cytokine, suggesting that either TNF-α could be responsible for the osteoclastogenesis or that protein A could directly induce osteoclast differentiation due to its ability to signal through TNFR1 and activate NF-κB [14,18]. The use of an anti-TNF-α neutralizing antibody allowed us to determine that osteoclastogenesis was indeed secondary to TNF-α. This cytokine is probably produced as a consequence of the initial interaction of protein A with TNFR1 [16,20]. The role of EGFR in osteoclast formation and distribution along the bone has been previously demonstrated using *egfr*$^{-/-}$embryos [38]. Moreover, *in vitro* assays have further established that EGFR is a critical regulator of osteoclast differentiation and survival through cross-talk with RANK signaling.

RANKL-RANK interaction trans-activates EGFR and induces its phosphorylation which is critical for osteoclast differentiation [38,39]. We have previously demonstrated the ability of protein A to induce EGFR phosphorylation and activation of downstream signaling [17] which may explain the role of EGFR in the osteoclastogenesis induced by this protein. An interesting finding of this study was that although the osteoclast differentiation induced by protein A was dependent on both receptors, the osteoclast activity was completely abolished in the absence of TNFR1 signaling. In this regard, previous work using RANKL as a trans-activator of EGFR has demonstrated that the osteoclast resorptive activity is not modulated by EGFR signaling [38] indicating that different signals control osteoclast differentiation and their function. Our findings are in agreement with a previous study demonstrating a critical role for TNFR1 in LPS/TNF-α mediated osteoclastogenesis [40].

Our results also demonstrate that other staphylococcal molecules, in addition to protein A, induce osteoclastogenesis [41]. This is likely through the induction of TNF-α based on the results of experiments using the anti-TNF-α neutralizing antibody and the SpA- mutant as stimuli. It has been demonstrated that surface-associated material from *S. aureus,* mostly constituted by proteins, is able to stimulate osteoclast maturation and activation in the absence of exogenously added RANKL [34,42] and the differentiation is inhibited in the presence of anti-TNF-α antibodies [34]. A role for *S. aureus* lipoproteins in osteoclast differentiation has also been recently described [43]. The staphylococcal surface protein Sbi has been recently identified as a novel inducer of TNFR1 and EGFR signaling in macrophages [44] and could potentially contribute to the osteoclastogenesis induced by *S. aureus*. An intriguing finding of this study, was that although the SpA-

mutant induced osteoclast differentiation through the induction of TNF-α by other bacterial molecules, it did not induce resorption pit formation suggesting that the signals that drive osteoclast activity are complex and far from being completely elucidated. In this regard, *in vivo* studies are highly valuable tools that can contribute to a better understanding of the molecular events that lead to bone resorption during osteomyelitis.

Several animal models have been proposed to study the pathogenesis of bacterial osteomyelitis *in vivo* [45,46]. Using a modification of a previously described mouse model of experimental osteomyelitis [29] we were able to demonstrate that the presence of protein A has a significant impact in osteoclast differentiation during the initial stages of *S. aureus* infection. Moreover, micro-CT analysis of the infected bone highlighted the importance of protein A in mediating cortical bone destruction. Our results are in agreement with previous studies demonstrating that *sae*, a positive regulator of SpA [47] contributes to bone destruction [29]. In addittion, using a rat osteomyelitis model [27,28] we were able to investigate the biological relevance of our findings during later stages of the disease. Increased number of osteoclasts as well as higher MMP-9 activity in tibias from rats inoculated with *S. aureus*, as compared with those inoculated with the SpA- mutant demonstrate that protein A contributes not only to osteoclast differentiation but also to osteoclast function *in vivo*. *S. aureus* intratibial infection induced histopathological changes that correlated with those previously described during bacterial osteomyelitis. The lack of congestion, edema, neutrophil exudate and bone sequestra in rats inoculated with the SpA- mutant demonstrate the involvement of protein A in the processes that lead to bone destruction during the progression of the disease.

Given the importance of the regulation of osteoclast activity in the maintenance of proper bone physiology, our data indicate the ability of protein A to drive osteoclast differentiation and activation through TNFR1 signaling which would be particularly relevant during the initial encounter of the host with bacteria contributing to accelerated bone destruction. Taken together, these findings suggest that protein A and TNFR1 could be considered as novel potential prophylactic/therapeutic targets to design agents aimed at preventing bone damage during *S. aureus* osteomyelitis.

Supplemental material

The following supporting information may be found in the online version of this article:

Movie S1: Cortical bone destruction in a representative tibia of mice inoculated with PBS.

Movie S2: Cortical bone destruction in a representative tibia of mice inoculated with *S. aureus*.

Movie S3: Cortical bone destruction in a representative tibia of mice inoculated with the SpA- mutant.

Acknowledgments

The authors thank Monica Pomerantz for technical assistance and Dr. Analia Trevani and Dr. Florencia Sabbione for assistance with PBMC isolation. This work was supported in part by grants from the Agencia Nacional de Promoción de la Ciencia y la Tecnología, Argentina (ANPCYT PICT 13-0941 to D.S., PICT10-2152, 11-2263 and 13-1233 to M.G.); the Secretaría de Ciencia y Técnica, Universidad de Buenos Aires,

Argentina (UBACyT 20020110100138 to M.G. and 20020130100331BA to D.S.) and the Florencio Fiorini Foundation (to M.G.). JEC is supported by a Burroughs Wellcome Fund Career Award for Medical Scientists and NIAID K08 AI113107 from the National Institutes of Health. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Figure Legends

Fig. 1. Osteoclastogenesis induced by *S. aureus* and protein A. Osteoclast precursors derived from bone marrow cells of BALB/c (black bars) and C57BL/6 (white bars) mice (A-C) or from human monocytes (D) were stimulated with purified protein A (SpA, 0,5

nM), GST (0,5 nM), RANKL (RL, 50 ng/ml) or heat- killed bacteria [*L. lactis* carrying a control vector (*L. lactis* CV), *L. lactis* expressing protein A (*L. lactis* SpA), *S. aureus* (SA) or a protein A deficient mutant (SpA-), 10^8 CFU/ml]. An anti-protein A neutralizing antibody (Sigma P3775, 500 µg/ml) was added as indicated (grey bars). On day 9, the number of osteoclasts quantified as multinucleated (more than 3 nuclei) TRAP positive cells (TRAP (+) MNCs) was determined. Pictures in B correspond to BALB/c mice. Bars represent cumulative data from three independent experiments with duplicates for each condition. Data were analyzed using 1-way ANOVA with the Bonferroni post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For images of TRAP staining the magnification was 20X.

Fig. 2. TNF-α production in response to *S. aureus*. Osteoclast precursors derived from bone marrow cells of BALB/c mice (A-B) or C57BL/6 mice (C-D) and from human monocytes (E-F) were stimulated with *S. aureus* (SA), the protein A deficient mutant (SpA-), *L. lactis* carrying a control vector (*L. lactis* CV) or *L. lactis* expressing protein A (*L. lactis* SpA) (10^8 CFU/ml), during 24 hours. In certain experiments an anti-protein A antibody (Sigma P3775, 500 µg/ml) was used as indicated (grey bars). The levels of TNF-α were quantified in the culture supernatant by ELISA. Bars represent cumulative data from three independent experiments with duplicates/triplicates for each condition. Data were analyzed using 1-way ANOVA with the Bonferroni post-test. *$p < 0.01$; ***$p < 0.001$.

Fig. 3. Role of TNFR1 and EGFR signaling in the osteoclastogenesis induced by protein A. Osteoclast precursors from C57BL/6 (black and white bars) or *tnfr1*$^{-/-}$ (grey bars) mice were stimulated with *S. aureus* (10^8 CFU/ml), SpA-D (0.5 nM), the L17A mutant (SpA-L17A, 0.5 nM), TNF-α (10 ng/ml as positive control) or RANKL (RL). An anti TNF-α neutralizing antibody (TN3-19.12 BDBiosciences, 10 µg/ml) was added as indicated (white bars). On day 9 cells were fixed and multinucleated TRAP (+) (TRAP (+) MNCs) were enumerated. Bars represent cumulative data from three independent experiments with duplicates/triplicates for each condition. (B) Data are presented as the percentage of the control (cells differentiated in response to SpA-D) for each condition. (A-D) Data were analyzed using 1-way ANOVA with the Bonferroni post-test.*$p < 0.01$; ***$p < 0.001$.

Fig. 4. Osteoclast activity in response to *S. aureus* and protein A. (A) Murine osteoclast precursors were stimulated with *S. aureus*, the SpA-mutant, *L. lactis* CV or *L. lactis* SpA (10^8 CFU/ml) for 9 days. Cathepsin K (CK) expression was quantified by real time RT-PCR and normalized to GAPDH expression. Data were analyzed using 1- way ANOVA with the Bonferroni post-test *P < 0.01. (B, C) Murine osteoclast precursors were stimulated with *S. aureus* or the SpA-mutant in the absence (black bars) or the presence (grey bars) of an anti-protein A neutralizing antibody (Sigma P3775, 500 µg/ml) for 9 days. MMP-9 was quantified by ELISA (B) and gelatinase activity in the supernatants of stimulated cells was detected by zymography (C). All samples were run in the same gel in an order different than shown which is indicated by the white lines in between the lanes. (B, C) The data shown are from one representative experiment of two performed.

Bars represent the average of two independent samples. (D) Functional activity of osteoclasts was determined by their ability to resorb dentine. Osteoclast precursors from C57BL/6 or *tnfr1*$^{-/-}$ mice were cultured on dentine discs and stimulated with *S. aureus*, the SpA-mutant, *L. lactis* CV or *L. lactis* SpA. After 9 days, cells were removed, and dentine resorption pits were determined by light microscopy. Samples were analyzed in a blind manner.

Fig. 5. Osteoclastogenic activity during experimental osteomyelitis. (A) Osteoclast precursors obtained 48 hours after inoculation with *S. aureus,* the SpA- mutant or PBS (Control, C) were cultured in the presence of M-CSF (30ng/ml) and RANKL (50 ng/ml). At day 5 the number of multinucleated TRAP (+) (TRAP (+) MNCs) was determined. Bars represent the mean and standard deviation of triplicate wells assessed for each mouse (n=3-4 mice per group). Data were analyzed using 1-way ANOVA with the Bonferroni post-test. $^{**}p < 0.01$. (B) Bacterial load in bone homogenates. Boxes and whiskers depict maximum and minimum values obtained from individual mice (n=12) and the horizontal line represents the median for each group. (C, D) Cortical bone destruction quantified by micro-CT analysis. Samples were analyzed in a blind manner. (C) Anteroposterior views of representative infected tibias of each group at 14 days post-inoculation. Left panel: microCT image; right panel: microCT reconstructions of the volume of cortical bone destruction are depicted. Each image was created by segmenting areas of intact cortical bone from areas of bone destruction, and then reconstructing only the areas of cortical bone destruction in three dimensions using the

manufacturer's software (D) Bars represent the median for each group [n=5 for *S. aureus* (min: 0.24; max: 0.59) and SpA- (min: 0.11; max: 0.79), n=3 for control (inoculated with PBS; min: 0.07, max: 0.14)]. Data were analyzed using Mann Whitney test. *P < 0.05. (E) The presence of bacteria in the bone was determined at 14 days post-inoculation. Boxes and whiskers depict maximum and minimum values obtained from individual mice (n=8-13) and the horizontal line represents the median for each group. Comparisons were performed by the Mann Whitney test.

Fig. 6. Histopathological changes induced by *S. aureus* protein A during experimental osteomyelitis. (A) Gelatinase activity in the supernatant of tibia homogenates from rats inoculated with *S. aureus* or the SpA- mutant was detected by zymography. Arbitrary units: ratio between the activity in the left infected tibia and the activity in the right control tibia. Each dot represents determinations made on an individual rat (n=7 for each group). Comparisons were performed by the Mann Whitney test. *p < 0.05. (B) Bone enlargement at the inoculation site was measured and the osteomyelitc index (OI) was calculated. Each dot represents determinations made on an individual rat (n=25 per group). Comparisons were performed using the Mann Whitney test. *p < 0.05. (C) Hematoxylin/Eosin and TRAP staining of tibia sections. Sections of dead bone (sequestra) with matrix bone reabsorption (middle, black asterisk), together with a large abscess (upper right, black arrow heads) and an intense inflammatory infiltrate of the bone marrow was observed in rats infected with the wild-type *S. aureus* consisting of a classic histological picture of osteomyelitis. Note the necrotic tissue surrounding the abscess infiltrating pieces of dead bone lacking cells and only composed of bone matrix

(sequestra). TRAP positive cells (black arrows) were present in sections from *S. aureus* infected bone. The infection with the SpA mutant was almost resolved as compared with the control bone and TRAP positive cells were not observed. Samples were analyzed in a blind manner, magnification: 45X. (D) The presence of bacteria in the bone was determined at 15 weeks post-inoculation. Boxes and whiskers depict maximum and minimum values obtained from individual mice (n=23-24) and the horizontal line represents the median for each group. Comparisons were performed by the Mann Whitney test. *$p < 0.05$.

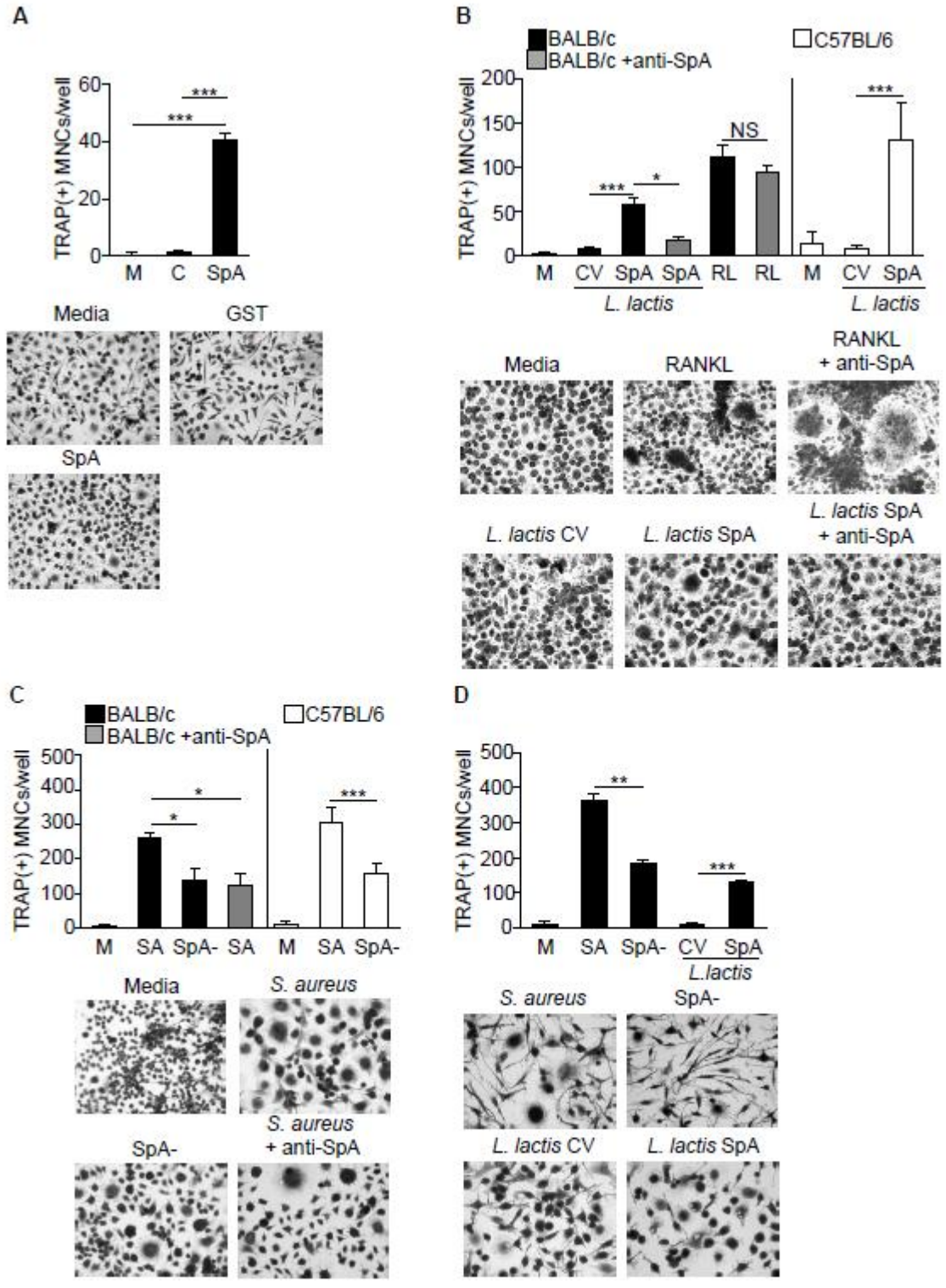
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Fig. 1

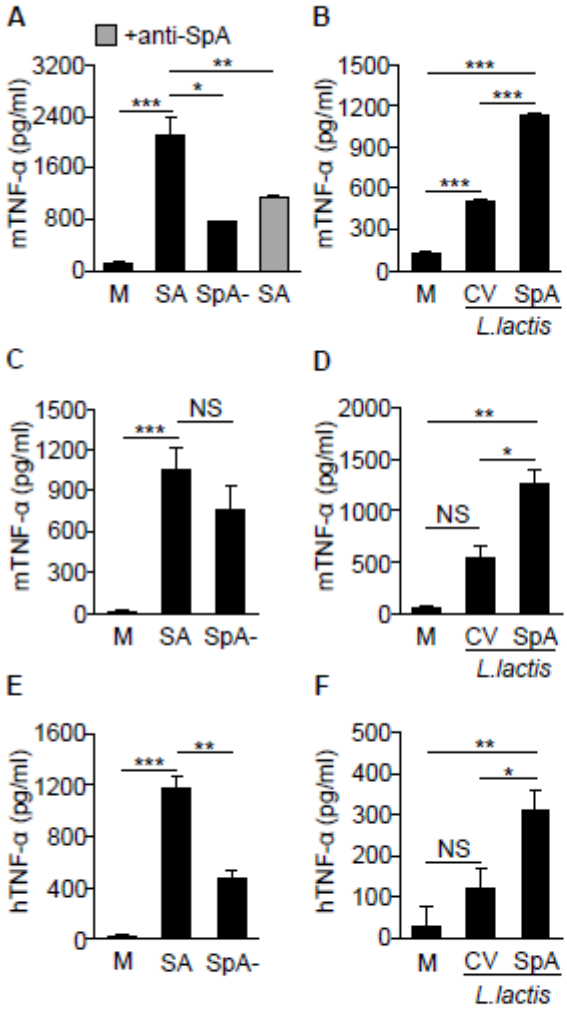

Fig. 2

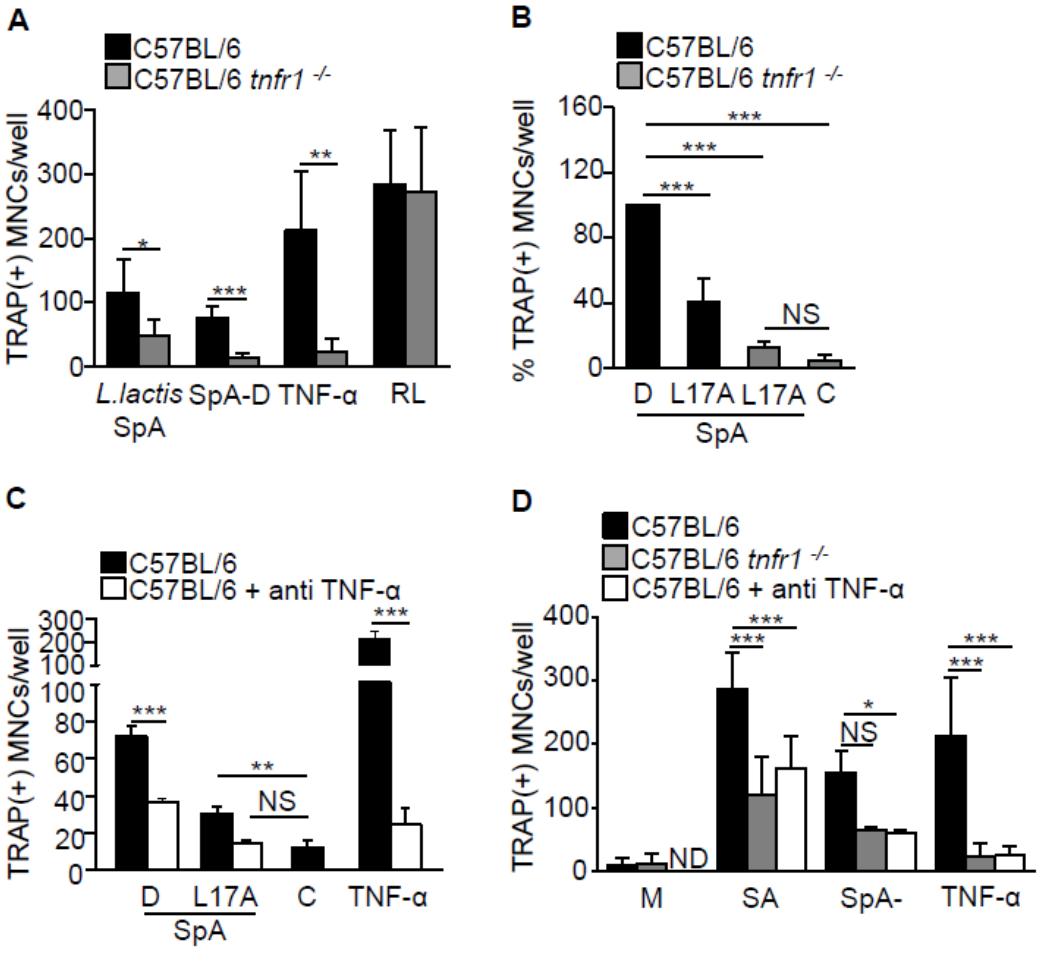

Fig. 3

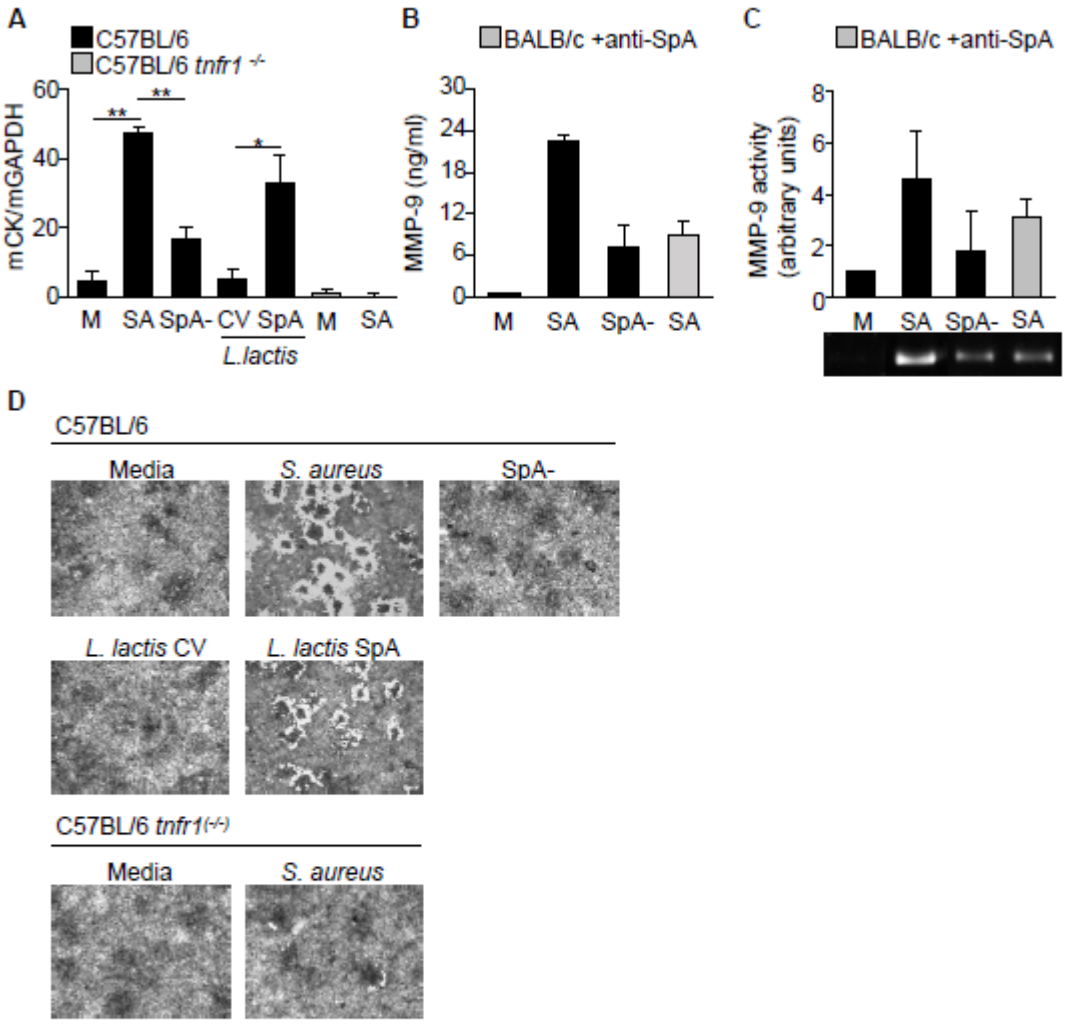
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Fig. 4

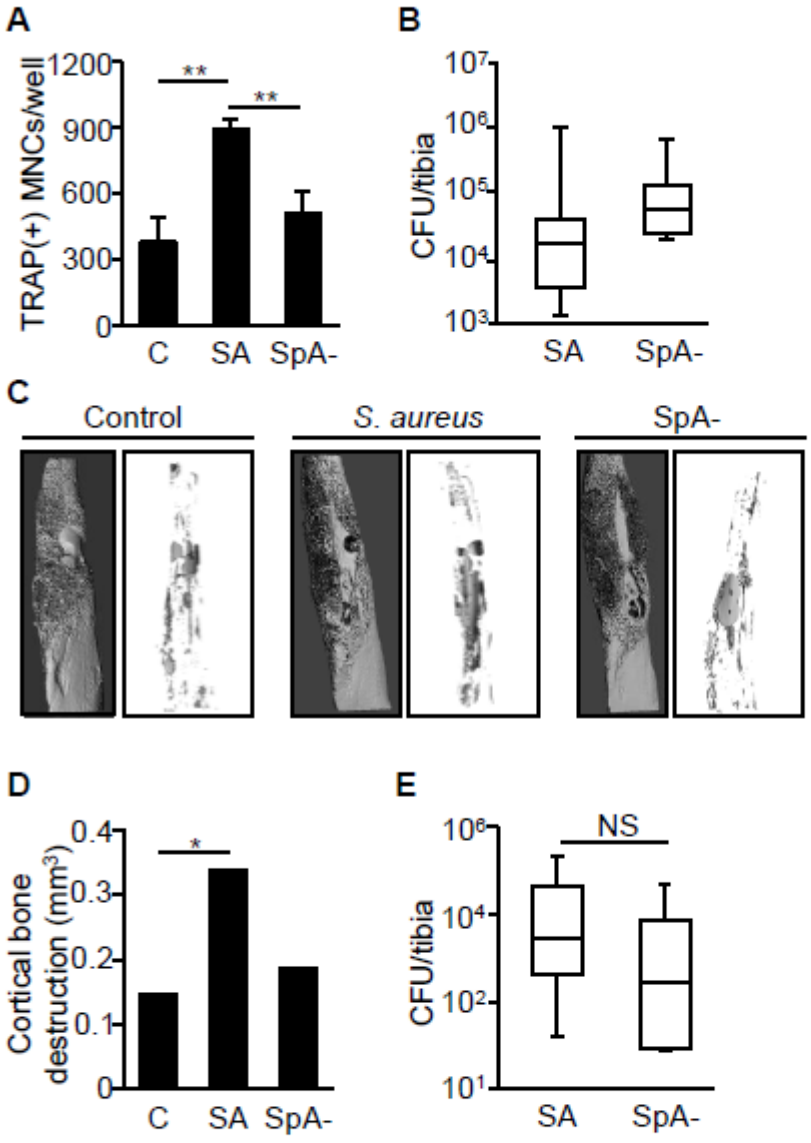
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Fig. 5

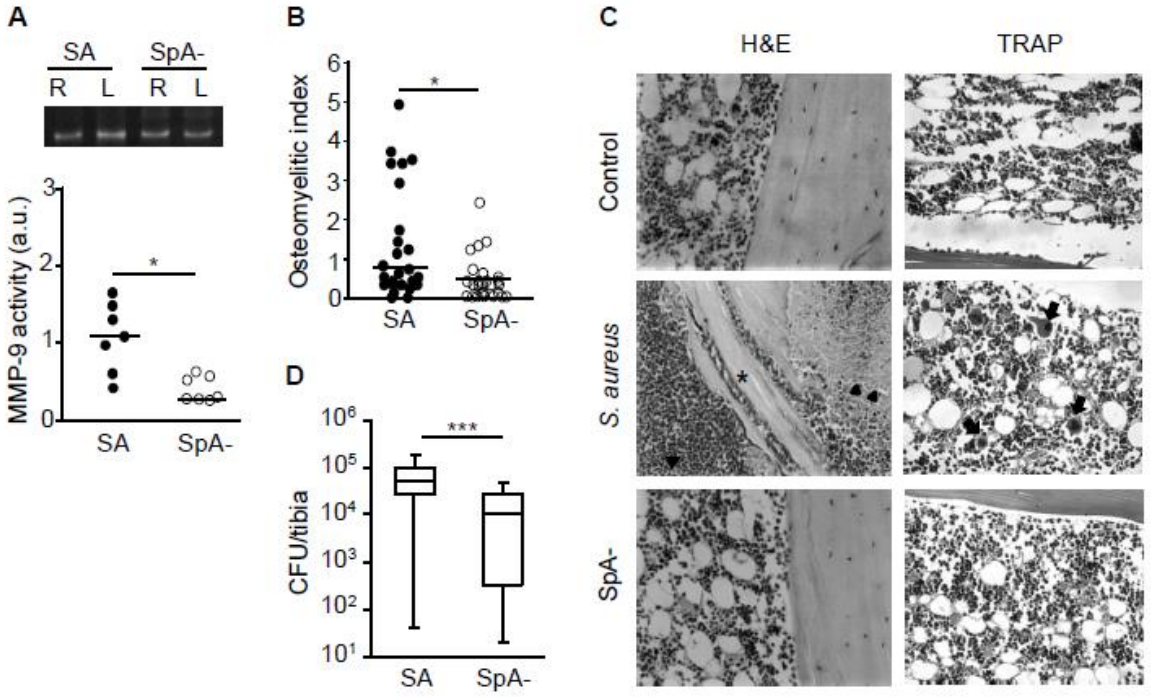

Fig. 6

Highlights

Staphylococcus aureus protein A plays a critical role in osteoclastogenesis.

Osteoclastogenesis induced by protein A is mediated by TNFR1 and EGFR signaling.

Protein A induces cortical bone destruction during experimental osteomyelitis.