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The good side of inflammation: *Staphylococcus aureus* proteins SpA and Sbi contribute to proper abscess formation and wound healing during skin and soft tissue infections.

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Abstract

Staphylococcus aureus is the most prominent cause of skin and soft tissue infections (SSTI) worldwide. Mortality associated with invasive SSTI is a major threat to public health considering

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the incidence of antibiotic resistant isolates in particular methicillin resistant *S. aureus* both in the hospital (HA-MRSA) and in the community (CA-MRSA). To overcome the increasing difficulties in the clinical management of SSTI due to MRSA, new prophylactic and therapeutic approaches are urgently needed and a preventive vaccine would be welcome. The rational design of an anti-*S. aureus* vaccine requires a deep knowledge of the role that the different bacterial virulence factors play according to the type of infection. In the present study, using a set of isogenic deficient mutants and their complemented strains we determined that the staphylococcal surface proteins SpA and Sbi play an important role in the induction of inflammatory cytokines and chemokines in the skin during SSTI. SpA and Sbi initiate signaling cascades that lead to the early recruitment of neutrophils, modulate their lifespan in the skin milieu and contribute to proper abscess formation and bacterial eradication. Moreover, the expression of SpA and Sbi appear critical for skin repair and wound healing. Thus, these results indicate that SpA and Sbi can promote immune responses in the skin that are beneficial for the host and therefore, should not be neutralized with vaccine formulations designed to prevent SSTI.

Keywords

Staphylococcus aureus; skin infections; abscess; SpA; Sbi

1. INTRODUCTION

Staphylococcus aureus is the most prominent cause of skin and soft tissue infections (SSTI) in the hospital and in the community worldwide. SSTI include superficial infections such as impetigo as well as more invasive infections such as cellulitis, folliculitis, subcutaneous abscess and infected ulcers and wounds [1,2]. Neutrophil recruitment to the site of infection is the hallmark of *S. aureus* infections and is required for bacterial clearance [3]. The coordinated action of innate immunity actors such as keratinocytes, endothelial cells, skin resident immune cells as well as recruited neutrophils is critical for proper abscess formation, the major host response to contain and ultimately eliminate the pathogen from the skin. During complicated SSTI *S. aureus* can disseminate to deeper areas of the skin and eventually reach the bloodstream evoking systemic disease and/or affecting distal organs. Mortality associated with complicated SSTI has a major impact in public health due to the increasing incidence of antibiotic resistant isolates in particular MRSA [4,5].

To overcome the increasing difficulties in the clinical management of SSTI new prophylactic and therapeutic approaches are urgently needed. In this regard, considerably attention has been given to the *S. aureus* virulence factors that interfere with abscess formation using *in vivo* animal models of skin infection [6–10]. The knowledge of those factors will allow the rational design of neutralizing strategies [11,12]. On the other hand, taking into account that abscess formation relies on the host immune response elicited after recognition of bacterial molecules by signaling receptors, immunotherapeutic approaches should also consider avoiding the neutralization of components that are critical for the induction of protective inflammatory responses. *S. aureus* protein A (SpA) and the staphylococcal immunoglobulin binding protein (Sbi) have been shown to induce inflammatory signaling through the engagement of the tumor necrosis factor receptor, TNFR1, and the epidermal growth factor

receptor, EGFR, in the lung [13], in the bone [14] and during systemic infections [15]. However, their role in the pathogenesis of SSTI has not been completely elucidated to date. Therefore, the aim of this study was to establish the importance of SpA and Sbi in the induction of inflammatory responses in the skin and their contribution to abscess formation during cutaneous infection.

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture conditions and α-hemolysis assay

S. aureus strains used are listed in Table 1. Bacteria were grown at 37°C with agitation until an OD_{600} of 0.8, washed and suspended in phosphate buffer (PBS). The medium generation time was equivalent among the wild type, the mutants and the complemented strains. To asses α-haemolysis bacteria were grown in TSB until an OD_{600} of 1. Bacteria was suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 5% fetal bovine serum and incubated for 24 hours to allow for α-haemolysis accumulation. The supernatant was then filtered and incubated with rabbit blood at 37°C during 1 hour and erythrocyte lysis was determined spectrophotometrically.

2.2. Animals and housing

Mice were obtained from the animal facility of the Department of Microbiology, School of Medicine, University of Buenos Aires (Buenos Aires, Argentina) or the animal facility of the Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine (Bronx, NY, United States). The procedures involving laboratory animals were approved by the Institutional Animal Care and Use Committees of the School of Medicine, University of Buenos Aires (CICUAL Approval number 809/17) or the Albert Einstein College of Medicine (Protocol number 20160808) and followed internationally accepted guidelines [16]. Animals were maintained in a conventional facility, with controlled temperature (22 ± 1°C), controlled humidity (55%), a 12:12 hour light/dark cycle and they were fed ad libitum. Procedures were performed in an experimental room within the mouse facility. Mice were euthanized using CO_2. The number of mice required for each experiment was determined based on preliminary experiments and the desired statistical significance. Cumulative data from independent experiments with small groups (control and experimental groups) is presented. The number of experimental repetitions and the n of each group is stated in the figure legends. The weight of the mice used was in accordance with their age. They showed good mobility and no differences in behavior were observed after manipulations. In this work molecular markers were evaluated in live animals and postmortem. The levels of pro-inflammatory cytokines in plasma from naïve mice were in the range of expected basal levels (IL-6: 0–50 pg/ml; TNF-α: 0–100 pg/ml).

2.3. Mouse models of subcutaneous and intradermal inoculation

2.3.1. Subcutaneous—BALB/c mice (8 weeks old) were anesthetized with ketamine (100 mg/kg) / xylazine (10 mg/kg) and subcutaneously inoculated in the shaved flank with 100 μl containing 1×10^8 CFU (*S. aureus* Newman) or 2×10^7 CFU (*S. aureus* USA300 LAC). The control groups were inoculated with PBS.

2.3.2. Intradermal—Eight week old C57Bl/6 mice under anesthesia with ketamine (100 mg/kg) / xylazine (16 mg/kg) were inoculated in the shaved lateral flank by intradermal injection of *S. aureus* Newman or the isogenic mutants in a small volume (30 μl) of PBS containing 5×10^8 CFU. Sham injections with PBS were done in control groups.

2.4. Skin infection

The progression of disease, at different time points after inoculation was monitored by measurement of lesion dimensions, length (L) and width (W), with a caliper. The dimensions were used to calculate the abscess area [$A = \pi (L/2) \times W/2$] [7,17,18]. Bacterial load was determined in abscess homogenates by serial dilution and culture on manitol salt agar plates. Homogenates were then centrifuged at 10.000 rpm for 10 minutes and the supernatant stored at −80°C for ulterior detection of cytokine/chemokines. For leucocyte characterization the complete abscess was cut in small pieces and treated with a protease cocktail (hyaluronidase 200 U/ml and collagenase II 250 U/ml) for 2 hours at 37°C and 5% of CO_2 atmosphere with slow agitation. Cell suspensions were filtered through a sterile stainless steel mesh and then used for surface antigen staining and viability assays and analyzed by flow cytometry. For mRNA quantitation, the complete abscess was homogenized in TRIzol regent and stored at −80°C for ulterior RNA isolation. In three independent experiments, one mouse per treatment group was used for histopathological analysis. The area of the skin lesion was recorded in mice used for bacterial/cytokine-chemokine quantitation, histopathological analysis, leukocyte characterization or mRNA quantitation to assure that data from all the groups of mice used throughout the study were represented in the abscess macroscopic evaluation.

2.5. Multi-channel fluorescence intravital microscopy

Twelve mice were imaged in 6 independent experiments. Groups of two mice were imaged in the same day. The treatments (PBS, *S. aureus*, SpA^- mutant, Sbi^- mutant) were distributed to include all the possible different pairs to be imaged in a certain day. Fifteen minutes prior the inoculation of bacteria mice received fluorescently conjugated anti-Ly6G (Alexa 647) antibodies (5 μg) by i.v. route. Immediately before imaging mice received TRITC Dextran (150.000 MW) by i.v. route to monitor proper blood flow. Images were taken from 15 minutes up to 5 hours after bacterial inoculation. During imaging mice were kept at body temperature using a heating pad. The skin was exteriorized in the right flank [19] and the tissue was super fused with warm PBS. Anesthesia (ketamine / xylazine at a quarter of the initial dose (ketamine (25 mg/kg) / xylazine (4 mg/kg)) was re-administered every 40–50 minutes as required. Under the microscope, neutrophil rolling, adhesion and transmigration in post-capillary venules (20–40 μm in diameter) were captured. Images and videos were captured using an Axio Examiner. D1 microscope (Zeiss) equipped with a Yokogawa CSU-X1 confocal scan head with four stack laser system (405nm, 488nm, 561nm, and 642nm wavelengths) and a 60X water immersion objective, and analyzed using Slidebook software (Intelligent Imaging Innovations). The following quantitative outcomes were determined: 1. Number of neutrophils that passed through, rolled or were adherent to a 100 μm fragment of a vein over a period of 30 minutes; 2. Fraction of free neutrophils (defined as the fraction of cells compared to total cells counted that were moving free along the blood vessel); 3. Fraction of rolling neutrophils (defined as the fraction of cells

compared to total cells counted that moved along the blood vessel wall at a velocity lower than free flowing cells); 4. Fraction of adherent neutrophils (defined as the fraction of cells compared to total cells counted that were arrested in the blood vessel wall for more than 30 seconds without movement of more than one cell diameter); 5. Neutrophil rolling velocity (defined as the distance traveled by the rolling cells in the blood vessel lumen per unit of time (μm/s); 6. Extravasated and tissue neutrophils [20,21].

2.6. Flow cytometry

Single-cell suspensions were incubated with fluorescently-labeled monoclonal antibodies for 30 min at 4°C. The following antibodies were used: phycoerythrin-labeled anti-F4/80 (Biolegend), Alexa 488-labeled anti-CD3 (Ligatis) and allophycocyanin-labeled anti-Ly6G (Biolegend). To evaluate cell viability, cells were stained with AnV (annexin V)-FITC (Biolegend) and Propidium Iodide according to the manufacturer's instructions. Cells were acquired within the 2 hours after staining on a FACSAria flow cytometer (BD Biosciences) and analyzed using Cyflogic software.

2.7. Keratinocyte cell culture and infection

The keratinocyte cell line HaCaT [22] was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum, penicillin ($100Uml^{-1}$) and streptomycin ($100Uml^{-1}$) (Gibco-BRL, USA). For bacterial infection 2×10^4 cells per were plated in 96-well plates. Cells were grown during 48 hours to achieve confluence and then stimulated for 4 hours with *S. aureus* Newman, or the isogenic SpA^- or Sbi^- mutants at a MOI: 100 in DMEM supplemented with 5% fetal bovine serum.

2.8. ELISA

IL-1β, TNF-α, IL-6, CXCL-1, CXCL-10 and IL-10 were quantified in skin lesion supernatants and standardized by the surface area of the respective lesion. IL-1β and IL-6 were quantified in culture supernatants. Enzyme-linked immunosorbent assay using matched antibody pairs (BD Biosciences (TNF-α, IL-6 and IL-10) or R&D Systems (IL-1β, CXCL-1 and CXCL-10)) was performed according to the manufacturer's instructions.

2.9. Real-time Polymerase Chain Reaction

RNA was isolated using TRIzol Reagent (Invitrogen). Complementary DNA (cDNA) was made from 1 μg of RNA using M-MLV Reverse Transcriptase (Promega). Primers used for gene quantification are listed in Table 2.

2.10. Statistics

The statistical tests used to analyze the data are indicated in the figure legends. GraphPad Prism software was used for the analysis.

3. RESULTS

3.1. SpA and Sbi contribute to decrease the severity of skin lesions and favor proper abscess formation during *S. aureus* SSTI.

Mice challenged by the subcutaneous route with the SpA$^-$ and Sbi$^-$ mutants developed larger skin lesions than mice challenged with the parental strain at early time points (12 hours and 3 days) after inoculation (Fig. 1A and E). Complementation of SpA and Sbi restored the phenotype observed in mice inoculated with the parental strain at day 3 post-inoculation (Fig. 1B, C and E), demonstrating the importance of both proteins in the development of SSTI. No significant differences in the bacterial burden in the lesions caused by the different strains were observed. Nonetheless, significantly lower bacterial counts - compared with those found at early time points after inoculation - were observed in mice challenged with the wild type strain at day 3 and day 7 post-inoculation (Fig. 1D). This decrease in bacterial burden was not observed in mice inoculated with the mutants (Fig. 1D). Considering that α-haemolysin (Hla) has been implicated in skin pathology [7,25,26] we have controlled the strains used in these experiments for Hla production. None of the mutants used presented higher production of Hla than the wild type strain (Fig. 1F).

We have used the strain Newman throughout the study taking advantage of the low production of α-haemolysin by this strain in order to evaluate the role of SpA and Sbi in the pathogenesis of SSTI separately from the well described role of Hla in skin dermonecrosis [7,25,26] which could mask the impact of SpA and Sbi during skin infection. Nonetheless, we repeated certain experiments using the USA300 LAC background to ascertain that the results obtained were not restricted to the strain Newman. During infection induced with the USA300 LAC strain it was observed that, in the absence of SpA the skin lesions were significantly larger than those induced by the parental strain equivalent to the results obtained using the strain Newman (Fig. 2A and C). Moreover, abscess from mice inoculated with the SpA$^-$ mutant presented significantly increased numbers of CFU in the skin at day 7 post-inoculation compared with those from mice inoculated with the wild type strain (Fig. 2B). No differences were found when comparing the skin lesions between mice inoculated with the Sbi$^-$ mutant and those from mice inoculated with the wild type strain (Fig. 2A, B and C) although a trend to increased bacterial burden in the abscess was observed (Fig. 2C). Production of Hla was increased in the Sbi$^-$ mutant compared to the wild type strain (Fig. 2D). However this small increase was not considered biologically relevant since the dermonecrosis induced by the Sbi- mutant was not different from that induced by the wild type strain (Fig. 2A and C).

To further evaluate the role of SpA and Sbi during *S. aureus* SSTI, we analyzed the histopathology of the skin lesions from mice challenged with the wild-type, SpA$^-$ or Sbi$^-$ *S. aureus* strains at day 3 post-inoculation. Skin lesions from mice inoculated with wild-type *S. aureus* strain Newman had the structure of an organized abscess with defined fibrous walls and focalized polymorphonuclear infiltrate surrounding the bacterial community (Fig. 3A and B). On the contrary, skin lesions from mice challenged with the SpA$^-$ or Sbi$^-$ mutants showed a disorganized structure with pour fibrous capsule formation, low neutrophil focalization and extended necrosis in dermis and epidermis which was more evident in the

case of the SpA⁻ mutant (Fig. 3A and B). Homogenization and disruption of collagen fibers was observed in the SpA⁻ and Sbi⁻ inoculated groups, respectively, whereas in mice challenged with wild-type *S. aureus* collagen fibers resemble those in the control group (Fig. 3C). The expression of MMP-9, a metalloprotease known to participate in the remodeling of the granulation tissue, was significantly increased in the abscess from all three groups of mice at day 3 post-inoculation compared with the control group (Fig. 3D). The expression of TGF-β, a fibrogenic growth factor that contributes to the deposition of extracellular matrix including collagen during the proliferative phase of skin wound healing [24], was upregulated at day 3 post-inoculation in response to cutaneous *S. aureus* infection (Fig. 3E). However, at this time-point TGF-β expression was significantly lower when the skin infection was produced by the SpA⁻ or the Sbi⁻ mutants compared with the wild type strain (Fig. 3E). Taken together these results indicate that the expression of either SpA or Sbi is critical for proper abscess formation and the induction of wound repair factors during the early phase of SSTI.

3.2. SpA and Sbi significantly participate in the induction of local immune responses during *S. aureus* skin infection.

We have previously established the importance of SpA and Sbi in the modulation of immune responses during *S. aureus* systemic infections [15,27,28]. During skin infections, the initial inflammatory response is critical for abscess formation, bacterial eradication and resolution of the infection [3,4]. Therefore, we next established the role of SpA and Sbi in the induction of inflammatory mediators in the skin. *S. aureus* induced the accumulation of IL-1β from early time points (12 hours) after inoculation (Fig. 4A). Interestingly, in mice challenged with the SpA⁻mutant the levels of IL-1β were significantly lower than those determined in the wild-type challenged group (Fig. 4A), highlighting the importance of SpA in the induction of IL-1β by *S. aureus* during skin infection. Mice challenge with the Sbi⁻ mutant showed a tendency to accumulate lower levels of IL-1β than the wild-type group but the levels of this cytokine were not as decreased as those in mice challenged with the SpA⁻ mutant and significant differences were only observed early 12 after the inoculation (Fig. 4A). Both proteins significantly participated in the induction of local IL-6 at twelve hours post-inoculation (Fig. 4B) whereas SpA also significantly contributed to the induction of TNF-α and CXCL-1 at day 3 post-inoculation (Fig. 4C and D). SpA also contributed to the local induction of the chemokine CXCL-10 (Fig. 4E). IL-10 was present in the abscess by day 3 after inoculation and significantly increased levels of this cytokine were quantified in abscess from mice inoculated with the SpA-mutant (Fig. 4F).

The skin is an important organ that recognizes pathogens and resident skin cells initiate the signaling cascades that are necessary to attract neutrophils and other immune cells that combat the infection. Therefore, we tested whether the expression of SpA and/or Sbi would affect the induction of inflammatory mediators in the skin at early stages upon infection. *In vitro* stimulation of keratinocytes with *S. aureus* during four hours induced increased levels of IL-1β and to a minor extent IL-6 (Fig. 5). Both mutants induced significantly lower levels of IL-1β and IL-6 than the wild-type strain (Fig. 5A and B). At the MOI used, viability of keratinocytes was not affected by the bacterial infection (data not shown).

3.3. SpA and Sbi significantly participate in the early recruitment of neutrophils to the site of infection during *S. aureus* SSTIs.

To evaluate the dynamics of neutrophil recruitment at early time points after the onset of *S. aureus* skin infection, we used intra-vital microscopy [21] to visualize the recruitment of neutrophils and their behavior in the skin venules using skin flaps from mice inoculated intradermally [19,29]. Since a dynamic portion of circulating neutrophils roll along the walls of post-capillary venules and search for the presence of host- and/or pathogen-derived chemotactic signals or chemoattractants [30], we recorded the number of neutrophils that passed through veins close to the intradermal inoculation site over a period of 30 min at two hours post-inoculation. Equivalent amounts of neutrophils were attracted to the area in response to either PBS (control) or wild-type *S. aureus* (Fig. 6A). On the contrary, a lower number of neutrophils was observed in mice challenged with the SpA$^-$ or the Sbi$^-$ mutants (Fig. 6A). *In vivo* imaging also revealed that neutrophils from mice inoculated with wild-type *S. aureus* were preferentially rolling at two hours after inoculation denoting the activation of the endothelium and that of the neutrophils themselves in response to the infection (Fig. 6B, supplementary video 1) whereas those from the control group were preferentially free (Fig. 6B, supplementary video 2). Not only a large fraction of neutrophils was rolling at that time point, but also the rolling velocity was significantly lower than that determined in the PBS group (Fig. 6D). At three hours post-inoculation the majority of *S. aureus* activated neutrophils were adherent to the endothelium (Fig. 6C, supplementary video 3) whereas in the control group all three phenotypes, free, rolling and adherent were observed (Fig. 6C, supplementary video 4). Interestingly, even though the number of neutrophils attracted to the infection site in mice inoculated with the SpA$^-$ or the Sbi$^-$ mutants was very low, a tendency of the Sbi$^-$ inoculated group to attract more neutrophils than those mice challenged with the SpA$^-$ mutant was observed particularly at three hours post-inoculation (Fig. 6A). Despite the low numbers, neutrophils recruited in response to the mutants presented an adherent phenotype from early time points (Fig. 6B, supplementary videos 5 and 6) and the rolling velocity was equivalent to that measured in neutrophils from mice inoculated with *S. aureus* (data not shown). Neutrophils extravasated to the skin tissue were evident at 5 hours after inoculation with *S. aureus* (Fig. 6E and F) whereas the amount of extravasated neutrophils was significantly lower in mice inoculated with the SpA$^-$ or Sbi$^-$ mutants as well as in the control group (Fig. 6 E and F).

Considering the low rate of neutrophil recruitment to the infection site in mice inoculated with the mutants, the re-directioning of neutrophils to the marginal pools was evaluated. No difference was observed in neutrophil numbers in the spleen or bone marrow among the groups at three hours post-intradermal inoculation. A small increase in the levels of neutrophils in the lungs was observed in mice inoculated with either of the mutants although differences did not reach statistical significance (data not shown).

At the very early time point after inoculation examined by intra-vital imaging, the expression of SpA and Sbi significantly contributed to TNF-α production in the skin (Fig. 6G). Interestingly, mice challenged with the SpA$^-$ mutant not only failed to produce increased levels of TNF-α compared with the control group, but also presented increased

expression of TGF-β in the skin (Fig. 6H) suggesting that in the absence of protein A an early anti-inflammatory microenvironment was favored.

Considering that in the above described experiments, in order to do the live imaging of the skin infection mice were inoculated by the intradermal route, we corroborated that mice inoculated with the SpA$^-$ mutant presented larger skin lesions at days 1 and 3 post-inoculation than the wild type inoculated group equivalent to the findings in the subcutaneous infection model (Fig. 6I). Mice inoculated with the Sbi$^-$ mutant showed larger skin lesions than the wild type group at day 1 post-inoculation (Fig. 6I) although differences were transient in this model (Fig. 6I day3) a feature also noticed in the subcutaneous model (Fig. 1A).

3.4. *S. aureus* lacking SpA or Sbi expression induce increased death of immune cells in the abscess microenvironment.

Proper abscess formation requires prolonged neutrophil survival within the abscess in the skin milieu [31,32]. Therefore, we characterized the immune populations present in the abscesses at 12 hours and 3 days after the inoculation and determined their viability. In all the groups, the predominant cell type was neutrophils with macrophages accounting for 10–20% of the population (Fig. 7A and B). Interestingly, whereas the neutrophils present in the abscess 12 hours post-inoculation were highly viable (Fig. 7C), the viability of macrophages in the lesions induced by wild-type *S. aureus* was in average 50% (Fig. 7D) denoting the early impact of infection on resident macrophages and/or newly recruited monocytes/macrophages. Skin wounds from mice inoculated with the SpA$^-$ mutant showed even lower viability of macrophages (average 24%) (Fig. 7D). Macrophages from mice inoculated with the Sbi$^-$ mutant presented increased mortality although did not reach statistically significance compared with those from wild type inoculated mice (Fig. 7D). At day 3 after inoculation, neutrophils continued being the major immune cells in the abscess (Fig. 7A). However, at this time point significantly reduced viability was observed in neutrophils from abscesses of mice inoculated with the SpA$^-$ or the Sbi$^-$ mutants compared with those from mice inoculated with wild-type *S. aureus* (Fig. 7C).

3.5. The inflammatory microenvironment induced by SpA and Sbi leads to infection resolution and healing.

We characterized the evolution of the skin lesions by day 7 after the inoculation. Mice challenged with the SpA$^-$ or the Sbi$^-$ mutants presented larger skin lesions than the wild-type inoculated group (Fig. 1A and E).

Histopathological analysis revealed that when the infection was induced with wild-type *S. aureus*, the skin exhibited evidence of wound healing, including epidermal regeneration, fibrosis as well as hair follicle and glands development by day 7 after inoculation (Fig. 8A and B). Conversely, mice inoculated with the SpA$^-$ or Sbi$^-$ mutants presented extended epidermal necrosis and ulceration, increased bacterial colonies and skeletal muscle compromise as well as poor annex development (Fig. 8A and B). Fibrosis was observed in mice inoculated with the Sbi$^-$ similarly to wild type inoculated mice which denoted a better

prognosis for tissue repair in this group compared with the SpA$^-$ inoculated group (Fig. 8A and B).

At this time point IL-1β was still detected in the abscesses and the levels of this cytokine were higher in mice inoculated with the wild-type strain compared with those determined in mice inoculated with the SpA$^-$ mutant (Fig. 8C).

The expression of MMP2, a metalloprotease associated to tissue damage, was significantly increased in abscess from mice inoculated with the SpA$^-$ or the Sbi$^-$ mutant compared with the levels found in control mice inoculated with PBS (Fig. 8D). At day 7 post-inoculation, the expression of MMP9 in abscess from mice inoculated with the Sbi$^-$ mutant remained elevated compared with the control and with mice inoculated with the wild type strain.

4. DISCUSSION

S. aureus SSTI represent a major threat to public health given the massive number of infections and as well as the widespread emergence of antibiotic resistant isolates such as MRSA both in the hospital and the community [33–36]. CA-MRSA are characterized by increased virulence and the capacity to initiate invasive staphylococcal infections even in healthy individuals [37–39] and whereas many SSTI are relatively minor and self-limiting, complicated SSTI can be life threatening [40–42].

To address the increasing problems related to the actual treatment of staphylococcal infections, several newer approaches using either preventive or novel therapeutic strategies are being evaluated [12,43–45]. The multiple staphylococcal antigens and the complexity of pathogenic pathways involved in staphylococcal diseases make it difficult to imagine a single and universal anti-*S. aureus* vaccine. Instead, vaccines targeting each type of staphylococcal infection have been proposed as the future approach [11]. In that direction, the rational design of vaccine formulations, either prophylactic or therapeutic, should consider the differential roles that staphylococcal virulence factors may play according to the type of infection. The induction, through vaccination, of antibodies that would neutralize staphylococcal virulence factors involved in skin damage and/or associated to deficient abscess formation are being considered in the case of SSTI [10,20,26,46]. In the present study we determined that *S. aureus* protein SpA, and to a lesser extent Sbi, are critical for the induction of inflammatory responses in the skin that lead to proper abscess formation and wound healing and therefore, attention should be given to not neutralize them with vaccine formulations designed to prevent SSTI.

It has been well established that an adequate inflammatory response is critical for abscess formation [3,47–49]. In this regard, our previous studies have established the capacity of SpA and Sbi to induce local and systemic inflammatory responses. SpA and Sbi signal through the host receptors TNFR1 and EGFR mimicking the downstream signaling induced by their natural ligands which leads to the activation of MAPKs and NFκB and the production of inflammatory cytokines and chemokines [13,15,27,50].

Upon the entry of *S. aureus* into the skin, many actors from the immune system such as keratinocytes, resident immune cells and endothelial cells sense the presence of the pathogen

and orchestrate the inflammatory response that conducts to the activation of the vascular endothelium and neutrophil recruitment and extravasation from the bloodstream, the first step in abscess formation. Among the soluble mediators involved in this process, IL-1β has been described as a central cytokine because of the functional and temporal association between neutrophil recruitment and IL-1/IL-1R activation [51] with IL-1β being the inducer of the chemokines that direct neutrophil migration [52]. Mice deficient in IL-1β fail to form abscess and eradicate the bacteria from the skin [51]. IL-1β production in the skin resulted partially dependent on signaling initiated by TLR2 and NOD2, PPRs that recognize tri-acyl and diacyllipopeptides and muramyl-dipeptide, a breakdown product of peptidoglycan, respectively [53,54]. In addition to those PRRs, in a recent study the importance of EGFR signaling in IL-1β production by keratinocytes was described [55] which may explain the deficient production of this cytokine in response to the SpA$^-$ and the Sbi$^-$ mutants both during the *in vitro* infection of keratinocytes as well as during *in vivo* skin infection.

TNF-α plays an important role in endothelial activation and neutrophil extravasation to tissues by inducing the expression of adhesion molecules in both endothelial cells and neutrophils and inducing chemokine production [56,57]. During SSTI, the importance of mast cell-mediated TNF-α production in the recruitment of neutrophils and bacterial clearance from the skin has been recently demonstrated [58]. Using intra-vital microscopy, we showed that *S. aureus* infection induces rapid recruitment and activation of neutrophils. In the absence of SpA or Sbi, however, neutrophils were not attracted to the infection site. Therefore, the contribution of SpA and Sbi to the early induction of TNF- α in the abscess microenvironment and the reported ability of these proteins to trigger TNFR1 signaling by themselves [13,15], may have a direct impact in the early recruitment of neutrophils to the skin. The levels of CXCL-1, a chemokine important for the induction of integrins that determine the conversion from rolling to firm adherence [59,60] were also decreased in the absence of SpA and likely negatively influenced neutrophil recruitment as well. Conversely, the expression of TGF-β was increased in the absence of SpA or Sbi which might contribute to the setting of an anti-inflammatory microenvironment within the abscess. In addition to driving neutrophil adhesion, TNF-α activates the coagulation cascade promoting fibrin clots that contribute to immobilize the bacteria [61]. The low levels of this cytokine in the skin of mice inoculated with the mutants may have impeded bacterial containment and favored dissemination to deeper tissues. In fact, the histological studies revealed increased number of bacterial colonies and a significant compromise of the skeletal muscle in mice challenged with the SpA$^-$ or the Sbi$^-$ mutants. Signaling induced by *S. aureus* lacking SpA or Sbi expression differed of sterile inflammation induced by PBS in which neutrophils were attracted to the area but the response was transient and the cells did not seem to extravasate. Whether the presence of bacteria in a non-pro-inflammatory milieu induced differential signaling that re-directed neutrophils to other organs, remains to be elucidated. In agreement with this notion, increased levels of neutrophils were found in the lungs of mice inoculated with the SpA$^-$ mutant but not with the complemented SpA$^-$(SpA) strain by day 3 after cutaneous infection (data not shown).

Resident macrophages also participate in the recognition of pathogens and the initiation of signaling cascades that recruit neutrophils. The perivascular subset, in particular, play a critical role in directing neutrophil extravasation at precise sites within the endothelium [20].

Staphylococcal α-haemolysin is an important evasion factor that interferes with the inflammatory response in the skin [9] and has been shown to lyse perivascular macrophages impairing the recruitment of neutrophils [20]. Our study was conducted with the strain Newman, a low producer of α-haemolysin, which allowed us to investigate the staphylococcal factors that positively modulate inflammation separately from those that impede abscess formation and favor dermonecrosis. Nonetheless, we reported here that *S. aureus* infection induced early death of macrophages within the abscess and mortality was increased in the absence of SpA mediated inflammation. Increased mortality of macrophages could be related to the increased levels of TGF-β induced by the SpA$^-$ mutant as a pro-apoptotic role for this cytokine has been previously reported [62]. The early impact of SpA on macrophage viability may have consequences in the ulterior neutrophil recruitment to the site of infection.

Abscess formation requires not only a robust neutrophil recruitment from circulation to the skin but also prolonged neutrophil survival within the tissue [31]. Inflammatory signaling is critical to sustain neutrophil viability [63]. Among them an anti-apoptotic role for IL-6, whose expression within the abscess was dependent on SpA and Sbi, has been described [64]. It has been demonstrated that during SSTI, the presence of *S. aureus* in the infected skin increases the lifespan of neutrophils at least three times [31] although the bacterial components responsible for that effect have not been completely described. A role for lipoteichoic acids in the anti-apoptotic signals has been proposed [65]. The work presented here suggests that the pro-inflammatory microenvironment induced by SpA and Sbi could help to prolong the lifespan of the arriving neutrophils whereas in the absence of these proteins the neutrophils recruited might die earlier.

In the subcutaneous skin infection model used, *S. aureus* starts to be significantly cleared from the skin by day seven [18]. Both the SpA$^-$ and the Sbi$^-$ mutants failed to be cleared at that time point. Moreover, in the experiments using the USA300 LAC background the bacterial burden in the skin at day 7 post-inoculation was significantly increased in mice inoculated with the SpA$^-$ mutant. In this regard is important to note the described role of SpA in neutrophil NETs production [66]. Therefore, SpA expression could contribute not only to neutrophil recruitment but also to bacterial clearance through NET trapping. Whether Sbi initiates NET production in neutrophils remains to be elucidated.

The successful resolution of SSTI implies not only the eradication of the bacteria but also the reconstitution of the epithelial barrier. Skin-wound healing starts immediately after injury and consists of three phases: inflammation, proliferation and maturation. In cutaneous wound healing keratinocytes play a central role, not only as a key regulatory cell type in skin repair, but also as the source of numerous growth factors, among which the EGF family members are prominent [67,68]. In this context, the regulation of EGFR ligands shedding by metalloproteases including ADAM17 is an important event in wound healing because keratinocyte proliferation and migration are in part mediated in an autocrine manner by EGFR-ligand interactions. We have previously demonstrated that both SpA and Sbi signal through the EGFR [14,15,50]. Therefore, the expression of these proteins during SSTI may contribute to early EGFR activation (even before EGFR ligands are induced) and favor tissue repair a hypothesis that is supported by the differences observed in the evolution of the skin

lesions in mice challenged with the SpA^{-}or Sbi^{-} mutants compared with those challenged with wild-type *S. aureus*. Remodeling of the granulation tissue is also an important component of the skin repair process and starts at the wound space approximately 3–4 days after injury. Macrophages, fibroblasts and endothelial cells move into the wound space at the same time. TGF-β derived from macrophages causes fibroblast infiltration which facilitates wound closing. Moreover, TGF-β has a prominent role in the induction of new collagen synthesis [24,69]. The differences observed in TGF-β induction at day 3 post-inoculation when the infection develops in the presence or absence of SpA or Sbi is also likely to contribute to the poor repair of the skin observed with the SpA^{-} and the Sbi^{-} mutants.

The proteins SpA and Sbi share certain functional properties [15,70] but also have some unique characteristics [71]. In this regard, the present study not only demonstrates that both SpA and Sbi significantly contribute to proper abscess formation and skin repair but also suggests that both proteins have some differential behavior during SSTI. Throughout the experiments it was possible to note a slightly milder phenotype when the infection was evoked by the Sbi^{-} mutant. These differences were even more noticeable in the USA300 LAC background, probably due to the differential expression of Sbi in *S. aureus* USA300 compared with *S. aureus* Newman [72]. Among the properties that may account for the differential role of SpA and Sbi in SSTI pathogenesis we observed that SpA had a more remarkable impact on macrophage survival early during the infection. The lesser mortality of macrophages in abscess from mice inoculated with the Sbi^{-} mutant compared with that in mice challenged with the SpA^{-} mutant could explain that in the Sbi^{-} group the levels of IL-1β are higher than those in the SpA^{-} group. Moreover, minor differences in neutrophil recruitment were also noticed by intra-vital imaging and the early induction of TGF-β was not as pronounced in the absence of Sbi suggesting that SpA could have a more potent pro-inflammatory activity in the skin than Sbi. In addition, a better prognosis for skin repair with higher fibrosis, less collagen alterations and more confined necrosis was observed in the Sbi^{-} group compared with the SpA^{-} group. It is also important to mention that SpA activates ADAM17 [27,28,50] and therefore it could potentiate the production of EGFR ligands in the skin. The direct impact of SpA on EGFR signaling (which shares with Sbi) but also its role on EGFR trans-signaling due to the induction of EGFR ligands is likely evidenced by the extended necrosis of the dermis and epidermis and poor fibrosis in mice inoculated with the SpA^{-} mutant compared with those inoculated with the Sbi^{-} mutant.

The role of SpA and Sbi in the pathogenesis of SSTI had not been established to date. Although it had been proposed that the predominant role, at least for SpA, would be immune evasion [11] in this work we have demonstrated that SpA and to a lesser extent Sbi elicit inflammatory responses in the skin that are necessary for abscess formation and skin recovery. Our results are in agreement with previous studies using *agr* mutants in which an attenuated phenotype was observed in the absence of a functional *agr* locus [8,26]. Although the attenuated phenotype clearly responded in part to the decreased levels of α-haemolysis expressed by the mutants, it is also noticeable that *agr* mutants present increased levels of SpA which in conjunction with our results suggests that the primary role of SpA during SSTI is not immune evasion. Our findings seem to indicate that Hla and SpA/Sbi would have opposite roles in the modulation of immune responses during SSTI. Whereas Hla lyses resident immune cells implicated in the production of inflammatory mediators that attract

neutrophils [9,20], SpA and Sbi induce the production of those inflammatory mediators and hence contribute to proper abscess formation.

Current evidence from pre-clinical studies indicates that targeting immune evasion factors of *S. aureus* for vaccine development holds promise [12]. In this regard, an anti-SpA antibody that neutralizes the immunoglobulin Fcγ-binding and B cell receptor crosslinking attributes of SpA has been shown to be protective in a neonatal mouse model of sepsis [73]. More recently, it was suggested, based on studies made in rodents, that SpA neutralizing antibodies could be useful for *S. aureus* decolonization in humans[74].

In conclusion, we agree that the neutralization of SpA would be a successful approach when SpA has a predominant immune evasion role such as in the colonization of healthy individuals [74] or sepsis [27,28] as well as in those cases were SpA-induced inflammation is deleterious for the host such as pneumonia [13] or osteomyelitis [14]. However, based on this study, we propose that SpA and Sbi should not be neutralized in pathologies in which a robust host inflammatory response is necessary and desirable to wall off and control the infection as well as to repair the affected tissue such as the case of SSTI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *S. aureus* proteins SpA and Sbi contribute to proper abscess formation.
- SpA and Sbi promote immune responses in the skin that are beneficial for the host.
- Vaccine formulations designed to prevent SSTI should not neutralize SpA and/or Sbi.

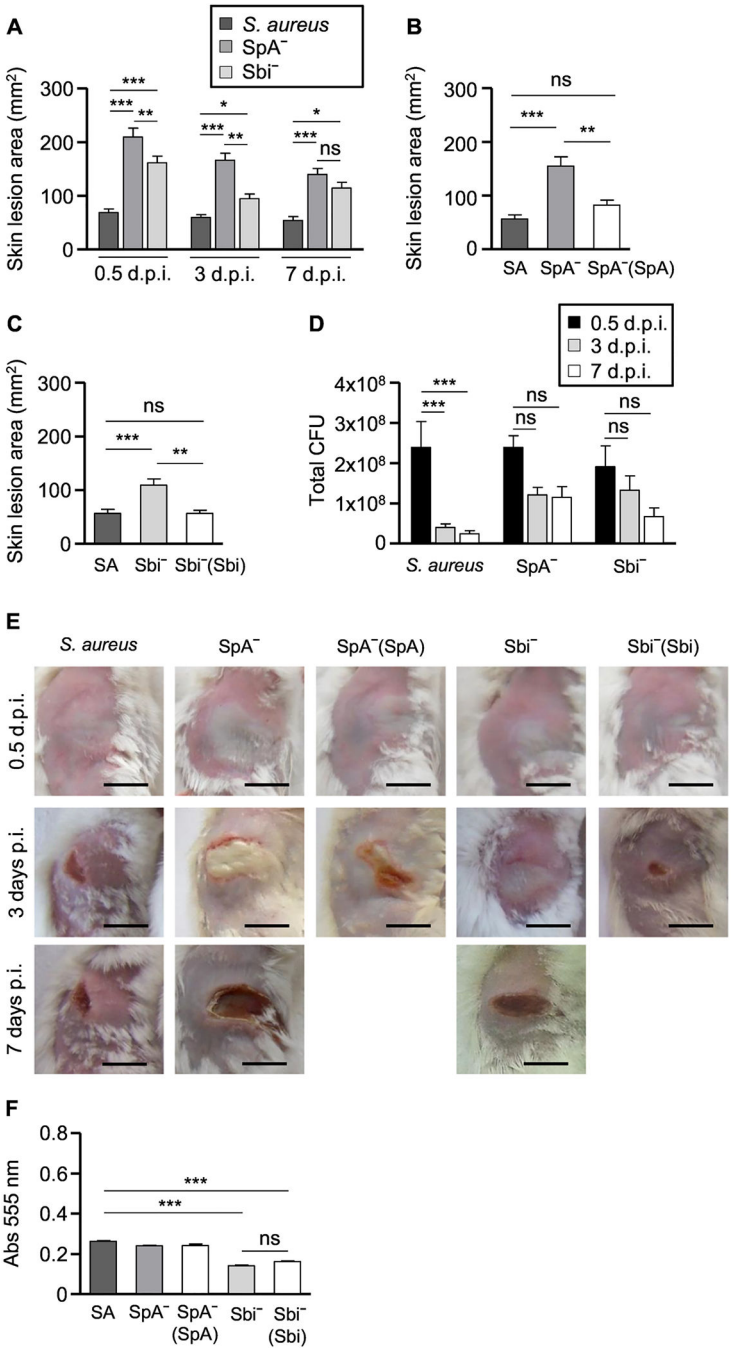

Figure 1. Role of SpA and Sbi in skin abscess formation by *S. aureus* Newman.

Groups of mice were inoculated with *S. aureus* Newman wild-type (SA), the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or the complemented isogenic strains SpA⁻(SpA) and Sbi⁻(Sbi) by the subcutaneous route. (A-C) The area of the skin lesion was determined at 12 hours (A), 3 days (A, B, C) and 7 days (A) after the inoculation. (D) Total bacterial counts in the skin lesion were determined at 12 hours, 3 days and 7 days after the inoculation. (E) Photographs of the skin lesions at the different time points after inoculation with *S. aureus* Newman, the isogenic mutants or complemented

strains. Black lines correspond to 1 cm. (F) Haemolysis produced by *S. aureus* Newman (SA), the isogenic SpA^{-} and Sbi^{-} mutants and the complemented strains. (A-D) Bars represent the mean and standard error for each group. Cumulative data from 7 (A-C) and 3 (D) independent experiments are shown. (A-C) n= 18 mice/group at day 0.5, 25 mice/group at day 3 and 18 mice/group at day 7. (D) n= 9 mice/ group at each time point. (d.p.i.): days post-inoculation. (A-D) Two way ANOVA and Bonferroni's multiple comparison test. (F) Bars represent the mean and standard error of triplicates. Data were analyzed with one way ANOVA and Bonferroni's multiple comparison test. (A-D, F) *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

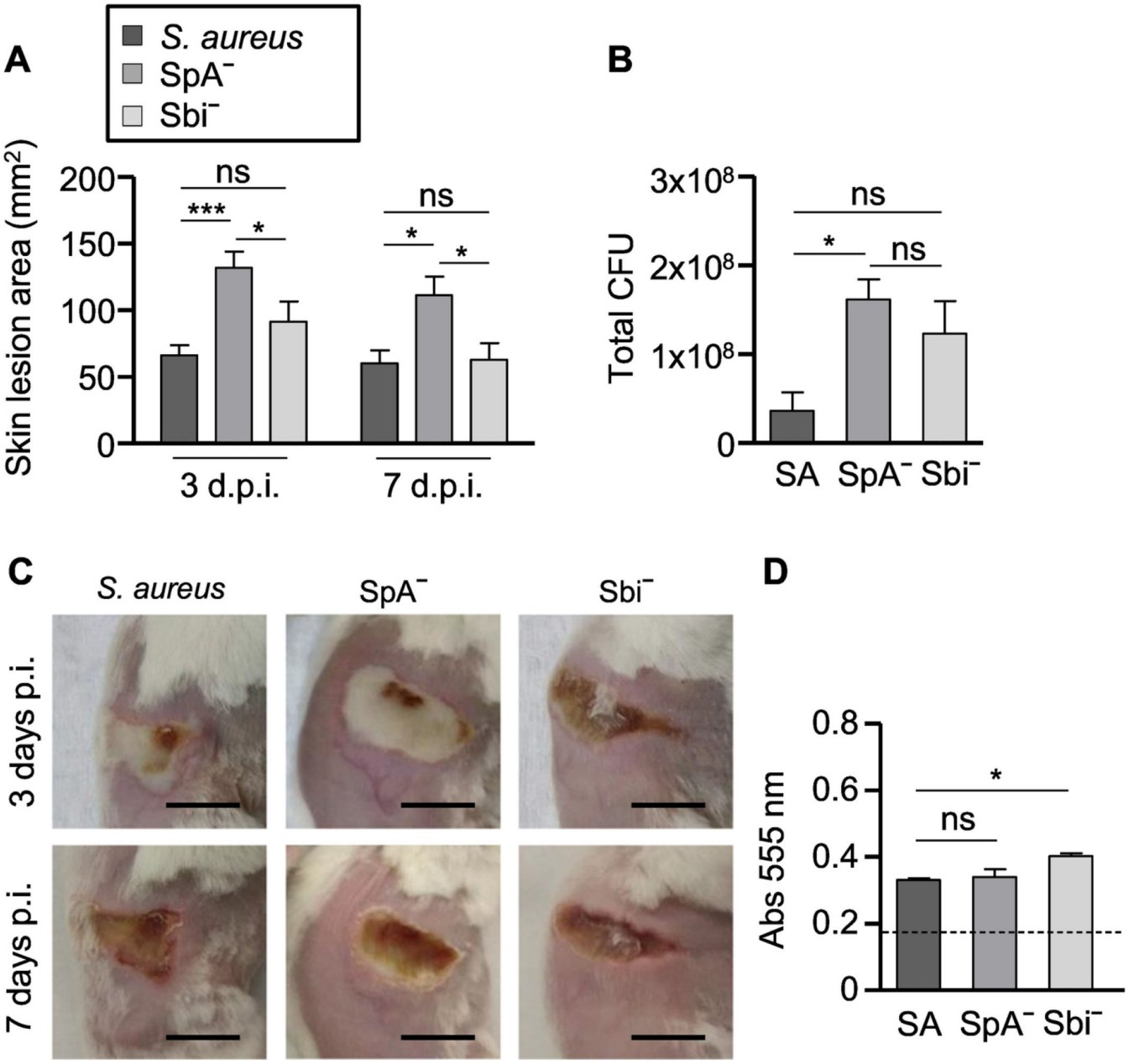

Figure 2. Role of SpA and Sbi in skin abscess formation by *S. aureus* USA300 LAC.

Groups of mice were inoculated with *S. aureus* USA300 LAC wild type (SA), the isogenic SpA deficient mutant (SpA⁻) or the isogenic Sbi deficient mutant (Sbi⁻) by the subcutaneous route. (A) The area of the skin lesion was determined at days 3 and 7 after inoculation. (d.p.i.): days post-inoculation. (B) Total bacterial counts in the skin lesion were determined at day 7 after the inoculation. (C) Photographs of the skin lesions at the different time points post-inoculation with *S. aureus* USA300 LAC or the isogenic mutants. Black lines correspond to 1 cm. (D) Haemolysis produced by *S. aureus* USA300 LAC, the isogenic SpA⁻ and Sbi⁻ mutants and the Δ Hla mutant (dotted line). (A, B) Bars represent the mean and standard error for each group. Cumulative data from 3 independent experiments are

shown. N=9 mice per group per time point. (A) Two way ANOVA and Bonferroni's multiple comparison test. (B) One way ANOVA and Bonferroni's multiple comparison test. (D) Bars represent the mean and standard error of triplicates. Data were analyzed with one way ANOVA and Bonferroni's multiple comparison test. (A, B, D) *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

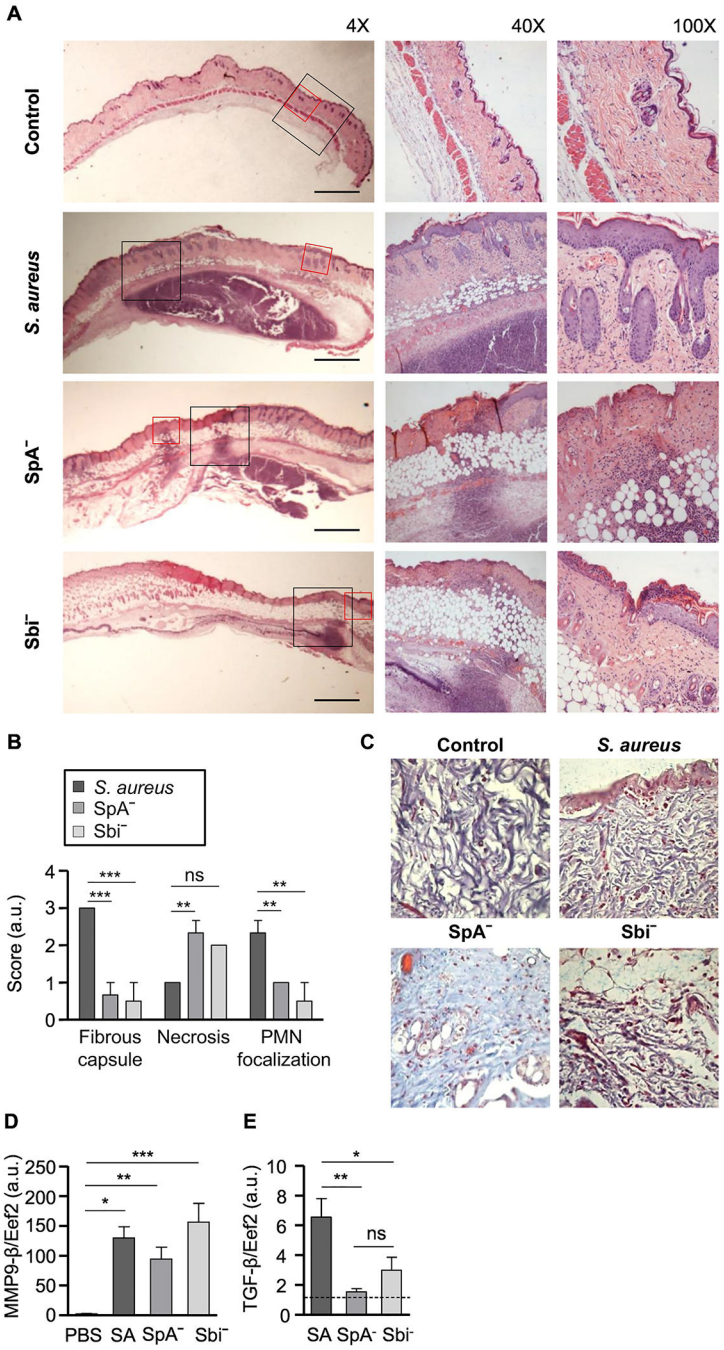

Figure 3. Histophatological alterations of the skin during SSTI.

Groups of mice were inoculated with *S. aureus* Newman wild-type, the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or PBS (control) by the subcutaneous route. (A, B, C) Histopathological analysis of skin lesions at day 3 post-inoculation. (A) Hematoxylin-Eosin staining. Black and red boxes in the 4X magnifications indicate the areas shown in the 40X and 100 X magnifications respectively. The black lines in the 4X magnification views correspond to 1 mm. (B) Bars represent the mean and standard error of the score obtained in each group (n=3 mice/group, from 3 independent

experiments). (C) Masson trichrome staining. 100X magnification. (D, E) MMP-9 (D) and TGF-β (E) mRNA levels at day 3 post-inoculation were quantified by real-time RT-PCR and normalized to Eef2 expression. (a.u.): arbitrary units. Bars represent the mean and standard error of cumulative data from 2 independent experiments (TGF-β: n=8 mice/group, MMP9: n=6 mice per group). (E) Dotted line indicates the TGF-β expression levels in the control group. (B) Two way ANOVA and Bonferronís multiple comparison test. (D-E) One way ANOVA and Bonferroni's multiple comparison test. (B, D-E) *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

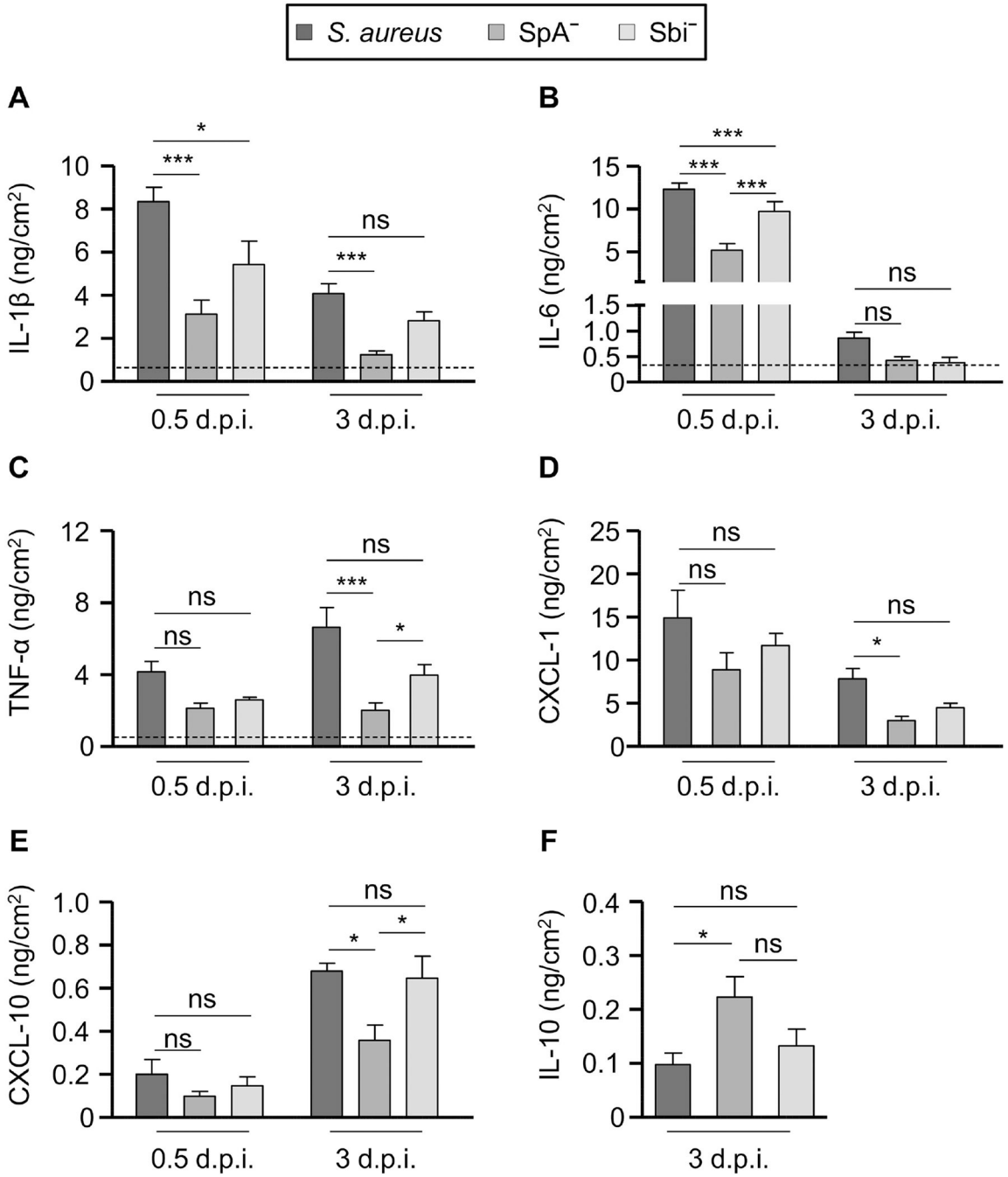

Figure 4. Cytokines and chemokines induced by *S. aureus* during SSTI.

Groups of mice were inoculated with *S. aureus* Newman wild-type, the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or PBS (control) by the subcutaneous route. (A-F) IL-1β (A), IL-6 (B), TNF-α (C), CXCL-1 (D), CXCL10 (E) and IL-10 (F) were quantified in abscess homogenates by ELISA at 12 hours and 3 days after the inoculation. Bars represent the mean and standard error of cumulative data from 3 independent experiments (n=9 mice/group at each time point). Dotted line in panels A-C depicts the values for basal production of cytokines/chemokines. The basal levels of

CXCL1, CXCL10 and IL-10 were under the limit of detection. (d.p.i.): days post-inoculation. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, Two way ANOVA and Bonferroni's multiple comparison Test (A-E), One-way ANOVA and Bonferroni's multiple comparison Test (F).

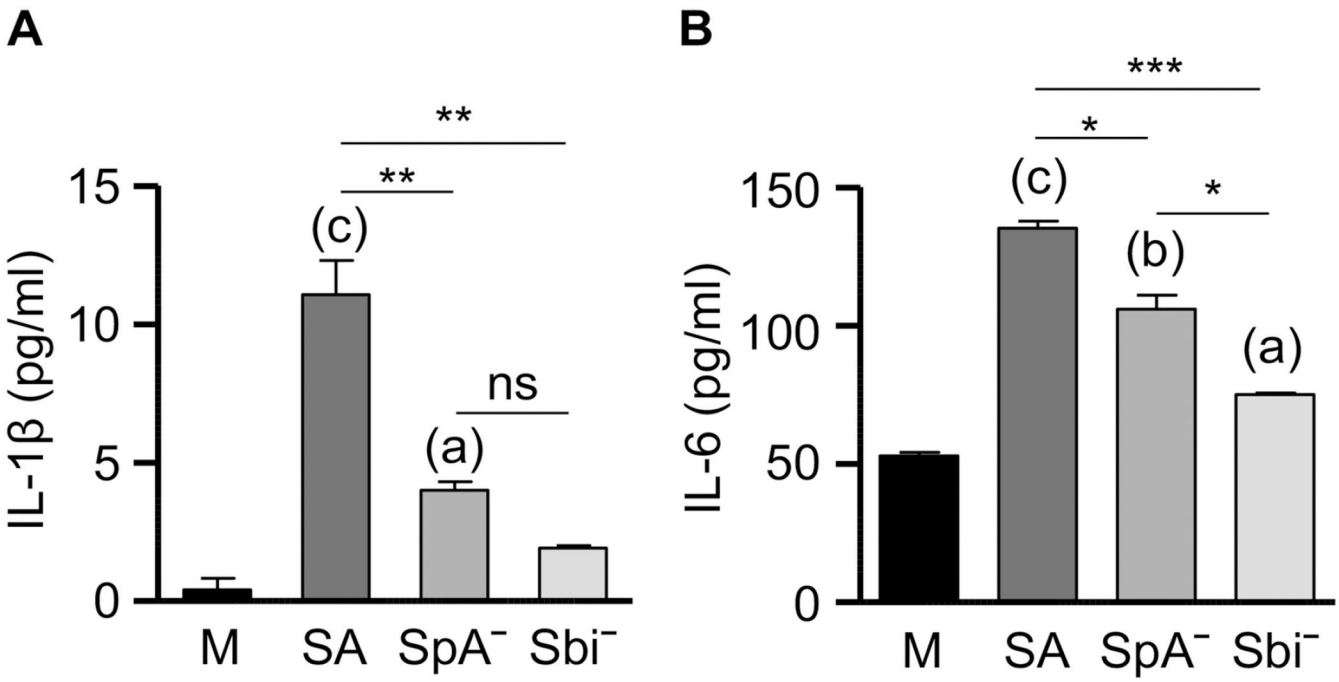

Figure 5. Induction of inflammatory cytokines in keratinocytes.

Human keratinocytes (cell line HaCaT) were stimulated for 4 hours with wild-type *S. aureus* (SA), the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or medium alone (M) and the production of IL-1β (A) and IL-6 (B) in the supernatant was determined by ELISA. (A-B) Bars represent the mean and standard error of cumulative data from 3 independent experiments (n=3 wells for each experiment). (a): $p< 0.05$; (b): $p< 0.01$; (c): $p<0.001$, compared with M, one way ANOVA and Bonferroni's multiple comparison test. *: $p< 0.05$, **: $p< 0.01$, ***: $p<0.001$, compared with SA; one way ANOVA and Bonferroni's multiple comparison test.

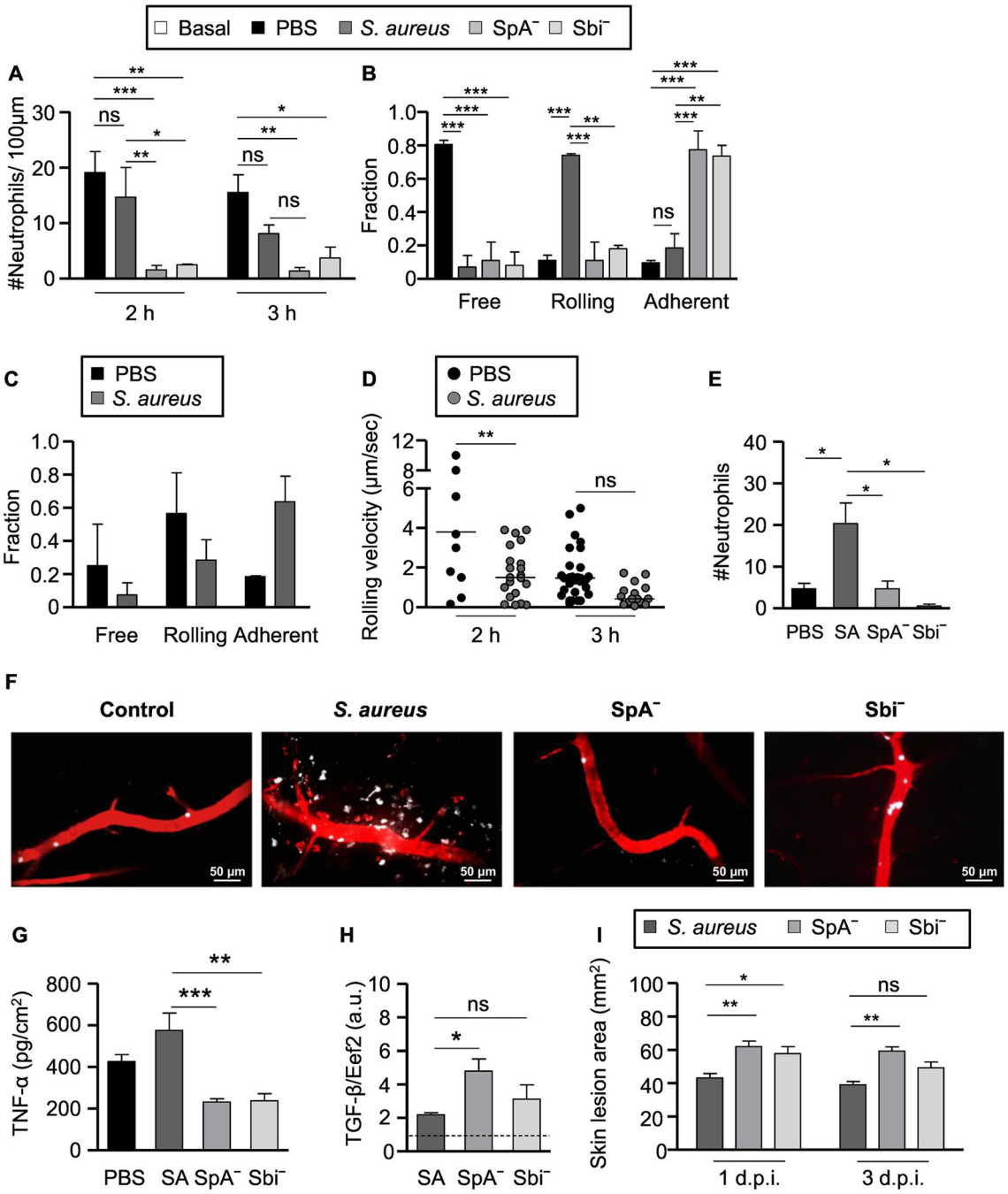

Figure 6. Role of SpA and Sbi in the early neutrophil infiltration during SSTI.

Groups of mice were inoculated with *S. aureus* Newman wild-type (SA), the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or PBS (control) by the intradermal route. (A) The number of neutrophils that passed through a vein section of 100 µm over a period of 30 min was recorded at the time points after inoculation indicated. (B, C) The fraction of neutrophils (from those counted in panel A) that were free, rolling or adherent was calculated for each group. (A, B, C) Bars represent the mean and standard errors from 6 independent experiments in which 300–400 µm were imaged (n= 3 mice/

group). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$; two way ANOVA and Bonferroni's multiple comparison test. (D) Circles represent the rolling velocity of individual rolling neutrophils and the horizontal lines the mean for each group. Cumulative data from 6 independent experiments in which 300–400 μm of veins were imaged (n=3 mice/group) are shown. *: $p<0.05$, **:$p<0.01$, two way ANOVA and Bonferroni's multiple comparison test. (E, F) Extravasating and tissue neutrophils in mice inoculated with wild-type *S. aureus*, the SpA-mutant, the Sbi- mutant or PBS at 5 hours post-inoculation. (E) Bars represent the mean and standard error (n=3 mice/group), *: $p<0.05$, one way ANOVA and Bonferroni's multiple comparison test. (G) TNF-α production in the supernatant of skin homogenates was quantified by ELISA at 3 hours post inoculation. Bars represent the mean and standard error of cumulative data from 2 independent experiments (n=5 mice/group). **: $p<0.01$, ***: $p<0.001$, one way ANOVA and Bonferroni's multiple comparison Test. (H) TGF-β mRNA levels at 3 hours post-inoculation were quantified by real-time RT-PCR and normalized to Eef2 expression. (a.u.): arbitrary units. Dotted line indicates the expression levels in the control group. Bars represent the mean and standard error of cumulative data from 2 independent experiments (n=5 mice/group). *: $p<0.05$, one way ANOVA and Bonferroni's multiple comparison Test. (I) The area of the skin lesion was quantified at days 1 and 3 post-inoculation. (d.p.i.): days post-inoculation. Data represent the mean and standard error from 3 independent experiments (n=6 mice/group at each time point). *: $p<0.05$, **: $p<0.01$, two way ANOVA and Bonferroni's multiple comparison test.

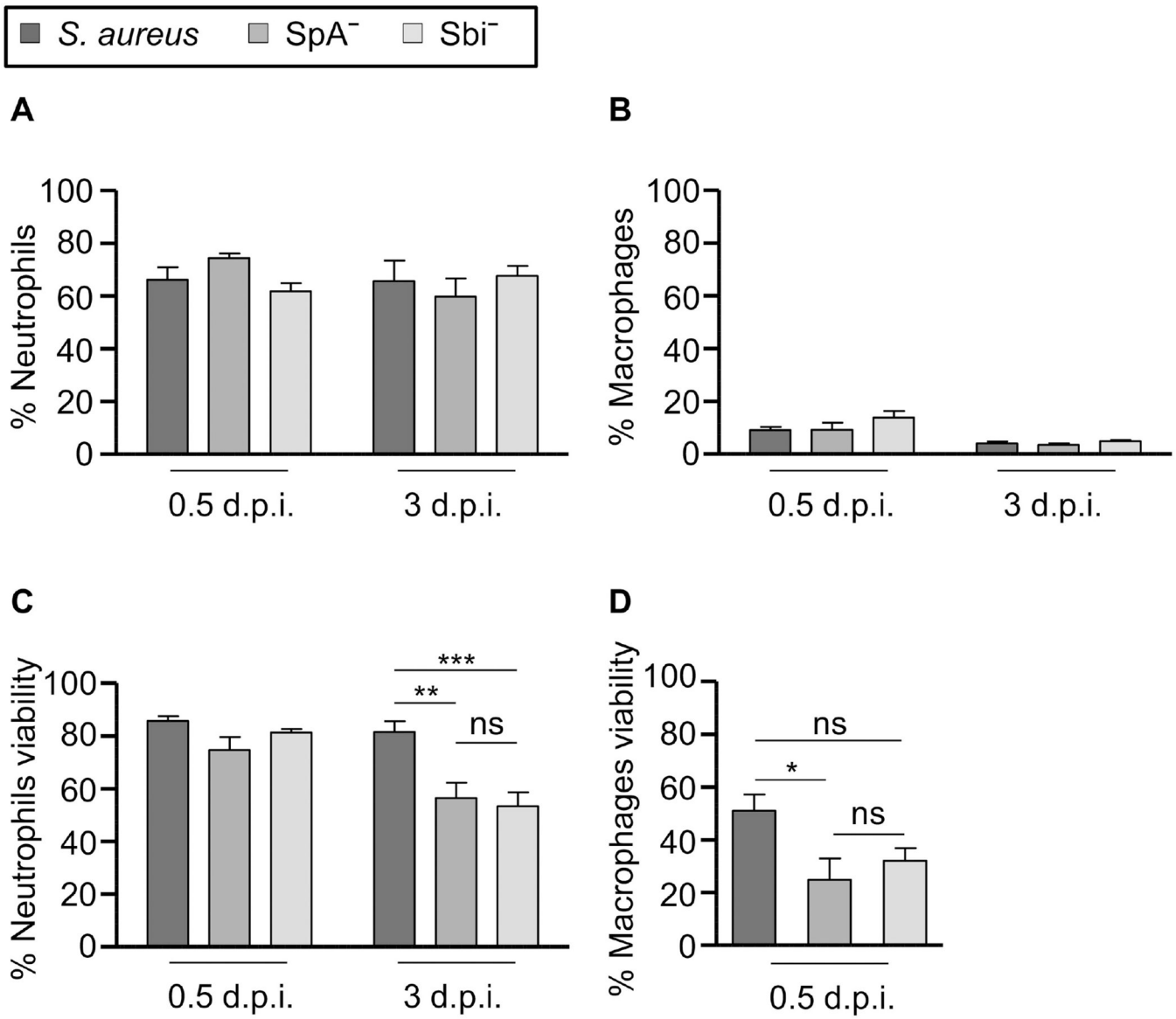

Figure 7. Distribution and viability of immune cells within the skin abscess.

Groups of mice were inoculated with *S. aureus* Newman wild-type (*S. aureus*), the isogenic SpA deficient mutant (SpA⁻) or the isogenic Sbi deficient mutant (Sbi⁻) by the subcutaneous route. (A, B) The percentage of neutrophils (A) and macrophages (B) present in the skin lesion were determined at 12 hours and 3 days post-inoculation by staining with specific antibodies and flow cytometry analysis. (C, D) Cell viability of neutrophils (C) and macrophages (D) present in the skin lesion was evaluated at 12 hours (C, D) and 3 days (C) post-inoculation by annexinV/propidium iodide staining and flow cytometry analysis. (d.p.i.): days post-inoculation. Bars represent the mean and standard error of cumulative data from 2 independent experiments (n= 6 mice/group at each time point). (A-C) Two way ANOVA and Bonferroni's multiple comparison test. (D) One way ANOVA and Bonferroni's multiple comparison test. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

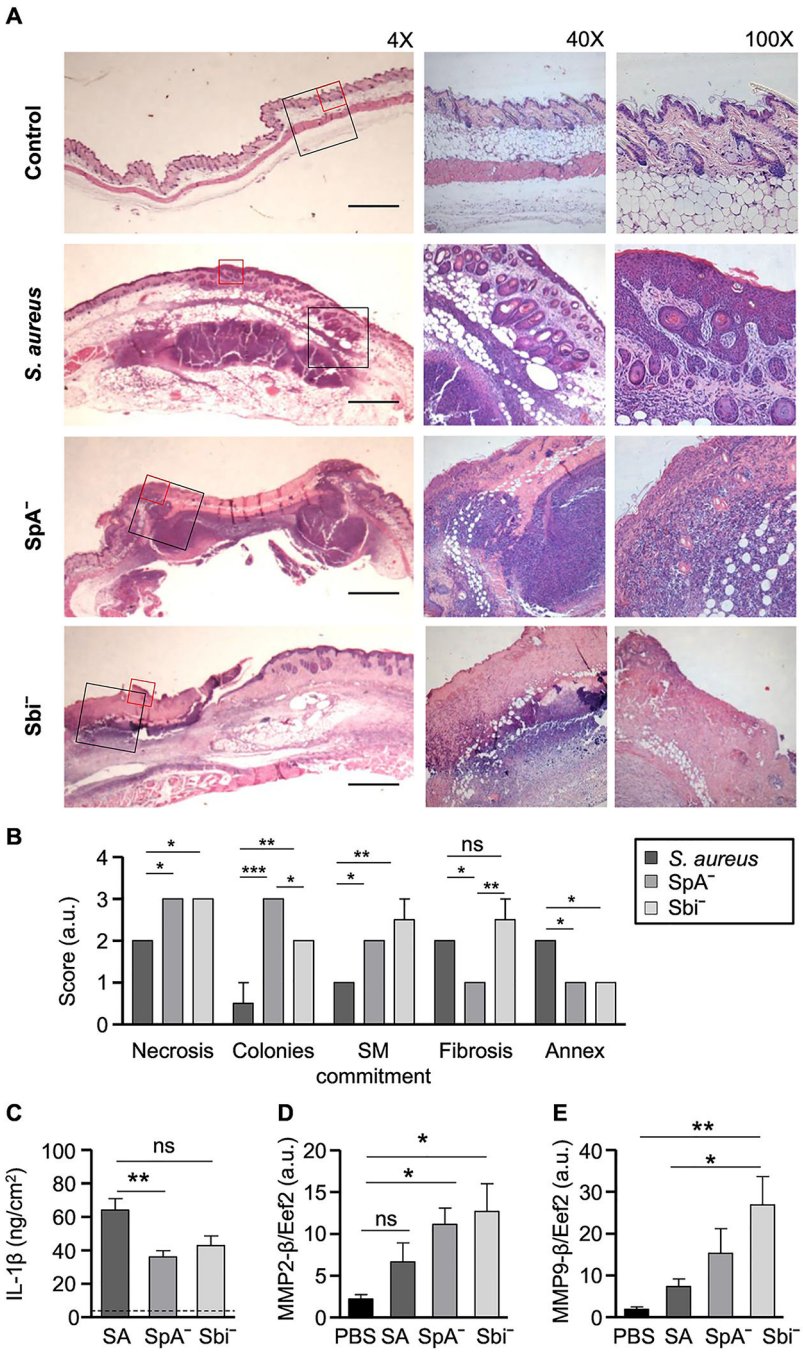

Figure 8. Role of SpA and Sbi in skin repair during SSTI.

Groups of mice were inoculated with *S. aureus* Newman wild-type (SA), the isogenic SpA deficient mutant (SpA⁻), the isogenic Sbi deficient mutant (Sbi⁻) or PBS (control) by the subcutaneous route. (A, B) Histopathological analysis of skin lesions at day 7 post-inoculation. (A) Hematoxylin-Eosin staining. Black and red boxes in the 4X magnifications indicate the areas shown in the 40X and 100 X magnifications respectively. The black lines in the 4X magnification views correspond to 1 mm. (B) Bars represent the mean and standard error of the score obtained in each group (n=3 mice/group from 3 independent

experiments). (C) IL-1β production was quantified in abscess homogenates by ELISA at day 7 after inoculation. Bars represent the mean and standard error of cumulative data from 3 independent experiments (n=9 mice/group). Dotted line indicates the mean of the control (PBS inoculated) group. **: $p<0.01$, one way ANOVA and Bonferroni's multiple comparison Test. (D, E) MMP-2 (D) and MMP-9 (E) mRNA levels at day 7 post-inoculation were quantified by real-time RT-PCR and normalized to Eef-2 expression. (a.u.): arbitrary units. Bars represent the mean and standard error of cumulative data from 2 independent experiments (n=6 mice per group).*: $p<0.05$, **:$p<0.01$, compared with control group, one way ANOVA and Bonferroni's multiple comparison Test.

Table 1.

Bacterial strains

Strain	Designation	Characteristics
S. aureus Newman (pCU1) *	*S. aureus*/SA	Cm^r
S. aureus Newman *spa*$^-$(pCU1) *	SpA^-	*spa*::Ka^r; Cm^r
S. aureus Newman *spa*$^-$ (pCU1-*spa*) *	SpA^-(SpA)	*spa*::Ka^r; Cm^r
S. aureus Newman *sbi*$^-$ (pCU1) *	Sbi^-	*sbi*::Em^r; Cm^r
S. aureus Newman *sbi*$^-$(pCU1-*sbi*) &	Sbi^-(Sbi)	*sbi*::Em^r; Cm^r
S. aureus USA300 LAC #∞	USA300 LAC	----
S. aureus USA300 LAC *spa*$^-$ #∞	USA300 LAC SpA^-	----
S. aureus USA300 LAC*sbi*$^-$ ∞	USA300 LAC Sbi^-	----
S. *aureus* USA300 LAC *hla*$^-$ #	USA300 LAC ΔHla	----

*: Provided by Dr. Timothy Foster, Trinity College, Dublin, Ireland.

∞: Provided by Dr. Joan Geoghegan, Trinity College, Dublin, Ireland.

&: [15]

#: Provided by Dr. Alice Prince, Columbia University, NY, United States.

Table 2.

Primers used for qRT-PCR

Gene	Primers	T°annealing	References
Eef2	5’- TGTCAGTCATCGCCCATGTG - 3’ 5’- CATCCTTGCGAGTGTCAGTGA - 3’	57°C	[23]
TGF-β	5’- CACCGGAGAGCCCTGGATA - 3’ 5’- T GTACAGCT GCCGCACACA - 3’	55°C	[24]
MMP-2	5’- CGGAGAT CTGCAAACAGGACA - 3’ 5’- CGCCAAATAAACCGGTCCTT - 3’	55°C	[24]
MMP-9	5’- CAGAC CAAGGGTACAGCCTGTT - 3’ 5’- AGTGCATGGCCGAACTC - 3’	49°C	[24]

* Eukaryotic translation elongation factor 2