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UNFOLDING THE IMMUNE RESPONSE AGAINST *STAPHYLOCOCCUS AUREUS*-MEDIATED SYSTEMIC *SEQUELAE* OF SKIN RECURRENCES

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ABSTRACT

Staphylococcal protein A (SpA) is a surface-associated virulence factor of *Staphylococcus aureus* (*S. aureus*) which binds human immunoglobulins via both Fc and Fab fragment masking the pathogen to the host immune system. This activity interacts with the normal maturation of the host immune system during an infection and allows *S. aureus* to cause recurrent infections as, for example, skin recurrences. Skin recurrences are not only bothersome superficial infections that require continuous treatments, but may also evolve in more complicated and systemic complications. Immunization with SpA protects animals against *S. aureus* systemic infections unmasking the pathogen to the host immune system, which turns out to recognize bacterial antigens otherwise hidden by SpA activity.

The aim of this project was to assess the protective effect of SpA_{mut} against skin recurrences and systemic complications in a mouse model of Skin and Soft Tissues Infections (SSTIs) set up in C57BL/6 mice, which are naturally susceptible to re-infections with *S. aureus*. Vaccination with SpA_{mut} adjuvanted with AS01 (SpA_{mut}/AS01) was able to limit bacterial spreading from the skin through the blood, abrogating *S. aureus* infiltration to the kidneys (target for systemic disease). *S. aureus*-specific protein microarrays were used to compare sera of mice vaccinated with SpA_{mut}/AS01 and then infected with those of mice only infected for their ability to recognize a selection of *S. aureus* antigens. Vaccination with SpA_{mut}/AS01 was able to unmask several *S. aureus* antigens to the immune system during SSTIs in mice. Interestingly, mice infected with *S. aureus* did not develop measurable antibodies against the mutated version of SpA, whereas infection in vaccinated mice significantly increased the avidity of antibodies against SpA_{mut} induced by previous immunization. Furthermore, only sera from vaccinated and infected mice allowed internalization of *S. aureus* by human phagocytes *in vitro*, suggesting a functional role in mediating the *in vivo* observed protection. Overall, these data support the essential role of vaccination with SpA, an immunomodulator antigen of *S. aureus*, in the induction of a functional specific antibody response during recurrences, contributing to the control of systemic bacterial dissemination, one of the main complications developed during *S. aureus*-mediated SSTIs.

I. INTRODUCTION

I. 1 *Staphylococcus aureus* – the "invisible" pathogen

Staphylococcus aureus (*S. aureus*) was firstly isolated in 1880 in the United Kingdom by the surgeon Sir Alexander Ogston, from a surgical abscess in a knee joint [1]. *S. aureus* is a Gram-positive, catalase and coagulase positive, nonmotile, non-spore forming coccus, which forms grape-like clusters when observed under a microscope (Figure 1) [2]:

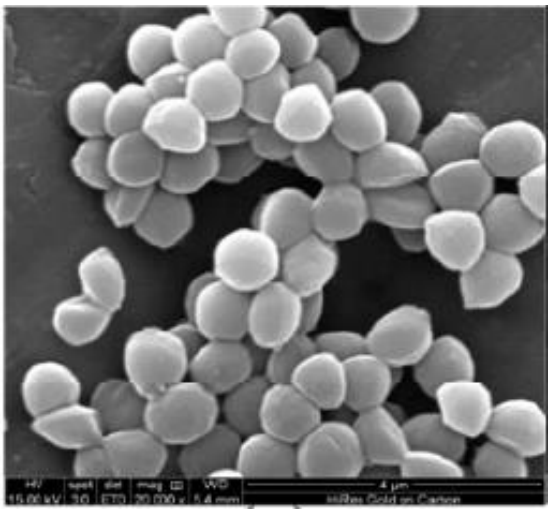


Figure 1. **Electron micrograph obtained through scanning electron microscopy of *S. aureus***. Bacterial cells are usually observed as grape-like clusters of cocci (adapted from Zhang *et al.* [2]).

The staphylococcal genome is composed by a single chromosome of 2.7-2.8 Mb, which is thought to carry about 2,500 genes [3]. *S. aureus* presents an external cell wall with a unique peptidoglycan structure, composed by glycopolymers and capsular polysaccharides, which play an important role in in colonization, pathogenesis and bacterial evasion from the host immune defenses [4].

S. aureus is a normal commensal that colonizes skin, nares and gastrointestinal tract of humans: it has been estimated that approximately 20-40% of the general population is stably colonized by this bacterium and around 90% is considered transiently colonized [5], [6]. Persistent or intermittent colonization with *S. aureus* has been linked to an increased risk of infections, especially in high-risk subjects who undergo surgical procedures, hospitalization or during intensive care stays [6], [7]. *S. aureus* can be responsible for a wide variety of diseases, ranging from mild and common conditions to life-threatening diseases (Figure 2):

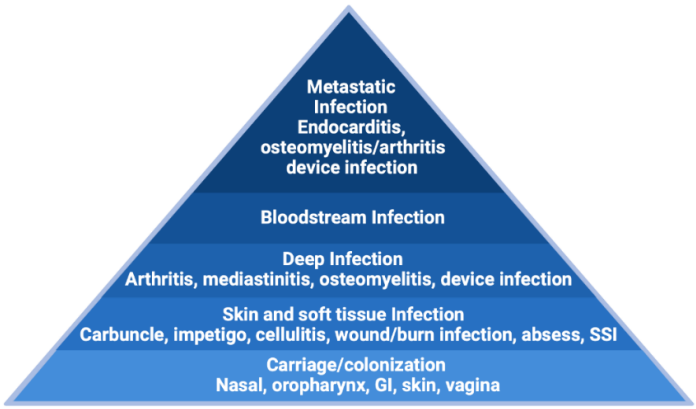


Figure 2. **The spectrum of *S. aureus*-mediated diseases**. The severity of infections increases from the bottom to the top of the triangle, while the frequency of infections decreases from the bottom to the top of the triangle (adapted from [8]).

One of the reasons why *S. aureus* can cause many different diseases is its wide variety of virulence factors that contribute to tissue colonization, infiltration, tissue damage and distant diseases. Furthermore, *S. aureus* is able to survive inside host innate immune cells and, after internalization, it may either persist inside the cells, escape the host defenses or further disseminate [9]. *S. aureus* also produces extracellular capsular polysaccharides, which protects the bacteria from phagocytosis and enhances bacterial virulence [10]. Type-5 and type-8 are capsular polysaccharides most frequently associated to human infections out of the eleven identified, and type-5 is also the most common capsular serotype of antibiotic-resistant strains [11]. *S. aureus* secretes several exotoxins, which can cause a wide variety of diseases, ranging from Skin and Soft Tissue Infections (SSTIs) to osteomyelitis or intoxications that can present as mild diseases (food intoxications) or life-threating situations (toxic shock syndrome).

SSTIs are the most common *S. aureus*-mediated infections, ranging from mild and self-resolving manifestations (impetigo, cellulitis) to immediate life-threatening diseases (necrotizing fasciitis, surgical site infections) (Figure 3) [12]:

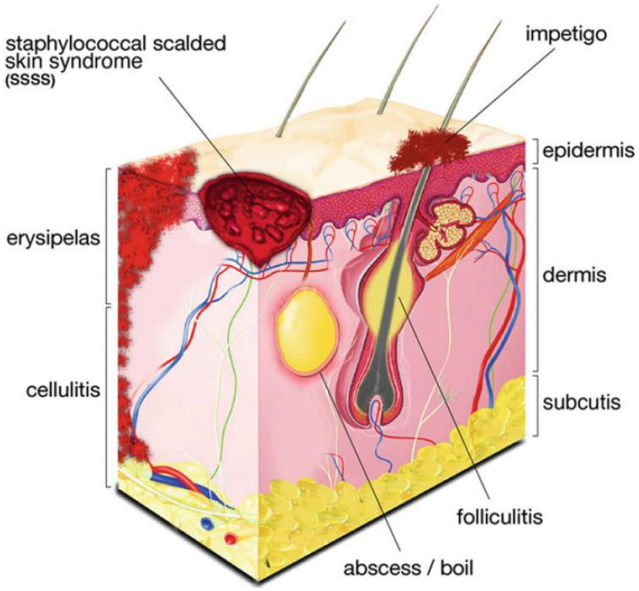


Figure 3. **Anatomical localization of the main superficial *S. aureus*-mediated SSTIs**. Structural components of the skin are indicated on the right, while *S. aureus*-mediated infections are indicated on the left. The epidermis can be affected by impetigo, staphylococcal scalded skin sy9ndrome (SSSS) and erysipelas. The dermis and subcutis can be affected by erysipelas and cellulitis. Manifestations affecting hair follicles include folliculitis, furuncles and carbuncles (adapted from [13]).

Uncomplicated SSTIs affect patients of all ages with a number of ambulatory care visits ranging between 11.6 and 14.2 million in the United States in the early 2000s, with *S. aureus* representing the most frequent cause of SSTIs in both adult and pediatric populations [14]–[16]. *S. aureus* SSTIs are also associated with development of serious complications, due to the ability of *S. aureus* to penetrate deep tissues, disseminate in the blood circulation and quickly spread to distant organs [17]. Moreover, the emergence of antibiotic resistant strains and the capability of *S. aureus* to evade the host immune system and impair the immune response are additional factors that could radically change the natural course of infection.

I. 2 Staphylococcal protein A: the immunomodulator antigen of *S. aureus*

The Staphylococcal protein A (SpA) is a 42 kDa protein that can be either anchored to the cell wall peptidoglycan by sortase A, or secreted during bacterial replication as peptidoglycan-linked SpA [18]. SpA is composed by three main α-helix segments and presents five highly homologous immunoglobulin-binding domains (IgBDs) in tandem, designated as domains E, D, A, B and C (Figure 4 A, B):

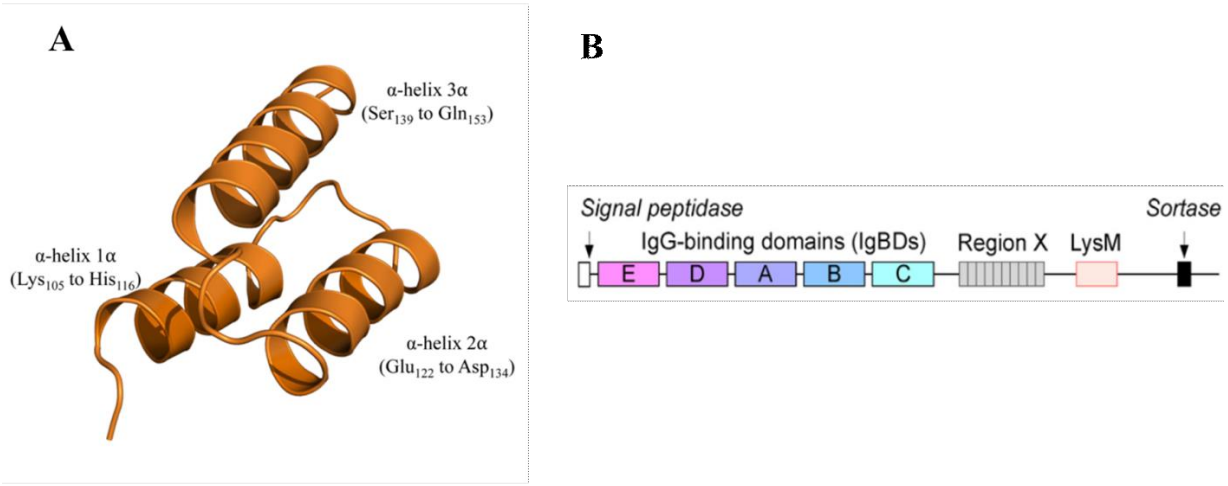


Figure 4. **Tertiary structure of SpA and its IgBDs**. **A**) Detailed secondary and tertiary structure of the engineered IgG binding domain of SpA, containing three α-helix segments (adapted from [19] , supplementary materials). **B**) Primary structure of SpA, with N-terminal signal peptidase cleaved upon secretion; IgBDs, a variable region X made of repeats, LysM domain and a C-terminal sorting signal for the attachment to the peptidoglycan by sortase A (adapted from [20]).

SpA is responsible for two distinct antibody binding activities: i) binding of Immunoglobulins (all but IgD, IgE and IgG3) through the Fc_γ domain, preventing the opsonophagocytosis by sequestering antibodies and displaying them on the bacterial surface in an incorrect orientation [21], [22]; ii) binding to the V_H3 domain of IgM/IgD present on the B cell surface, triggering B cell superantigen activity and causing B cell apoptosis [23], [24] (Figure 5).

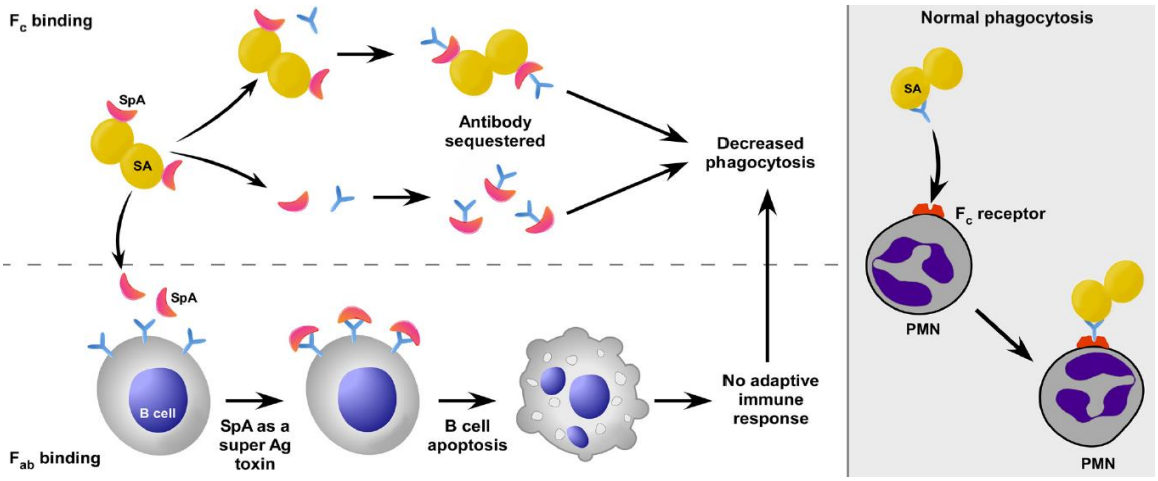


Figure 5. **Mechanisms of action of the immunomodulator antigen SpA**. On the left panel, representation of the two SpA antibody binding activities: SpA present on the surface of *S. aureus* (SA) or freely secreted can sequester the circulating antibodies by binding of the F_c region, thereby impairing the normal phagocytosis (depicted on the right panel). Alternatively, SpA can bind the F_{ab} regions of the B-cell receptor, acting as a toxic superantigen and eventually leading to B cell apoptosis (Adapted from Kobayashi and DeLeo [25]).

One of the main roles of the SpA is to facilitate the bacterial survival and to allow *S. aureus* to escape the host immune system. SpA, indeed, as an immunomodulator antigen can impair the activation of the immune response, affecting the expansion of effector T and B lymphocytes and the development of memory cells. Eventually, *S. aureus* exploits these mechanisms to mediate persistent and recurrent infections, without being effectively recognized and eliminated by the immune system [26], [27] (Figure 6):

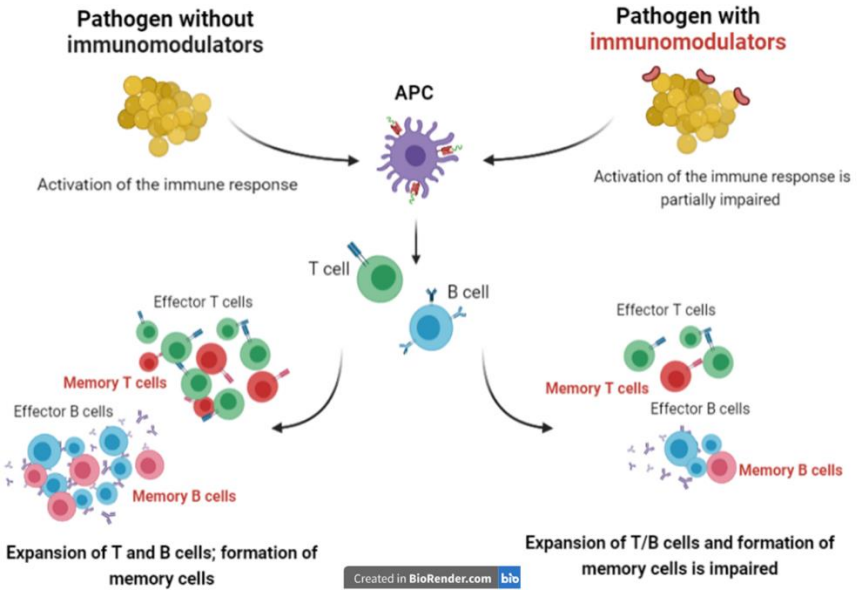


Figure 6. **Diagram representing the differences in the immune responses against pathogens with or without immunomodulator antigens**. Immunomodulator antigens can partially impair the immune response, the expansion of T and B effector cells and the formation of memory cells, allowing pathogens as *S. aureus* to cause persistent and recurrent infections (created with BioRender.com). APC: Antigen Presenting cell.

I. 3 Vaccination against *S. aureus*: thinking out of the box

Vaccines throughout the last century have contributed to the almost complete eradication of several infectious agents, significantly improving the human welfare and well-being [28]. One of the most challenging aspect of treating staphylococcal infections is the capability of *S. aureus* to quickly adapt and become resistant to antibiotics. A possible option to reduce the likelihood of the development of resistance could be represented by vaccination: in the past two decades different types of vaccines against *S. aureus* infections were developed, but no vaccine candidate succeeded in showing efficacy against infection in later clinical trial phases [29], [30]. However, vaccines tested so far in efficacy trials targets single antigens and contained no adjuvants, so they were likely insufficient to cope with the complexity of this pathogen. Promising new vaccine candidates including SpA, pore-forming toxins and new adjuvants to stimulate cell-mediated immunity and increase vaccine efficacy are between late pre-clinical and early clinical development phase [31], [32]. Among the vaccines that

reached the clinical trial, V710 was a vaccine developed by Merck targeting the iron-scavenger protein IsdB, targeting especially patients receiving hemodialysis after renal failure [33]–[38], while StaphVAX was a glycoconjugate vaccine developed by Nabi Biopharmaceuticals, targeting capsular polysaccharides type 5 (CP5) and type 8 (CP8) [39]. Recently, Pfizer developed a new promising four-component (SA-4Ag) vaccine composed by CP5, CP8 and the two surface protein antigens Clumping factor A (ClfA) and Manganese transport C protein (MntC), but it failed in reaching the desired efficacy [29], [40]. Finally, GlaxoSmithKline (GSK) developed a five-component (SA-5Ag) vaccine against *S. aureus* recurrent SSTIs, including bioconjugates of Hla-CP5 and ClfA-CP8, together with a detoxified version of SpA, which presents amino acid substitutions in the immunoglobulin binding domains which abolish the binding to the Fcγ and Fab fragments of IgGs (Justia Patents, application n. 20210023192). In this context, an innovative way of designing vaccines against *S. aureus* could be represented by the selection of the immunomodulator antigen SpA as a vaccine candidate. Indeed, the protective role of SpA as an immunomodulator against *S. aureus* was already observed in a systemic model of infection in guinea pigs, as well as its effect as an immunomodulator antigen [41]. Animals were immunized with a nontoxigenic form of SpA, denominated SpA_{KKAA}, engineered by substituting 20 amino acids residues essential for its association with Ig Fcγ and Fab [42] (Figure 7, panel A). Immunized animals not only were protected from the systemic infection (Figure 7, panel B and C), but they developed high levels of antibodies against several *S. aureus* antigens after infection, respect to only infected animals (Figure 7, panel D):

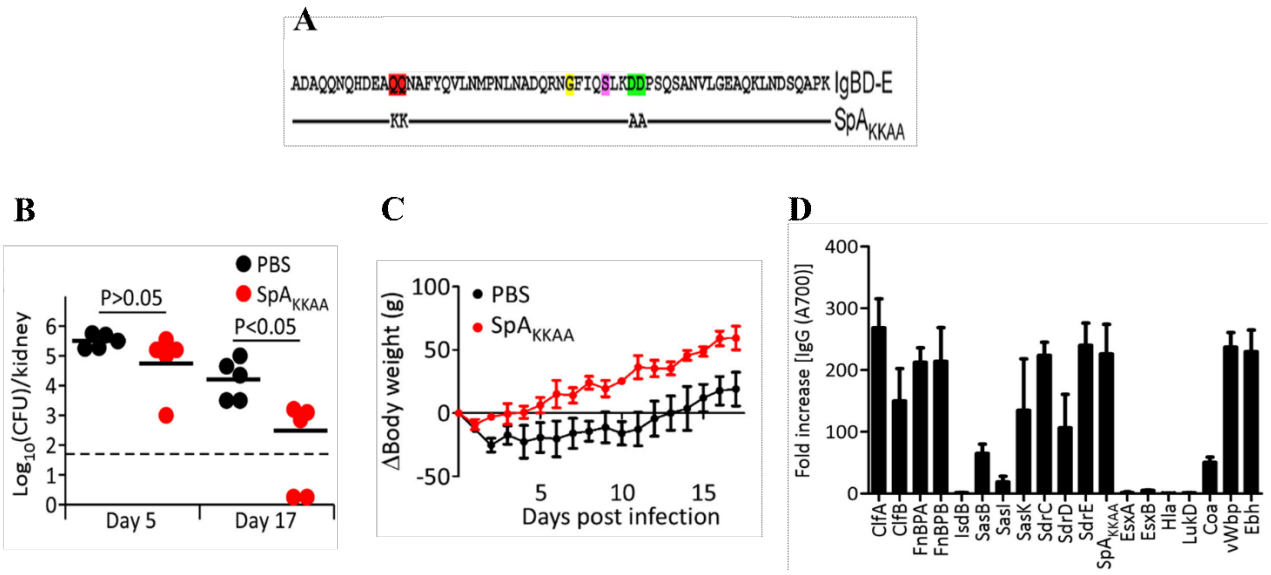


Figure 7. **Primary structure of nontoxigenic SpA_{KKAA} and its protective and unmasking effect of *S. aureus* in guinea pigs after infection. A**) Primary structure of the IgBD E of SpA_{KKAA}, in which the glutamine (Q, red) and aspartate (D, green) residues were substituted with lysine (K) and alanine residues, respectively (adapted from [20]). **B**) Bacterial load evaluated in the kidneys five or seventeen days after infection, in immunized or

not immunized animals. Data are represented as mean (black line) of single values (single dots). **C**) Variations in the animal body weight, measured over a 17-day period after infection. Data are represented as mean values, with lines representing standard errors of means. **D**) IgG titers against different staphylococcal antigens were higher in the serum of immunized and infected animals, respect to IgG titers in the serum of infected-only animals (adapted from [41]).

I. 4 Mouse models of recurrent *S. aureus* SSTIs

The adversities in developing an effective vaccine against S. aureus could be due to the wide variety of infections caused by *S. aureus*, the lack of correlates of protection and the differences between preclinical models and humans. Animal models to mimic diseases aim to understand the pathogenesis and the mechanisms whereby protective immunity is achieved [43]. However, the use of mice models to simulate *S. aureus* infections is a hotly debated issue for several reasons, since mice are not natural hosts for *S. aureus*, they are resistant to superantigens, their skin has a different structure respect to the human skin and there are few differences between human and mouse adaptive immune responses [44], [45]. To induce skin infections in mice, usually the hair on the back or the flank of animals is shaved using a razor or depilatory cream; then a suspension of 10^7-10^9 CFU *S. aureus* in PBS is injected into subcutaneous tissues [46], [47]. Within 24 hours, bacteria elicit inflammatory responses and cause swelling, which can increase in the next 5-7 days to a size of 30-100 mm^2 [47]. Dermonecrotic lesions usually resolve over the next 7-9 days, with the closure of the open wound and, sometimes, with the formation of a scar [46], [48]. To mimic the recurrent skin infection in mice, 6-8 weeks after the first skin infection a second infection is performed, usually in the opposite flank of the animal, with the same or a higher infective dose of the first infection [49]. In order to have an effective re-infection, it is crucial to choose the right strain of mice: some strains, as the BALB/c mice, are not susceptible to re-infections, while the C57BL/6 mice will develop a secondary skin infection [49]. This susceptibility seems to be driven by the different major histocompatibility complex (MHC) haplotypes in the mice strains, that cause different immune response to *S. aureus* and make the C57BL/6 mice one of the few susceptible mice strains to *S. aureus* recurrent infections, which make C57BL/6 mice a suitable model for studying recurrent SSTIs [50].

II. AIM OF THE THESIS

S. aureus is the main cause of SSTIs which are associated with the development of serious systemic complications and recurrent infections thanks also to the capability of *S. aureus* to evade the immune system due to its main immunomodulator antigen SpA. Therefore, the aim of this project was to evaluate the "hidden effect" of the Staphylococcal protein A (SpA) as a vaccine candidate in a mouse model of skin recurrences for its protective effect on recurrences and systemic complications and for the unmasking of the pathogen to the host immune system. This work will be useful to better understand the added value of the SpA in the vaccine formulated against *S. aureus*-mediated skin recurrences, which can be useful to prevent systemic complications of skin recurrences, one of the most common and most serious complications of skin and soft tissue infections (SSTIs).

III. MATERIALS AND METHODS

III. 1 In vivo model of *S. aureus* skin recurrence

Bacterial strains and preparation of inoculum for infection

S. aureus USA300 lac strain was used for the *in vivo* model of skin infection and skin recurrence. Bacteria were grown in tryptic soy broth (TSB) at 37° C in agitation at 250 rpm until reaching an optical density at 600 nanometers (OD_{600}) equal to 2.0 (early exponential phase) and diluted 1:1 in a freezing solution, composed by phosphate-buffered saline (PBS) + 10% bovine serum albumin (BSA) + 10% L-glutamic acid. Aliquots were conserved at -80° C in cryovials until use.

For the inoculum preparation, frozen stocks of bacteria were thawed and diluted 1:25 in TSB (obtaining an initial OD_{600} approximately equal to 0.04) and incubated at 37°C at 250 rpm until bacteria reached the early exponential phase, at OD_{600} equal to 2.0, approximately 1.5-2 × 10^9 Colony Forming Units (CFUs) / ml. Bacteria were then washed once in sterile PBS and diluted with sterile PBS to obtain 4 × 10^8 Colony Forming Units CFU / ml or 8 × 10^8 CFU / ml for the skin infection or the skin recurrence, respectively.

S. aureus USA300 lac GFP-expressing strain was used for the internalization assays. Bacteria were streaked on a tryptic soy agar (TSA) plate and incubated O/N at 37° C + 5% CO2. The following day, single colonies were diluted in 3 ml of TSB in a 50 ml tube with a loosen cap. Three pre-inocula were prepared and they were incubated overnight at 37°C at 250 rpm. The following day each pre-inoculum was diluted in 30 ml of TSB in a 50 ml tube, with a final OD_{600} of 0.05. Inocula were incubated with loosen cap at 37°C and 250 rpm for about 3 hours, until they reached OD_{600} of 2.0. Bacteria were centrifuged at 2400 rpm and 4° C for 10 minutes and washed twice with RPMI 1640 GlutaMAX + 25 mM HEPES + 0.05 % BSA. After the final resuspension, OD_{600} of bacteria was measured again and adjusted to OD_{600} of 1.0. Bacteria were aliquoted in 2 ml cryovials and stored at -20°C until use.

Ethical Statements

Animal husbandry and experimental procedures were ethically reviewed and carried out in accordance with European Directive 2010/63/EU, Italian Decree 26/2014 and GSK Vaccines' Policy on the Care, Welfare and Treatment of Animals, and were approved by the Italian Ministry of Health (authorization 123/2015-PR). Upon arrival, animals will be randomly distributed in different experimental groups (up to five) in individually ventilated cages (IVC, Sealsafe Plus GM500 by Tecniplast). The acclimation will last for a period of 5 days. At the end of the acclimation period, each animal will be identified by an individual tattoo. All animals ad libitum access to GMP-grade

food (Mucedola 4RF25 TOP CERTIFICATE) and bottled, filtered, tap water. Certified, irradiated cellulose bags containing Mucedola SCOBIS UNO bedding, and carboard tunnels (ANTRUM) or plexiglass mouse house will be provided within the cages. A few food pellets in the cage will be also used as enrichment for forging and additional gnawing. Cage and bedding changes will be performed once every two weeks. Air supplied in IVC will be 100 % fresh air filtered by EPA filter by the IVC system, with 60-75 air changes per hour. The animal room conditions will be as follows: temperature 21°C (+/- 3°C), relative humidity 50% (range 30-70%) and 12h/12h light/dark cycle. Pressure, temperature and relative humidity will be recorded continuously by room probes, while the IVC system will be recorded individual motors' performance. The light cycle settings will be ensured by a qualified, alarmed system.

Immunization

Five-week-old female Specific Pathogen Free (SPF) C57BL/6 mice were immunized twice intramuscularly (i.m.), 28 days apart. Animals were immunized with 10 µg of a mutated form of the staphylococcal protein A (SpA_{mut}), adjuvanted with AS01, in a final volume of 50 µl, 25 µl/posterior leg. The control group was immunized with the formulation buffer alone (10 mM Na2HPO4, 150 mM NaCl, pH 6.1).

Prior to administering the immunization, both formulations (the adjuvanted antigen and the control one) were visually inspected for the absence of particulates and tested for several acceptance criteria, as the integrity of the antigen via SDS-PAGE, the absence of aggregates in the adjuvant via light-scattering, the absence of biological contamination via plating on TSA plates of the formulates and the control of pH and osmolality.

Mouse model of skin infection and skin recurrence

For the infection model set up experiments, the day before the skin infection or the skin recurrence the back of mice was shaved under anesthesia (3.5% isoflurane) using electric razor and depilatory cream. The day after shaving, mice were infected. For the primary skin infection, mice were infected subcutaneously (s.c.) in the right flank with a high dose (4×10^7 CFU / 50µl), a medium dose (2×10^7 CFU / 50µl) or a low dose (1×10^7 CFU / 50µl) of *S. aureus* USA300 lac strain under anesthesia (3.5% isoflurane). Six weeks after the first inoculum mice have usually completely recovered from skin infection and they were shaved and infected again as previously described in the opposite flank with 4×10^7 CFU / 50µl of *S. aureus* USA300 lac strain, regardless of the infective dose of the first infection, to establish the model of skin recurrence.

For the *in vivo* vaccine efficacy studies, four weeks after the second immunization the back of mice was shaved under anesthesia (3.5% isoflurane) using electric razor and depilatory cream.

The day after shaving, mice were infected. For the primary skin infection, mice were inoculated subcutaneously (s.c.) in the right flank with 2×10^7 CFU / 50µl of *S. aureus* USA300 lac strain, under anesthesia (3.5% isoflurane). Six weeks mice were shaved and infected again as previously described in the opposite flank with 4×10^7 CFU / 50µl of *S. aureus* USA300 lac strain to establish the model of skin recurrence.

Mice were observed daily for clinical signs of disease as reported in the following table up to 14 days after infection (Table 1). Mice were scored on the basis of pre-established readouts as reported below, and they were euthanized in case they exhibited pre-established humane endpoints in agreement with local Animal Welfare Policies.

At day 7 or 14 after skin infection, a subgroup of animals was sacrificed for evaluation of bacterial load in skin and kidneys and for the collection of blood for serological analysis.

SA Skin Infection Model	Clinical Score							
Clinical symptoms	0	0,5	1	1,5	2	2,5	3	4
1. Scruffy coat 2. Kyphosis 3. Reduced deambulation 4. Skin lesion 5. Inactivity (muribond state)		Mild Redness	Moderate Mild Motile lesion	Severe Moderate Mild Non-motile lesion	Severe Moderate Necrotic lesion small	Severe Necrotic lesion medium	Necrotic lesion large	Yes
6. Weight loss					5%	10%	15%	20%
								Immediately humanely euthanised

Final Score: Sum of A + B
A. Maximum score from points 1, 2, 3, 4, 5
B. Score from point 6

SMALL NECROTIC LESION SIZE	MEDIUM NECROTIC LESION SIZE	LARGE NECROTIC LESION SIZE
< 15 mm²	< 50 mm²	> 50.1 mm²

Table 1. **Table reporting the signs of disease for assignment of clinical scores**. Score A is defined by the maximum score from points 1 to 5; while score B is defined by the score from point 6. Different colors identify the severity of symptoms; green: healthy-like; yellow: mild; orange: moderate; red: severe.

III. 2 Sample collection

Serum samples

Blood samples were collected for serological analysis at different time points as reported below: i) day 0 (Pre-immune); ii) day 52 (SpA_{mut}/AS01 post II); iii) day 72 (two weeks after skin infection); iv) day 97 (4 weeks after skin infection); v) day 107 (1 week after skin recurrence).

For serum sampling, blood samples were incubated at room temperature for 3-4 hours and then centrifuged at room temperature for 12 minutes at 1,000 *Xg*. Serum was then collected, filtered using 0.22 µm filters (Millipore) and stored at -80° C until use.

Skin biopsies and kidneys collection

At the defined time points, mice were euthanized by cervical dislocation and kidneys and skin biopsies were collected. The whole skin lesion was excised to ensure the full recovery of bacteria present in the site. Skin biopsies and kidneys were homogenated in 5 mL of sterile PBS using a GentleMacs instrument and following manufacturers procedures. For CFU counts homogenated samples were filtered using a 70nM filter (Millipore) to remove cellular debris and serially diluted as better explained in the "Experimental readouts" section. CFU counts in graphs have been reported as total counts/organ.

III. 3 Experimental Readouts

Assessment of dermonecrotic lesion severity

For the infection model set up experiments, the area of dermonecrotic lesions in each flank was evaluated daily at day 4 after the skin infection or the skin recurrence. For the *in vivo* vaccine efficacy studies, dermonecrotic lesions in each flank were photographed and measured daily from day 4 to day 7 post infection. Mice were anesthetized with 3.5% isoflurane and lesions were photographed; pictures were then analyzed using the ImageJ software (ImageJ 1.49v, National Institute of Health, USA) and expressed as the area of the dermonecrotic lesion (mm^2). Cumulative data analysis of the area of dermonecrotic lesions was performed, calculating the Area Under Curve (AUC) for the whole period of observation, as described below in section "Area Under the Curve analysis".

Assessment of disease severity

Mice were scored daily for seven days after infection based on the clinical score table reported in the section "Mouse model of skin infection and skin recurrence". The values of each score were then summed together to give a total score used as marker of disease severity. These values were finally represented as cumulative data through the calculation of the AUC for the whole period after the infection.

Bacterial load in skin and kidneys, dissemination severity index

In order to evaluate the local and systemic bacterial load, skin biopsies, kidneys homogenates were enumerated for Colony Forming Units (CFUs) as follows.

Filtered homogenates were serially 10-fold diluted in PBS, up to 10^{-7}. 10µl of each diluted sample were spotted in duplicate onto TSA + 5% sheep blood plates and incubated overnight at 37°C + 5% CO_2. For the skin homogenates, colonies were enumerated the day after and CFUs were reported as the $\log_{10}$ of the CFUs in the entire homogenated sample (5 mL).

For the evaluation of bacterial dissemination after the skin recurrence the whole homogenated kidneys samples were centrifuged and the pellet was resuspended in 100 µl of PBS: this concentrated sample was plated on TSA + 5% blood plates, in order to evaluate bacterial dissemination in the whole organ. Based on the number of colonies present in the kidneys, a dissemination severity index was assigned to each animal (see table 2).

Dissemination index	Number of CFUs
No dissemination	< 10
Minimal dissemination	10 - 500
Mild dissemination	500 – 1500
Heavy dissemination	> 1500

Table 2. **Table reporting the criteria for the dissemination index assignment**. The different dissemination severity indexes were assigned based on the number of bacteria recovered from the whole homogenate. A threshold of 10 colonies was determined, under which the sample was considered not disseminated, due to the presence of possible contaminants in the homogenate.

III. 4 Immunological readouts

Magnetic beads coupling with staphylococcal antigens

Staphylococcal antigens of interest were coupled to magnetic beads using an automatized approach or a manual approach for staphylococcal proteins or capsular polysaccharides, respectively. For the bacterial proteins, 5×10^6 magnetic beads were coupled to 20 µg of SpA_{mut}. The carboxyl groups present on the beads surface were activated and covalent bonds were created between the magnetic beads and the amino groups of the staphylococcal proteins, through a fully automatized process. Coupled beads were stored at 4°C in the dark up to 21 days.

Multiplex and automatized Luminex assay for antibody titers evaluation

For the total IgG titers in mice sera, multiplex and automatized Luminex assays were carried out in 384-well plates, through a Workstation Hamilton Star (Hamilton). Briefly, the Hamilton robot performed a first 1:100 dilution in PBS of each serum sample and then 2-fold serial dilutions for 12 points, comprehensive also of a mouse standard serum. 25 µl of each dilution of sample or standard were dispensed in the appropriate wells, along with blanks (PBS). 10 µl of SpA_{mut}-coupled beads were added to the samples and the reaction was incubated for 1 hour, RT, at 1200 rpm. After an automatized cycle of washes with TPBS, 25 µl of a 1:100 dilution of anti-mouse IgG secondary antibody conjugated with R-Phycoerythrin were added to each well. The assay was incubated for 30 minutes, RT, at 1200 rpm; after an automatized cycle of washes with TPBS the assay was resuspended

in PBS and the signals were acquired with a FLEXMAP 3D reader (Luminex, Biorad). Fluorescence intensities (FI) signals of the blanks were subtracted to the fluorescence intensities of the samples and interpolated to the standard curve of each analyte, expressing the IgG titer as Relative Light Unit/ml (RLU/ml). For each sample tested, the experiment was considered valid if a titer was observed in at least 3 points of the dilution and if these titers were interpolated in the linear portion of the standard curve; the final IgG titer of each value was expressed as the median value of the observed titers.

Determination of different IgG subclasses in mice sera

For the IgG subclasses analysis, the assay was performed manually in 96-well plates, testing pools comprehensive of single sera collected after immunization with SpA_{mut}/AS01 (SpA_{mut}/AS01 post II) or two weeks after infection (1st infection 2 weeks) from each mice group. A mouse serum standard was diluted 1:500 and 3-fold dilutions were performed for six points. Pools were firstly diluted 1:10000 and 3-fold serial dilutions were performed in 8 points. 10 µl of SpA_{mut}-coupled magnetic beads mix were added to each well and the assay was incubated for 1 hour at 900 rpm at room temperature. Each assay was repeated in five different plates and, after a cycle of automatized washes with TPBS, 25 µl of a 1:100 dilution of a specific anti-mouse secondary antibody conjugated with R-Phycoerythrin diluted was added to each well of the respective plate: i) IgG, ii) IgG1, iii) IgG2a, iv) IgG2b, v) IgG3. After an incubation of 30 minutes at 900 rpm at room temperature and a cycle of automatized washes with TPBS, the assays were resuspended in PBS and the signals were acquired using a Lx (200) reader (Luminex, Biorad).

Results were reported as the ratio between the RLU/ml of the different IgG subclasses.

Characterization of antibody affinity and avidity

Ammonium thiocyanate was used to determine the avidity of IgGs to SpA_{mut} at 2M concentration, which is able to detach the antibody from its specific antigen.

Serum samples and a mouse standard serum were 3-fold serially diluted in PBS in 8 points in a 96-well plate, in duplicate. 25 µl of diluted serum samples and standard were added to the appropriate wells and incubated with 10 µl of magnetic beads coupled with SpA_{mut} for 1 hour, RT, shaking at 900 rpm. After washing the plate for three times using an automated plate washer, 50 µl of 2M ammonium thiocyanate was added to one half of the plate, while the other replicates of the samples were incubated with 50 µl of PBS for 30 minutes, RT, shaking at 900 rpm, as depicted in the image below (Figure 8):

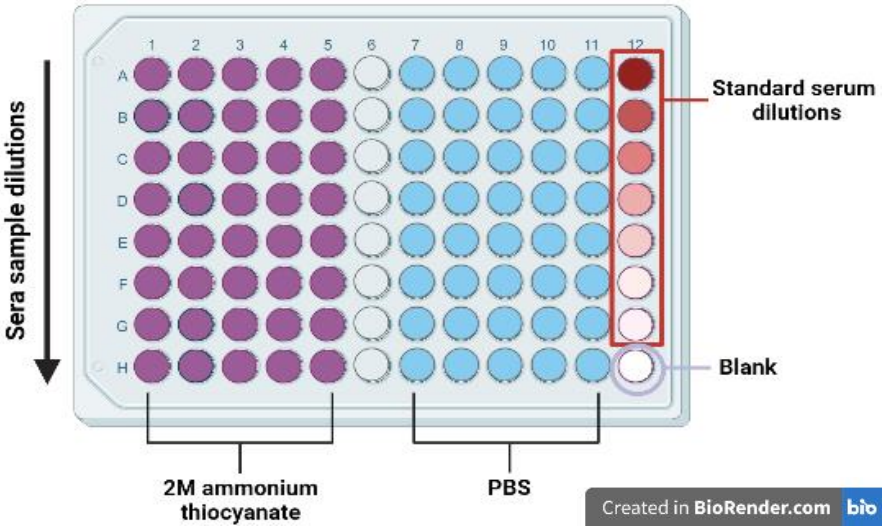


Figure 8. Representation of the sample distribution for the antibody affinity and avidity analysis. Serum samples and a mouse standard are 3-fold serially diluted in duplicate, one replicate is incubated with the 2M ammonium thiocyanate or the PBS.

After washing the plate three times, 25 µl of a 1:100 dilution of a phycoerythrin-goat anti-mouse IgG secondary antibody were added to all the samples and the assay was incubated for 15 minutes, RT, at 900 rpm. After washing the plate three times, the signal was acquired using a Lx (200) reader (Luminex, Biorad). Results were reported as avidity percentage, calculated on the basis of the ratio of the antibody titers recovered after incubation with ammonium thiocyanate respect to titers observed after incubation with PBS.

III. 5 Internalization of bacteria mediated by THP-1 cells

THP-1 cell culture

THP-1 cells (ATCC TIB-202) were grown in suspension in RPMI 1640 with GlutaMAX + 10% fetal bovine serum (FBS) + penicillin-streptomycin-glutamine (PSG, 100 units of penicillin, 100 ug of streptomycin and 29.2 mg/ml of L-glutamine, Gibco). Frozen vials containing about 4 million of cells in 90% FBS + 10% DMSO were quickly thawed in a water bath at 37° C and centrifuged for 7 minutes at 400 g at room temperature. Cells were resuspended in the cell medium and transferred to a 75 cm^2 cell flask, maintaining cells were at a concentration of 4×10^5 cells/mL. Cell suspensions were incubated at 37° C + 5% $C0_2$. Two days prior to the internalization assay, THP-1 cells were cultured in a 175 cm^2 cell flask in 40 mL of the same cell medium at a concentration of 2×10^5 cells/mL.

THP-1 mediated internalization assay

Single sera collected from animal studies were heat-inactivated at 56° C for 30 minutes. Frozen live aliquots of *S. aureus* USA300 GFP strain were thawed in a water bath at 37° C. Treated sera were diluted with the PBS + 0.05% BSA, and 20 µl of each dilution were transferred to the appropriate well of a 96-well plate, along with 20 µl of *S. aureus* USA300 GFP strain. Bacteria and sera were incubated at 37° C, 750 rpm for 15 minutes to allow pre-opsonization of bacterial cells. THP-1 cells were centrifuged at 400 g per 7 minutes at room temperature and resuspended in PBS + 0.05% BSA reaching a concentration of 3.75×10^6 cells/mL. 10 µl of the cell suspension were added to the pre-opsonized bacteria and the assay was incubated at 37° C, 750 rpm for 30 minutes to allow internalization of opsonized bacteria. 10 µl of 60 µg/ml lysostaphin (Sigma Aldrich) were added to the assay, to lyse non internalized bacteria bound to the cell surface. Finally, to fix bacteria and cells, 100 µl of 2% formaldehyde were added to each well and the plate was incubated on ice for at least 1 hour, to ensure bacteria inactivation.

Samples were acquired with a CANTO II without HTS flow cytometer, gating on: i) THP-1 cells; ii) singlet cells; iii) FITC-A, representative of the GFP expressed by bacteria, representative of cells that have effectively internalized GFP-expressing bacteria.

Results were represented as the percentage of GFP^+ THP-1 singlet cells and their median fluorescence intensity (MFI), normalized versus the median signal given by pre-immune sera.

III. 6 Staphylococcal protein microarray

Selection and expression of suitable *S. aureus* antigens

S. aureus proteins suitable to be spotted onto microarray slides were selected and their genes were amplified by Polymerase Chain Reaction (PCR) from *S. aureus* NCTC 8325 or Newman strains and cloned as N-terminal 6-histidine-tagged (His-tag) or tagless constructs, as described previously [51], [52]. Through the Polymerase Incomplete Primer Extension (PIPE) technique, the His-tagged products were cloned into the pET-15b+ vector, while the untagged PCR products were cloned into the pET-24b vector; both constructs were transformed in competent BL21 (DE3) *E. coli* cells. Other selected antigens were amplified from the *S. aureus* Newman strain and cloned as Glutathione S-transferase (GST) into the pgex-tev vector and transformed into the BL21 *E. coli* cells.

Recombinant *E. coli* cells were plated on Luria Bertani (LB) supplemented with ampicillin plates and incubated at 37° C O/N. The day after, single colonies were diluted in LB with vegetable proteins (LB-PTK) reaching an OD_{600} between 0.03 and 0.05 and they were incubated at 37°C at 160 rpm for about 7 hours, until bacteria reached a stationary phase. Bacteria were then diluted 1:100 in LB-PTK broth and incubated at 20° C at 160 rpm O/N. The day after, recombinant protein expression was

induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), bacteria were incubated at 20° C, 160 rpm for 24 hours. Induced bacteria were then centrifuged at 3600 rpm, 4° C for 20 minutes and the pellet was stored at -20° C until use.

Recombinant *S. aureus* protein purification

S. aureus pellets were thawed and resuspended in CelLytic Express (Sigma-Aldrich) and incubated for 1 hour, shaking, at room temperature. For His-tag proteins, lysates were centrifuged at 30,000 g, 4° C for 10 minutes, and supernatants were load into NI-NTA agarose columns (ThermoFisher) equilibrated with wash buffer A (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). After three washes with wash buffer A and three washes with wash buffer B (20 mM Imidazole, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0), proteins were eluted with elution buffer (250 mM Imidazole, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0) in Eppendorf tubes containing 1 mM of dithiothreitol (DTT).

GST-tag proteins were purified using GSTrap FF columns (Cytiva), following manufacturer's procedures. Proteins were eluted with 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 in Eppendorf tubes containing 1 mM of DTT.

Eluted proteins were quantified with 660 nm Protein Assay Kit (Pierce) following manufacturer's procedures and, if necessary, concentrated using Vivaspin units (GE Healthcare) and analyzed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on NuPAGE 4-12 % Bis-Tris Midi Gel (Thermo Fisher).

Extraction, purification and characterization of *S. aureus* native proteins and capsular polysaccharides were described elsewhere [51], [52] and in supplementary material of Rigat *et al*.[53].

Integrity characterization of purified proteins by SDS-PAGE analysis

S. aureus recombinant proteins, with a final concentration of 2 ug, were mixed with the 4X lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher) and the 10X NuPAGE reducing agent (ThermoFisher), in a final volume of 30 ul with 1X PBS. After vortexing, the samples were boiled at 100° C for about 5 minutes, cooled down on ice for 2 minutes and let reach room temperature. 20 ul of sample were loaded into 4-12% Bis-tris Midi Gel and the SDS was run with 1X MES at 180V for about 40 minutes. Finally, gels were rinsed with demineralized water, stained with Simply Blue SafeStain (ThermoFisher), decolored with demineralized water and images were acquired with the GelDoc (BioRad). The molecular weight of each protein was evaluated by comparing the bands with the marker (Novex Sharp, Thermofisher), for each antigen the presence of aggregated or degraded forms were also evaluated.

Protein microarray design, spotting and immunogenicity scanning

The staphylococcal protein microarray was generated by spotting relevant *S. aureus* antigens that were previously selected, expressed in competent *E. coli* cells, purified and characterized [53]; antigens were resuspended in 40% glycerol at a final concentration of 0.36-0.5 mg/ml. As a positive control, mouse IgGs were 2-fold diluted in 8 points, with a concentration from 0.5 mg/ml up to 0.004 mg/ml. To delineate the edges of each pad on the microarray, 2-fold serial dilutions of BSA marked with Cyanine 3 and Cyanine 5 (BSA-Cy3/Cy5) in 8 points were also spotted. For the controls instead, mouse IgM, rabbit IgG and human IgG were diluted in the same way of the mouse IgG and spotted, while the tetanus toxoid (-tt) was used as a control for the CP5-tt and CP-8 tt capsular polysaccharides. Finally, PBS + 40% glycerol was used as a blank.

100 pl of all the antigens and controls were spotted randomly in at least four replicates on ultra-thin nitrocellulose-coated glass slides (Maine Manufacturing) through an ink-jet spotter (Arrayjet) in a cabinet at 18° C and 40% humidity. Slides were conserved in the dark at 4° C until use.

Prior to testing mice sera, slides were saturated with a blocking buffer (BlockIt, Arraylt) for 1 hour at room temperature in the dark in a humid chamber. Sera samples were diluted in blocking buffer and slides were incubated for 1 hour in the dark in a humid chamber. After performing three washes with PBS + 0.1% Tween-20, slides were incubated for another hour with a 1:800 dilution of Alexa Flour 647-conjugated anti-rabbit or anti-mouse IgG secondary antibody. Finally, after other three washes with PBS + 0.1% Tween-20, slides were rinsed in deionized water and let to dry.

Slides were scanned using an InnoScan 710 AL (INNOPSYS) and images were generated by the Mapix Microarray Image Analysis Software. Fluorescence intensities of spots were determined using ImaGene 9.0 software (Biodiscovery Inc.) and data analysis was performed using R scripts.

For each serum sample, the mean fluorescence intensity (MFI) of each replicate was determined by subtracting the average MFI of all the blanks to the mean of the MFI of the replicates for each antigen. A stringent cutoff was determined based on the average of MFIs obtained from pre-immune mouse sera, adding three times the standard deviation of the replicates. The obtained cutoff value was equal to 500: if a tested sera showed a MFI value below this threshold it was considered as negative and this value was substituted with 500; if the MFI value obtained was over 500, the value was considered positive and it was maintained.

III. 7 Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software version 8.0.0. The non-parametric Mann-Whitney U-test was used to assess significant differences between two groups, as for the evaluation of IgG titers against SpA_{mut} (Figure 7 and 13). The one-way ANOVA Kruskal-

Wallis and uncorrected Dunn's post test was used to assess significant differences between three or more groups, as in the *in vivo* results or in the internalization assay (Figure 10 B and 12). Finally, the Fisher's exact t test was used to assess significant differences between frequences, as in the dissemination severity index or the protein microarray results (Figure 11 B and 15).

A p value ≤ 0.05 was considered significative (legend: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001; ns: non significative, p > 0.05).

Area Under the Curve analysis

The Area Under the Curve (AUC) was used to perform a cumulative data analysis on the dermonecrotic lesion areas.

The trapezoidal area under the curve of the dermonecrotic lesion areas, for a single day, was calculated using the following formula (Figure 9):

$$AUC_{0-1} = \frac{(B_{t0} + B_{t1}) \text{ x } (t_0 - t_1)}{2}$$

Figure 9. **Formula used to calculate the AUC for a single point**. B: greater base; b: smaller base; t: timepoint.

The AUC was calculated for each time point after infection in the single animal and eventually summed together to have a final AUC value representing the whole time period after the infection, as depicted below (Figure 10):

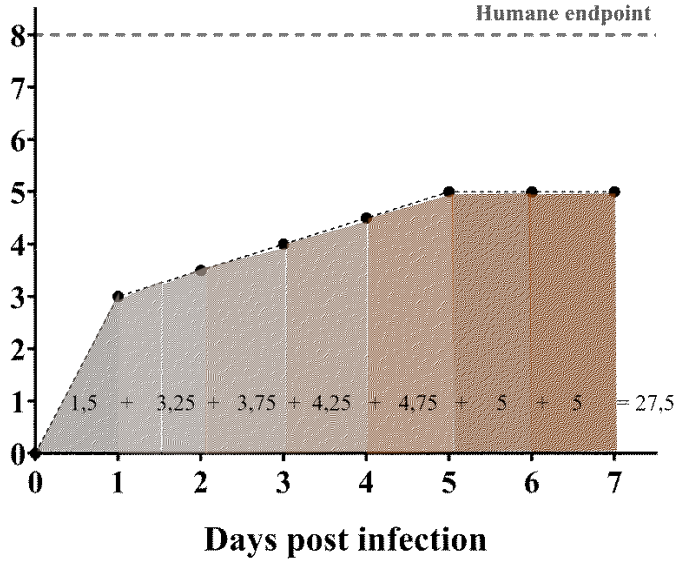


Figure 10. **Example of AUC calculation**. For each day post infection, the AUC of the single animal was calculated using the formula reported above and eventually the values are summed together.

IV. RESULTS

IV. 1 Set up of a mouse model of *S. aureus*-mediated skin infection and recurrences

The model of skin infection and recurrence was set up in 8-week-old female C57BL/6 mice. Mice were infected on the right flank (after shaving) with 4×10^7, 2×10^7 or 1×10^7 CFUs/50 µl of *S. aureus* USA300 lac strain. Mice were observed daily up to one week after infection and dermonecrotic skin lesions were photographed and measured at day 4, which represents the maximum peak of development of the lesion (Figure 11, panel A). To develop a model of skin recurrences, six weeks after the first skin infection (when animals have already completely cleared bacteria) mice were infected again subcutaneously, but on the opposite flank (after shaving) with 4×10^7 CFUs/50 µl regardless of the first infective dose. Mice were observed daily up to one week after the infection, pictures of the dermonecrotic skin lesions were photographed and measured at day 4 after the infection (Figure 11, panel B):

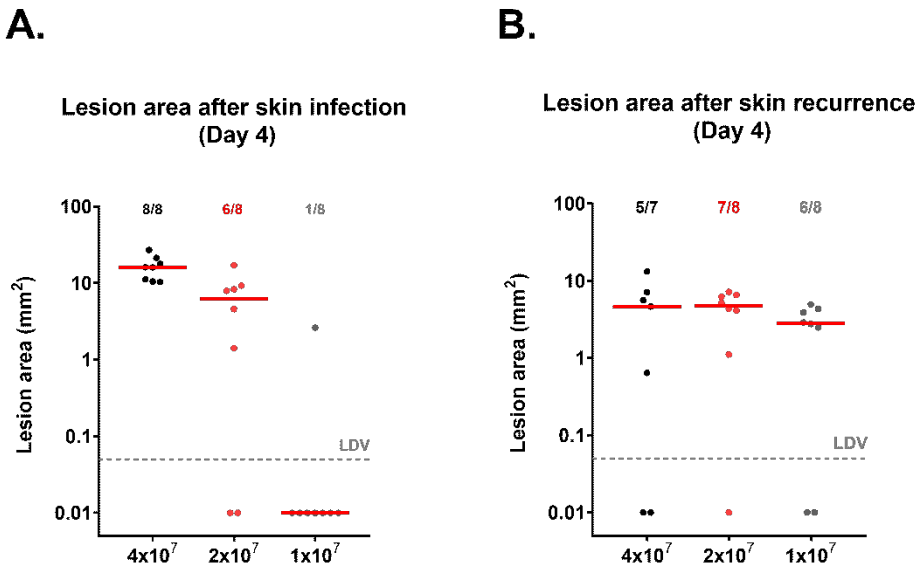


Figure 11. **Analysis of areas of dermonecrotic lesions developed by mice at day 4 after skin infection with different doses of *S. aureus* USA300 lac strain (A) and after skin recurrence with 4×10^7 CFUs/mouse (B)**. Lesion area data are represented in a logarithm base 10 ($\log_{10}$) scale. Data are represented as the median (red line) of the single value for a single mouse, represented as single dots. Above each group, the frequence of mice that develop a dermonecrotic skin lesion after skin infection is represented, respect to the total number of mice. The lower detectable value (LDV) is reported as a grey dotted line: each negative value is represented below the LDV.

After skin infection, all the mice that received the highest dose (4×10^7 CFUs/50 µl) developed dermonecrotic skin lesions, with a median value of 16.06 mm^2. Six out of eight (6/8) mice that received the medium dose (2×10^7 CFU/50 µl) developed a measurable dermonecrotic lesion, with a median value of 6.27 mm^2. Finally, only one mouse out of eight (1/8) that received the lowest dose (1×10^7 CFU/50 µl) developed a measurable dermonecrotic skin lesion (Figure 11, panel A).
After the second infection aimed to mimic skin recurrences, five out of seven (5/7) mice that had received the highest dose during the first infection developed dermonecrotic skin lesions with a median value on day 4 post infection of 4.65 mm^2. Seven out of eight (7/8) mice infected with the medium dose during the first infection also developed a skin lesion (median value of 4.75 mm^2), while the frequency of mice treated the first time with the lowest dose of bacteria that developed dermonecrosis after the second infection was six out of eight (6/8), with a median value of 2.82 mm^2 (Figure 11, panel B).

Along with the development of dermonecrotic lesions, also the disease severity was assessed, in order to select the best combination of infective doses to set up the infection model, making sure to choose a combination that allowed the development of lesions after both the first infection and the skin recurrence, but without causing a severe disease with the need to euthanize the animals before having reached the pre-defined endpoint (Figure 12):

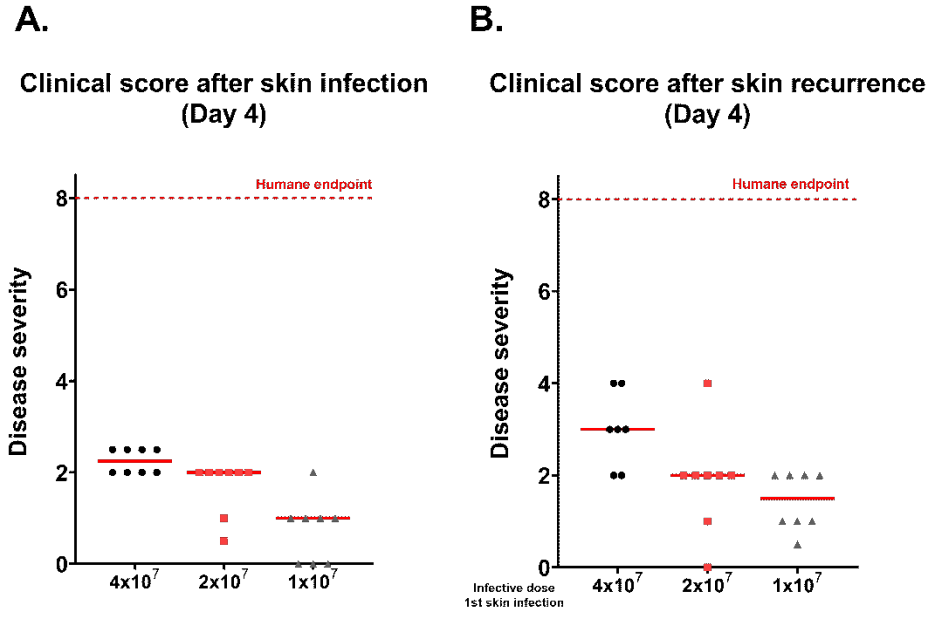


Figure 12. Clinical score evaluation at day 4 after first skin infection (panel A) or after skin recurrence (panel B). Data are represented as the sum of the two clinical score (see materials and methods, section III.3). Data are represented as the median (red line) of single values (single dots).

In accordance with the development of lesions and their area, after the first skin infection mice that received the highest dose (4×10^7 CFUs/50 µl) and the medium dose (2×10^7 CFU/50 µl) had similar

clinical scores (median value of 2.3 and 2.0, respectively), while mice that received the lowest dose (1×10^7 CFU/50 µl) had the lowest clinical scores (median value of 1.0). After the skin recurrence, mice that received the highest dose as first infection showed the highest clinical scores (median value of 3.0), while mice that received the medium dose as first infection showed lower clinical scores (median value of 2.0); finally, mice that received the lowest dose as first infection had the lowest clinical scores (median value of 1.5).

For these reasons, the best combination to be selected should be the one which ensured the highest frequency of mice which developed dermonecrotic lesion after the first and the second infection, but which cause the least severe disease as possible. Therefore, the combination 2×10^7 CFU/50µl and 4×10^7 CFU/50µl was chosen for further analysis.

IV. 2 Vaccine efficacy studies in a mouse model of skin recurrence

Evaluation of SpA_{mut}/AS01 immunogenicity

Once the skin recurrences infection model in mice was setup, animals have been immunized with our candidate in order to evaluate the vaccine efficacy in this preclinical model of *S. aureus* infection.

5-week-old female C57BL/6 mice were immunized twice twenty-eight days apart intramuscularly as described in material and methods. Before infection, sera were collected to determine the presence of anti-SpA_{mut} specific antibodies. As reported in Figure 13, mice mounted a strong immune response against SpA_{mut}, indicating that we could proceed with the challenge.

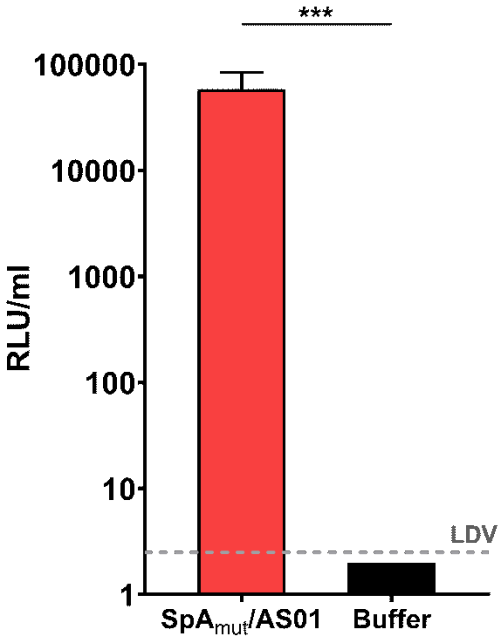


Figure 13. **Evaluation of SpA_{mut}/AS01 immunogenicity via Luminex analysis.** Anti-SpA_{mut} antibodies were titrated in mouse sera collected after the second dose of immunization. Bars represent the median value of Relative Light Unit/ml (RLU/ml) of SpA_{mut}-specific IgG titers, calculated by interpolating the median fluorescence intensities (MFI)

of tested sera with a mouse standard curve, with interquartile range as error (log_{10} scale). LDV: lower detectable value, grey dotted line. Statistical analysis: Mann-Whitney U test. ***: p value = 0.0002.

Determination of protective efficacy of SpA_{mut} against skin infections

To evaluate the protective effect of SpA_{mut} against skin infection, mice were infected on the right flank (after shaving) with 2×10^7 CFU/50 µl of *S. aureus* USA300 lac strain. Mice were observed daily and dermonecrotic skin lesions were photographed and measured up to seven days after infection. Two weeks after infection a subgroup of mice was sacrificed, skin biopsies were collected and bacterial burden evaluated (Figure 14 A, B). To determine the systemic dissemination of bacteria after infection, mice were weighted daily up to 7 days after infection and two weeks after infection kidneys were collected from a subgroup of mice and bacterial burden was evaluated (Figure 15 A,B):

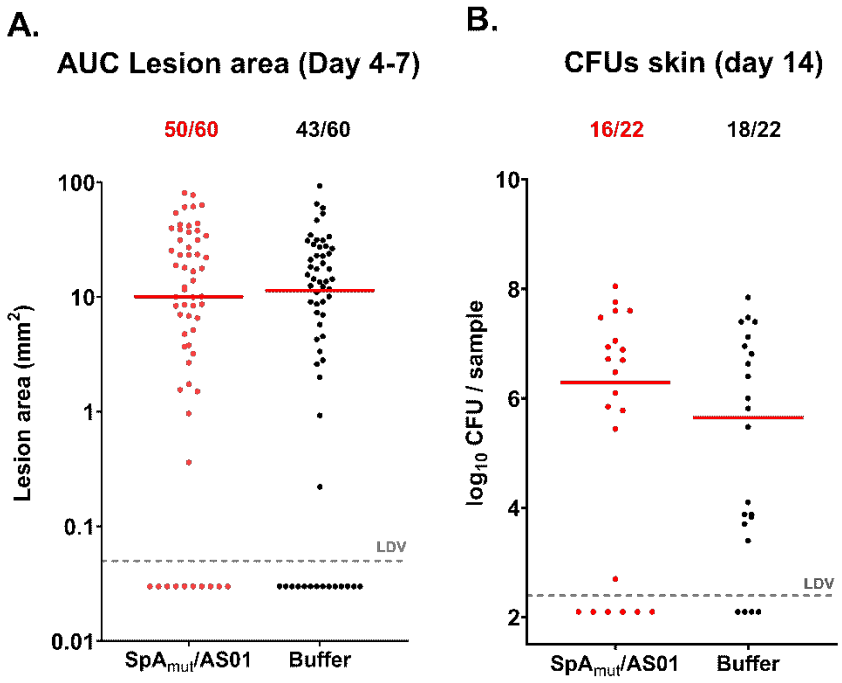


Figure 14. **Local evaluation of protective effect of immunization with SpA_{mut}/AS01 against skin infection**. **A)** AUC of dermonecrotic lesion areas in immunized and non-immunized animals, from day 4 to day 7 after skin infection (log_{10} scale). Data are represented as the median (red line) of single values (single dots). Above the single values, the frequence of mice that developed a dermonecrotic lesion is reported, over the total number of mice. The LDV is reported as a grey dotted line: each negative value is represented below the LDV. **B)** Log_{10} of CFUs evaluated in skin biopsies collected two weeks after skin infection in immunized and non-immunized animals. Data are represented as median (red line) of single values, represented as single dots. LDV: lower detectable value, grey dotted line.

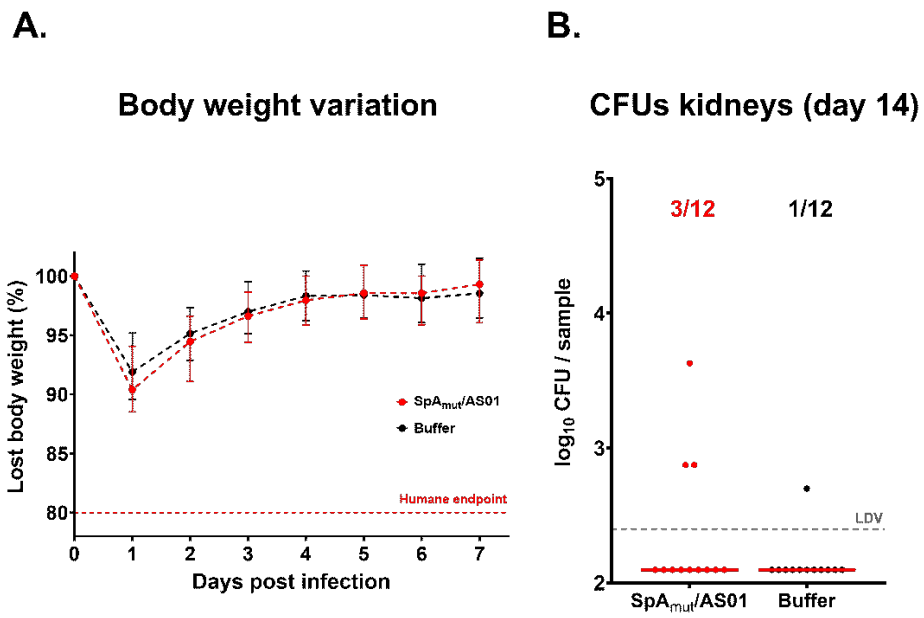


Figure 15. Systemic evaluation of protective effect of immunization with SpA$_{mut}$/AS01 after skin infection. **A)** Percentage of lost body weight in immunized and non-immunized animals up to 7 days after infection, respect to the initial weight of each animal. Data are represented as the median of single values per group and interquartile range as error. The red dotted line represents the humane endpoint (loss of 20% of the initial body weight). **B)** Log_{10} of CFUs evaluated in kidneys collected two weeks after skin infection in immunized and non-immunized animals. Data are represented as median (red line) of single values (single dots). Above each group the frequences of mice that showed bacteria in the kidneys is reported, respect to the total number of mice. LDV: lower detectable value, grey dotted line.

After the skin infection, no significative differences were observed between immunized and non-immunized mice, both in terms of development of dermonecrotic skin lesions or quantity of bacteria recovered in skin biopsies. Regarding the systemic dissemination of bacteria, no significative differences were observed between immunized and non-immunized animals. Very few bacteria were recovered in the kidneys of the mice, this could be due to the low dose used for the skin infection.

Immunization with SpA$_{mut}$/AS01 protects against systemic complications of skin recurrences

Six weeks after the first infection, mice were re-infected in the left flank (prior to shaving) with 4×10^7 CFU/50 µl of *S. aureus* USA300 lac strain. Mice were observed for one week after the skin recurrence, dermonecrotic skin lesions were photographed daily and measured (Figure 16, A). One week after the skin recurrence mice were sacrificed and bacterial burden was evaluated in skin biopsies (Figure 16, B). For evaluating the systemic complications of skin recurrences, mice were weighted daily up to seven days after infection and one week after infection the bacterial burden was evaluated in kidneys. Since after skin recurrence few bacteria were usually recovered from the

kidneys, a dissemination index was assigned to each animal on the basis of the number of colonies recovered in the whole organ (Figure 17 A, B).

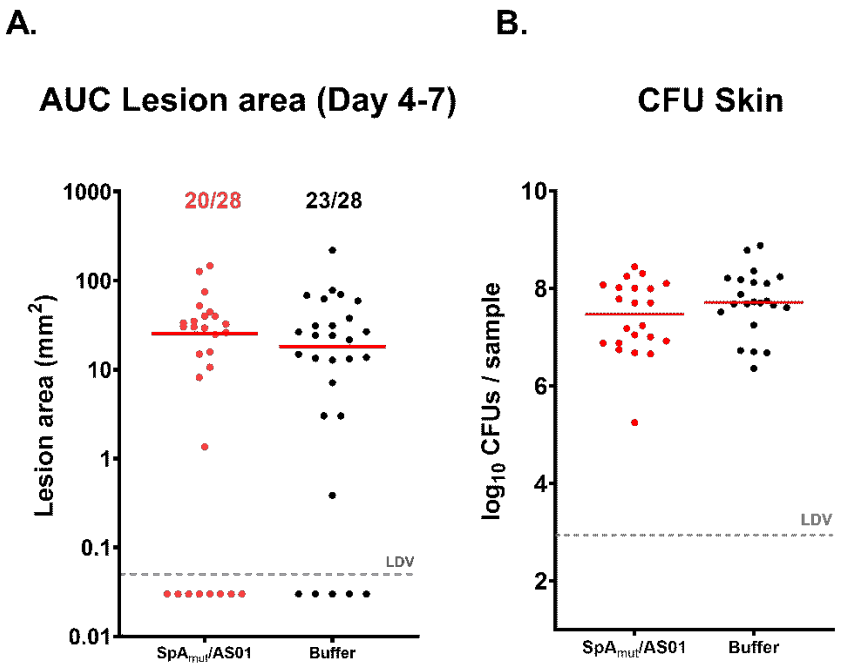


Figure 16. **Local evaluation of protection mediated by SpA_{mut}/AS01 immunization against skin recurrence.** **A**) AUC of dermonecrotic lesion area in immunized and non-immunized animals (log_{10} scale). Data are represented as the median (red line) of single values (single dots). Above each group, the frequence of mice that developed a dermonecrotic lesion, respect to the total number of mice, is reported. **B**) Log_{10} of CFUs evaluated in skin biopsies one week after infection. Data are represented as median of single values (red line). LDV: lower detectable value, grey dotted line. Statistical analysis: one-way ANOVA Kruskal-Wallis, uncorrected Dunn's post test.

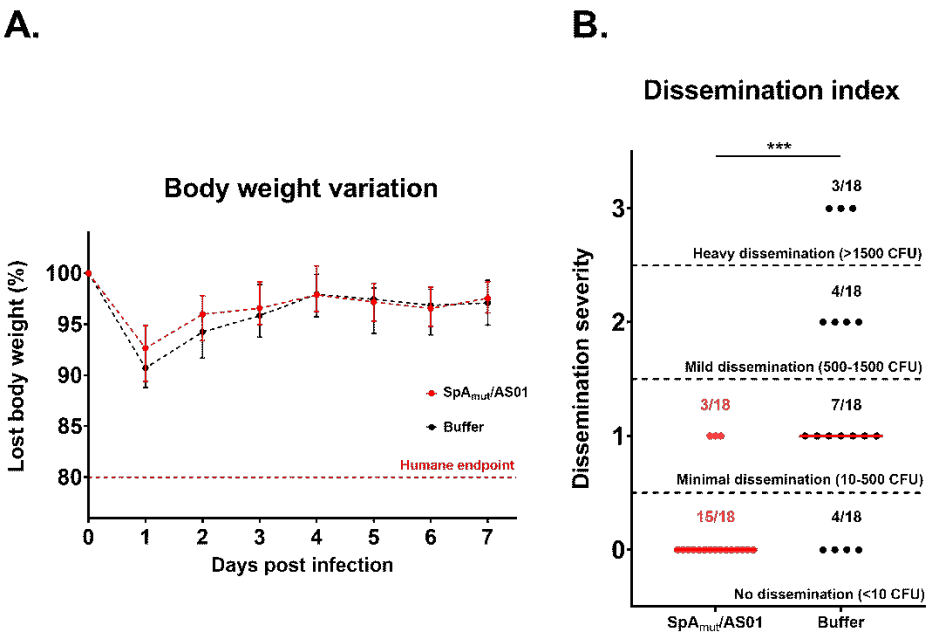


Figure 17. **Protective effect of immunization with SpA_{mut}/AS01 against systemic complication of skin recurrences. A)** Percentage of lost body weight in immunized and non-immunized animals up to 7 days after infection, respect to the initial weight of each animal. Data are represented as the median of single values per group and interquartile range as error. The humane endpoint is represented as a red dotted line. **B**) Dissemination severity index, based on bacterial burden in kidneys of immunized and non-immunized mice and assigned to each animal one week after skin recurrence. Each threshold defines a dissemination severity index; data are represented as median (red line) of single values (single dots). Above each group, the frequence of mice that developed a dermonecrotic lesion, respect to the total number of mice, is reported. Statistical analysis: Fisher's exact t test. ***: p value = 0.0006.

After the skin recurrence no significative differences were observed between immunized and non-immunized animals neither for the development of dermonecrotic skin lesions nor for the number of bacteria recovered in skin biopsies. Also, for the body weight variation, no significative differences were observed between immunized and control animals. Interestingly, almost none of the immunized and infected animals presented bacteria in the kidneys (15 out of 18 mice), and the three animals which presented bacteria in these organs were only minimally invaded (Figure 17, B). On the other hand, not immunized mice were more heavily infected both in term of frequency (14 out of 18) and in term of magnitude (7 out of 18 mildly invaded or higher). This difference was statistically significant indicating that vaccination with SpA_{mut}/AS01 helped the immune system to mount a stronger and more effective immune response. To try to better understand why there were no differences in the local conditions but a large difference in systemic outcome when animals were vaccinated before the first infection, we focused our attention in the humoral response of mice after immunization and infection, to propose a possible mechanism of protection.

IV. 3 Immunization with SpA_{mut}/AS01 enhances bacterial phagocytosis

The THP-1-mediated internalization assay was performed as described in materials and methods (section III.5). For the assay the following serum samples were tested: i) sera obtained from normal mice (pre-immune), as control; ii) sera obtained 20 days after the second dose of immunization with SpA_{mut}/AS01 (post II); iii) sera obtained two weeks after skin infection from non-immunized mice (infection 2 W); iv) sera obtained two weeks after skin infection from immunized mice (SpA_{mut}/AS01 infection 2W). Results were normalized on the median signal given by pre-immune sera, as percentage of THP-1 singlet cells that internalized *S. aureus* USA300 GFP (Figure 18):

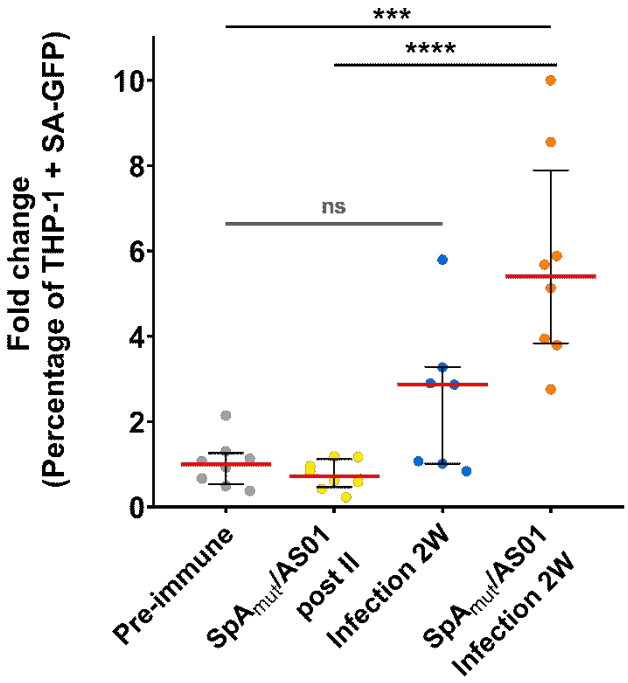


Figure 18. *S. aureus* internalization by THP-1 cells is mediated by sera obtained from immunized and infected mice. Data are represented as the fold change of percentage of THP-1 cells that resulted positive to FITC-A signal, respect to the median value of pre-immune sera. Data are represented as median (red line) of single values (single dots), with interquartile range as error. Statistical analysis: one-way ANOVA Kruskal-Wallis, uncorrected Dunn's post test. ns: not significant; ****: p value <0.0001; ***: p value = 0.0007.

Sera obtained after the second dose of immunization (SpA_{mut}/AS01 post II, yellow dots) had a median value of fold change equal to 0.7-fold (0.47-fold as 25% percentile, 1.1-fold as 75% percentile) respect to the pre-immune sera. Sera obtained after infection only (Infection 2W, blue dots) had a median value equal to 3-fold (1-fold as 25% percentile, 3.3-fold as 75% percentile), respect to the pre-immune sera. Finally, the sera obtained after immunization and infection (SpA_{mut}/AS01 Infection 2W, orange dots) had a median value equal to 5-fold (3.8-fold as 25% percentile, 7.9-fold as 75% percentile) respect to the pre-immune sera.

Sera from immunized and infected mice showed the highest fold-change of percentage of THP-1 cells that effectively internalized GFP-expressing *S. aureus* respect to the pre-immune group ($p = 0.0007$, ***) or to sera obtained after immunization with SpA_{mut}/AS01 ($p < 0.0001$, ****). Interestingly, even if an increase in the phagocytosis was observed in sera obtained after only infection, this increase was not significant respect to pre-immune sera.

IV. 4 Analysis of anti-SpA_{mut} antibodies in mouse sera

To better understand the potential impact of infection on SpA_{mut}/AS01 vaccinated or not vaccinated mice on SpA_{mut} IgG titers, sera from animals immunized and infected or only infected were analyzed

at Luminex assay. Due to the sticky features of SpA protein, analysis have been performed against the same mutated version of the antigen used to vaccinate animals which is unable to bind IgG through both the Fc and the Fab (V_H3 family) portions. Vaccination with the *S. aureus* antigen induced a strong immune response as reported above (Figure 13) but, interestingly, this response was not boosted by infection (Figure 19). Notably, infection seemed not to induce any IgG response against the antigen we used for the assay that, even if significantly mutated as compared to the wild type protein, still retained 92.5% of aminoacidic identity for the region in common (the mutated version lacks the C-terminal domain called X region) and its 2D structure is highly conserved [20].

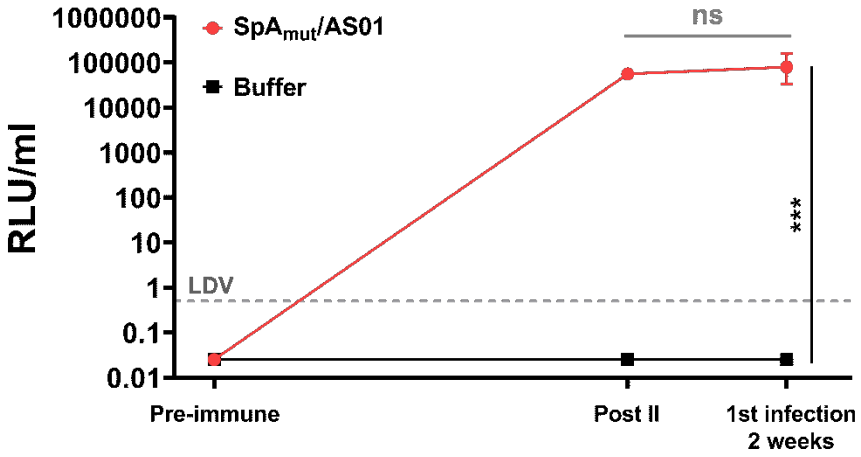


Figure 19. **Analysis of anti-SpA$_{mut}$ antibodies in mouse sera**. IgG titers were evaluated in sera collected from normal mice (Pre-immune), after the second dose of immunization (Post II) or two weeks after skin infection (1st infection 2 weeks), from immunized mice (red line) or non-immunized mice (black line). Data are represented as the median of single values, with interquartile range as error. LDV: lower detectable value, grey dotted line; ns: not significant. Statistical analysis: one-way ANOVA Kruskal-Wallis, uncorrected Dunn's post test. ns: not significant; ***: p value = 0.0002.

In order to further analyze the antibody response to SpA$_{mut}$ from a more qualitative point of view, IgG subclasses were also analyzed along with IgG avidity for the antigen. In Table 3, proportions among the different IgG subclasses after immunization and infection were reported. We did not observe any difference in ratios at least as high as a two-fold change and therefore concluded that infection did not cause a major switch in IgG subclasses as compared to vaccination alone. Negative control samples were not analyzed since no IgG titers were measured against SpA$_{mut}$.

	Ratio		
Group	**IgG1/IgG2a**	**IgG1/IgG3**	**IgG2a/IgG3**
Post II	0.59	0.81	1.36
1st infection 2 weeks	0.75	1.34	1.78

Table 3. SpA_{mut}-specific IgG subclasses composition analysis after immunization and infection. Data are represented as the ratio between the observed titers of the different subclasses after immunization with SpA_{mut}/AS01 (Post II) and two weeks after the first skin infection (1^{st} infection 2 weeks).

On the other hand, subcute infection with *S. aureus* strongly increased IgG avidity for the antigen of interest, underlying that infection boosted the humoral response induced by vaccination (Figure 20):

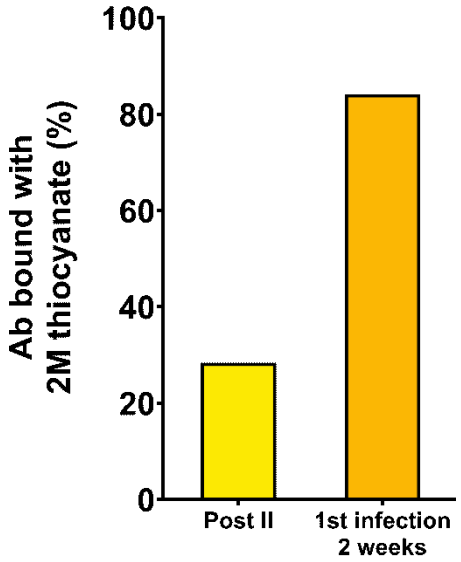


Figure 20. **Analysis of SpA_{mut}-IgG avidity**. Sera collected after second dose of immunization (Post II) and after infection (1st infection 2 weeks) were tested in combination with 2M ammonium thiocyanate or PBS, to evaluate the avidity of the antibody to its specific antigen. Bars represents the median percentage of avidity, calculated on the ratio of observed titers with ammonium thiocyanate respect to titers observed with PBS.

IV. 5 Role of SpA_{mut} in unmasking *S. aureus* antigens

In order to assess possible differences in the humoral response between immunized and non-immunized mice after infection, sera obtained two weeks after skin infection from both groups were tested on a staphylococcal protein microarray, which was designed and validated as described in the materials and methods (section III.6). Eventually, 96 different staphylococcal antigens were spotted onto the protein microarray, reported in the table 4 below:

Antigens name			
Aap (53-608)-His	HlgC-His	Sta002(19-187)-His	Sta056-His
Baa(petTEV)-His	IsdA-His	Sta003-His	Sta057C-His
ClfA-His	IsdA(40-184)-His	Sta006-His	Sta059-His
ClfB-His	IsdB-His	Sta006-Cys+	Sta060-His
ClfB(45-552)-His	IsdI-His	Sta006-Cys-	Sta065-His
ClfB-N3-His	LysM069A-His	Sta006F1-His	Sta066-His
ClfB-N12/2-His	LysMSpa-His	Sta006F3-His	Sta069-His
ClfB-N23-His	LukE-His	Sta011-His	Sta070-His
ClfB-N3-SdrD-N3-His	LukF-His	Sta011-Cys+	Sta072-His
CoA-His	NM403-His	Sta011-Cys-	Sta073 N-term-His
CP8-tt	NM405-His	Sta013-His	Sta082-His
CP5-tt	NM406-His	Sta014-His	Sta083-His
Eap-GST	NM407-His	Sta015-His	Sta088-His
EbpS(1-189)-His	NM408-His	Sta017-His	Sta097-His
EfbP-His	NM409-His	Sta018-His	Sta098-His
FnBA-His	NM411-His	Sta019-His	Sta099-His
FnbpAN2N3(194-511)-His	Nuc-His	Sta021-His	Sta100-His
GST-STOP(pgex)	NW_1-His	Sta022-GST	Sta101-His
GST-Sbi(41-253)-His	NW_2-His	Sta024-His	Sta102-His
Hla D151L2-His	NW_8-His	Sta026-His	Sta105-His
Hla H35L-His	NW_6-GST	Sta027-His	Sta107-His
Hla H35LBL21	NW_SdrE(53-632)-His	Sta028-His	Sta108-His
Hla H35LT7Express	SasD-His	Sta029-His	Sta111-His
Hla N49A-His	SdrC-His	Sta030-His	Sta112-His
Hla N49W-His	SdrC(51-518)-His	Sta031-His	Sta113-His
Hla PSGS-His	SdrD(53-592)-His	Sta037-His	Sta115-His
Hla PSGS2	SdrD-N3-His	Sta038-His	Sta116-His
Hla PSGS3-His	SpaDEABC-His	Sta042-His	Sta118-His
Hla Q97L-His	Spa-wt	Sta048-His	Sta121-His
Hla T22L-His		Sta049-His	Sta122-His
HlA Y101L-His		Sta051-His	Sta123-His
		Sta052-His	tt

Table 4. List of the 96 different staphylococcal antigens spotted onto the microarray slides. A total of 124 staphylococcal antigens were spotted onto the microarray slides, but some of those antigens were mutated or truncated forms of the same proteins, which are grouped in an orange box in the table.

Single sera (n = 9 per group) obtained from immunized and infected mice or infected-only mice were assessed against 96 different staphylococcal antigens. An antigen was defined as recognized by the sera from a group when at least 5 out of the 9 sera tested recognized that antigen as described in material and methods. In the following table (Table 5) antigens that reacted with sera were reported.

Antigen	SpA_{mut}/AS01		Buffer	
	+	-	+	-
Aap (53-608)-His	7	2	1	8
ClfA-His	8	1	4	5
ClfB-His	9	0	7	2
EfbP-His	7	2	5	4
FnBA-His	9	0	7	2
FnbpAN2N3(194-511)-His	6	3	2	7
HlaH35L-His	9	0	3	6
HlgC-His	7	2	2	7
IsdA-His	6	3	2	7
IsdB-His	7	2	1	8
LysM069A-His	7	2	0	9
NM403-His	8	1	3	6
NM407-His	5	4	0	9
NM408-His	8	1	4	5
NW_8-His	6	3	4	5
NW_SdrE (53-632)-His	9	0	9	0
SdrC-His	5	4	2	7
SdrD-N3-His	9	0	8	1
SpA_{mut}-His	9	0	2	7
Sta002 (19-187)-His	8	1	3	6
Sta003-His	9	0	3	6
Sta006F1-His	5	4	0	9
Sta006-His	9	0	9	0
Sta011 Cys-	8	1	0	9
Sta011 Cys+	9	0	9	0
Sta011-His	9	0	9	0
Sta013-His	9	0	0	9
Sta014-His	9	0	1	8
Sta015-His	9	0	0	9
Sta017-His	9	0	3	6
Sta018-His	5	4	0	9
Sta019-His	9	0	1	8
Sta021-His	8	1	4	5
Sta022 GST	9	0	9	0
Sta024-His	9	0	8	1
Sta026-His	9	0	9	0
Sta027-His	9	0	2	7
Sta029-His	9	0	0	9
Sta031-His	9	0	8	1
Sta038-His	9	0	8	1
Sta042-His	6	3	2	7
Sta048-His	9	0	8	1
Sta052-His	7	2	2	7
Sta056-His	7	2	0	9
Sta059-His	5	4	1	8
Sta060His	9	0	3	6
Sta082-His	5	4	5	4
Sta083-His	6	3	6	3
Sta097-His	8	1	4	5
Sta098-His	8	1	6	3
Sta099-His	5	4	4	5
Sta107-His	8	1	6	3
Sta108-His	9	0	9	0
Sta112-His	9	0	9	0
Sta121-His	7	2	1	8
Sta122-His	6	3	0	9
Sta123-His	8	1	6	3

Table 5. List of the 57 staphylococcal antigens recognized by at least five out of nine single sera obtained from immunized (SpA_{mut}/AS01) or non-immunized (Buffer) mice, obtained two weeks after skin infection.

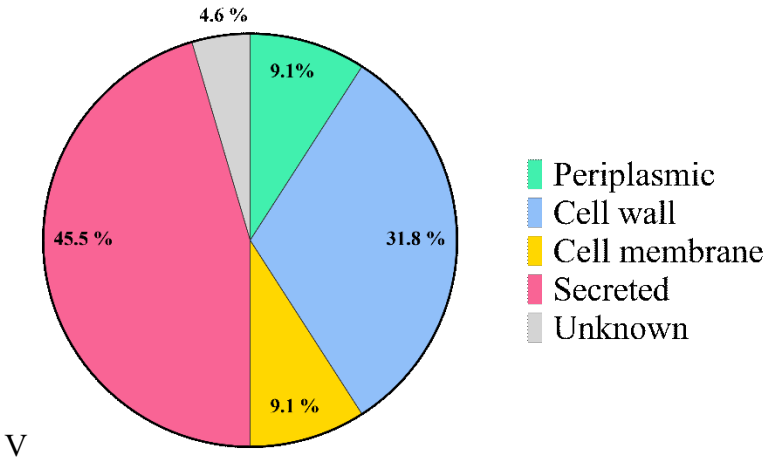
Furthermore, out of the 96 antigens spotted onto the microarray, 22 antigens reacted with higher frequence with sera obtained from immunized and infected mice, respect to sera obtained from infected-only mice (Figure 21):

N. of sera	Aap (53-608)-His		Hla H35L-His		Isdb-His		LysM069A-His		NM403-His		NM407-His		SpADEABC-His		Sta002 (19-187)-His		Sta003-His		Sta006F1-His		Sta011 Cys-	
1																						
2																						
3																						
4																						
5																						
6																						
7																						
8																						
9																						
p val	0.0152 (*)		0.0090 (**)		0.0152 (*)		0.0023 (**)		0.0498 (*)		0.0294 (*)		0.0023 (**)		0.0498 (*)		0.0090 (**)		0.0294 (*)		0.0004 (***)	

N. of sera	Sta014-His		Sta015-His		Sta017-His		Sta018-His		Sta019-His		Sta027-His		Sta029-His		Sta056-His		Sta060-His		Sta121-His		Sta122-His	
1																						
2																						
3																						
4																						
5																						
6																						
7																						
8																						
9																						
p val	0.0004 (***)		<0.0001 (****)		0.0090 (**)		0.0294 (*)		0.0004 (***)		0.0023 (**)		<0.0001 (****)		0.0023 (**)		0.0090 (**)		0.0152 (*)		0.0090 (**)	

Figure 21. **List of staphylococcal antigens that are more reactive with sera from immunized and infected animals (SpA_{mut}/AS01, orange box) respect to sera from infected only mice (Buffer, blue box)**. Each antigen was tested with 9 single sera per group, each colored box represents a reactive sera. Significative differences were evaluated through the Fisher's exact t test.

The cellular localizations of these 22 antigens were analyzed and are summarized in the pie chart below (Figure 22):



V

Figure 22. **Pie chart representing the cellular localization of the 22 previously reported antigens**. Each staphylococcal antigen was searched in protein data banks, in which also the cellular localization is reported.

Interestingly, almost half of the selected antigens (10 out of 22, 45.5%) are secreted antigens, 7 out of 22 (31.8%) are cell-wall anchored antigens and both cell membrane and periplasmic associated antigens are 2 out of 22 (9.1%). Finally, cellular localization of only one of these antigens is unknown, since its sequence was not annotated in the protein data banks.

V. DISCUSSION

S. aureus is the main cause of Skin and Soft Tissue Infections (SSTIs) which often are associated with the development of systemic complications and recurrent infections. *S. aureus* carries a wide variety of virulence factors and among these, the Staphylococcal protein A (SpA) is a main immunomodulator antigen that allows the bacterial survival, impairing bacterial killing by sequestering circulating antibodies and causing B cell apoptosis acting as a superantigen [25]. In previous work, the protective effect of SpA was evaluated in a systemic model of infection in guinea pigs. An interesting "unmasking" effect was observed since animals immunized before infection developed a broader antibody repertoire respect to animals only infected [41]. The aim of the present work was to evaluate the possible "hidden effect" of the SpA as a vaccine candidate against *S. aureus*-mediated skin recurrences and to propose possible mechanisms of protection with a special focus on antibody response and unmasking effect on *S. aureus* antigens expressed during infection. In the context of designing a vaccine against *S. aureus*, selecting an immunomodulator antigen as SpA could represent an innovative way of thinking giving the immune system the possibility of better recognizing the pathogen elicit a broader and, possibly, more efficient immune response. In particular, since SpA is able to impair the adaptive immune response by sequestering circulating antibodies and blocking B cell functions, a vaccine containing an inactivated form of this antigen could help the immune system to clear faster bacteria and to induce a more efficient and mature adaptive immune response. Whenever a SSTIs is established by *S. aureus,* bacteria proliferate in the skin and deeper tissues, causing disease and inflammation. Due to the inflammatory status, the blood vessels undergo a vasodilation and the endothelium cells lost their tight formation. Thank to this, *S. aureus* is able to enter the blood vessels, spread into the blood circulation and disseminate to distant organs as the kidneys, that were used in our studies as indicator of systemic dissemination of *S. aureus*, as indicated in the image below (Figure 23):

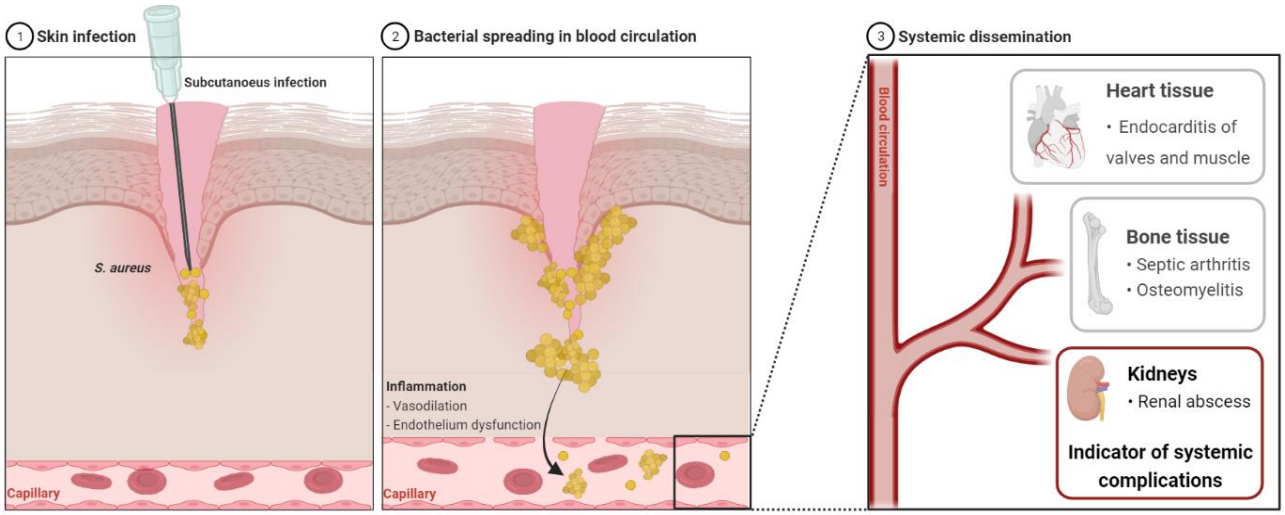


Figure 23. Diagram showing the normal spreading in the blood circulation of *S. aureus* after a skin infection. *S. aureus* after establishing a skin infection is able to exploit the vasodilation due to the inflammatory status and spreading in the blood circulation. From there, it can disseminate to distant organs causing systemic complications, that are often associated with SSTIs. The dissemination in the kidneys in our studies was used as an indication of bacterial spreading to the blood circulation after skin infection and recurrence (created with BioRender.com).

From our in *vivo* efficacy studies, it was observed that even if immunization with SpA_{mut}/AS01 did not protect mice from skin disease neither after the first nor after the second infection (Figure 14 and 16), it actually was able to strongly prevent bacteria dissemination during skin recurrences (Figure 17 panel B). Interestingly, this was not the case during the first infection, meaning that the protection observed is not directly dependent on vaccination but SpA_{mut}/AS01 vaccine helps the immune system to better perform its work (Figure 15 panel B). This is perfectly in line with data obtained in the *in vitro* internalization assay, a surrogate of *in vivo* opsonophagocytic activity, and broader IgG repertoire and increased avidity of IgG against SpA could at least partially explain this effect. Indeed, a possible mechanism of protection that could explain the *in vivo* data is represented by an enhanced bacterial phagocytosis. It was observed that sera from immunized and infected animals showed the highest internalization of bacteria by human monocytic cells as compared to all the other groups in analysis, different that was statistically increased when compared with both sera from pre-immune and only vaccinated mice (Figure 18). Interestingly, mice only infected with *S. aureus* did not developed antibodies against the inactivated form of SpA (Figure 19). Natural infection could preferentially induce antibodies against the X region that is not present in SpA_{mut} and/or against the Fc and F_{ab} binding sites, but it is certainly noteworthy that there are almost no antibodies against a protein that retains two thirds of the total length of the native antigen and 92.5% of its sequence. On the other hand, infection boosted the immune response against the antigen used to vaccinate the mice significantly increasing the avidity of antibodies for SpA_{mut} (Figure 20). This maturation of response

is mainly attributable to the exposure to *S. aureus* and not to the natural maturation of the germinal centers, as the increase of affinity was observed between the immunization and two weeks after infection, in less than 16 days. This improvement in the affinity of the SpA_{mut}-antibodies could be, at least in part, responsible for the mediation of the increased bacterial internalization, along with the development of a different antibody repertoire due to the unmasking of the pathogen. In fact, immunized and infected animals recognized more staphylococcal antigens as compared to infected-only animals (Figure 21). Among these 22 antigens recognized with higher frequence when tested with sera from immunized and infected animals, there are important virulence factor of *S. aureus*: i) a mutated version of the α-hemolysin (HlaH35L), an important toxin for *S. aureus* in the development of dermonecrotic lesions in SSTIs [54]; ii) an accumulation-protein (Aap), which is involved in biofilm formation of *Staphylococcus epidermidis* [55]; iii) iron-regulated surface determinant B (IsdB), which promotes adherence to endothelial cells and was previously selected as a vaccine candidate against *S. aureus* sepsis [56]. Overall, these data could be of great importance in the design of a vaccine against *S. aureus*, which should include the immunomodulator antigen SpA, among others: antibodies elicited after immunization can represent a defense line against a subsequent exposure to *S. aureus*, preventing its systemic dissemination and protecting against one of the most common and life-threating sequelae of *S. aureus* infections.

VI. CONCLUSIONS

The aim of this work was to evaluate the protective effect of SpA against *S. aureus*-mediated skin recurrences, with a special focus on antibody response and the unmasking of the pathogen to the immune system. A mouse model of skin recurrences was set up in C57BL/6 female mice and employed to evaluate the immunogenicity of a nontoxigenic SpA adjuvanted with AS01 (SpA_{mut}/AS01), which was able to elicit high titers of antibodies after immunization. The results reported in this thesis have shown that immunization with SpA_{mut}/AS01 before *S. aureus* skin infection in mice: i) allows the unmasking of several staphylococcal antigens (n=22, out of 96), which reacted with higher frequence with sera from immunized and infected animals when tested on the protein microarray slides; ii) increases the quality of SpA_{mut}-specific antibodies, in terms of affinity, after infection with *S. aureus*, rather than the total antibody titers observed after infection; iii) increases the bacterial internalization by human phagocytic cells of *S. aureus*, especially after infection. These results could explain, at least partially, the ability of immunized mice to limit bacterial dissemination in blood circulation during skin recurrence: immunization with SpA_{mut}/AS01 did in fact unmasked the pathogen to the host immune system eliciting a broader antibody repertoire mainly directed to extracellular staphylococcal antigens that, combined with the improvement of the quality of SpA_{mut}-specific antibodies could mediate the enhanced phagocytosis of *S. aureus* in the blood circulation, limiting the bacterial dissemination from the primary site of infection to distant organs. These results can significantly help in advancing the development of a vaccine against *S. aureus* which should include the immunomodulator antigen SpA, giving the possibility to the immune system to recognize the pathogen and elicit an effective and protective response against systemic dissemination of *S. aureus* during skin recurrences, one of the most common and threatening complications of *S. aureus*-mediated SSTIs.

Transparency and Conflicts of Interest

This work was sponsored by GlaxoSmithKline Biologicals SA. Andrea Paola Mandelli is a PhD Student of the University of Siena and participates in a post graduate studentship program at GSK.

VII. REFERENCES

[1] A. Ogston, "On abscesses," *Rev. Infect. Dis.*, vol. 6, no. 1, p. 122—128, 1984, [Online]. Available: http://europepmc.org/abstract/MED/6369479.

[2] K. Zhang, H. Zhang, C. Gao, R. Chen, and C. Li, "Antimicrobial Mechanism of pBD2 against Staphylococcus aureus," *Molecules*, vol. 25, no. 3513, 2020.

[3] A. Młynarczyk, G. Młynarczyk, and J. Jeljaszewicz, "The Genome of Staphylococcus aureus: a review," *Zentralblatt für Bakteriol.*, vol. 287, pp. 277–314, 1998, doi: 10.1016/S0934-8840(98)80165-5.

[4] D. Keinhörster, S. E. George, C. Weidenmaier, and C. Wolz, "Function and regulation of Staphylococcus aureus wall teichoic acids and capsular polysaccharides," *Int. J. Med. Microbiol.*, vol. 309, no. 6, p. 151333, 2019, doi: 10.1016/j.ijmm.2019.151333.

[5] J. Kluytmans, A. van Belkum, and H. Verbrugh, "Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks," *Clin. Microbiol. Rev.*, vol. 10, no. 3, pp. 505–520, 1997, doi: 10.1128/cmr.10.3.505.

[6] H. Wertheim *et al.*, "The role of nasal carriage in Staphylococcus aureus infections," *Lancet Infect. Dis.*, vol. 5, no. December, pp. 751–762, 2005, doi: 10.1016/S1473-3099(05)70295-4.

[7] M. A. Halablab, S. M. Hijazi, M. A. Fawzi, and G. F. Araj, "Staphylococcus aureus nasal carriage rate and associated risk factors in individuals in the community," *Epidemiol. Infect.*, vol. 138, no. 5, pp. 702–706, 2010, doi: 10.1017/S0950268809991233.

[8] J. Broughan, R. Anderson, and A. S. Anderson, "Strategies for and advances in the development of Staphylococcus aureus prophylactic vaccines," *Expert Rev. Vaccines*, vol. 10, no. 5, pp. 695–708, 2011, doi: 10.1586/erv.11.54.

[9] J. Bien, O. Sokolova, and P. Bozko, " Characterization of Virulence Factors of Staphylococcus aureus : Novel Function of Known Virulence Factors That Are Implicated in Activation of Airway Epithelial Proinflammatory Response ," *J. Pathog.*, vol. 2011, pp. 1–13, 2011, doi: 10.4061/2011/601905.

[10] K. O'Riordan and J. C. Lee, "Staphylococcus aureus Capsular Polysaccharides," *Clin. Microbiol. Rev.*, vol. 17, no. 1, pp. 218–234, 2004, doi: 10.1128/CMR.17.1.218-234.2004.

[11] J. C. Lee, "The prospects for developing a vaccine against Staphylococcus aureus," *Trends Microbiol.*, vol. 4, no. April, pp. 162–166, 1996.

[12] S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland, and V. G. Fowler, "Staphylococcus aureus Infections : Epidemiology , Pathophysiology , Clinical Manifestations , and Management," *Clin Microbiol Rev*, vol. 28, no. 3, pp. 603–661, 2015, doi: 10.1128/CMR.00134-14.

[13] R. Olaniyi, C. Pozzi, L. Grimaldi, and F. Bagnoli, "Staphylococcus aureus-Associated Skin and Soft Tissue Infections: Anatomical Localization, Epidemiology, Therapy and Potential Prophylaxis," in *Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis*, F. Bagnoli, R. Rappuoli, and G. Grandi, Eds. Cham: Springer International Publishing, 2017, pp. 199–227.

[14] L. F. McCaig, L. C. McDonald, S. Mandal, and D. B. Jernigan, "Staphylococcus aureus-associated skin and soft tissue infections in ambulatory care," *Emerg. Infect. Dis.*, vol. 12, no. 11, pp. 1715–1723, 2006, doi: 10.3201/eid1211.060190.

[15] A. L. Hersh, H. F. Chambers, J. H. Maselli, and R. Gonzales, "National trends in ambulatory visits and antibiotic prescribing for skin and soft-tissue infections," *Arch. Intern. Med.*, vol. 168, no. 14, pp. 1585–1591, 2008, doi: 10.1001/archinte.168.14.1585.

[16] G. T. Ray, J. A. Suaya, and R. Baxter, "Incidence, microbiology, and patient characteristics of skin and soft-tissue infections in a U.S. population: A retrospective population-based study," *BMC Infect. Dis.*, vol. 13, no. 1, pp. 1–11, 2013, doi: 10.1186/1471-2334-13-252.

[17] P. Tattevin *et al.*, "Concurrent Epidemics of Skin and Soft Tissue Infection and Bloodstream Infection Due to Staphylococcus aureus," *Clin. Infect. Dis.*, vol. 55, no. 6, pp. 781–788, 2012, doi: 10.1093/cid/cis527.

[18] S. Becker, M. B. Frankel, O. Schneewind, and D. Missiakas, "Release of protein A from the cell wall of Staphylococcus aureus," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 4, pp. 1574–1579, 2014, doi: 10.1073/pnas.1317181111.

[19] H. G. Lee, S. Kang, and J. S. Lee, "Binding characteristics of staphylococcal protein A and streptococcal protein G for fragment crystallizable portion of human immunoglobulin G," *Comput. Struct. Biotechnol. J.*, vol. 19, pp. 3372–3383, 2021, doi: 10.1016/j.csbj.2021.05.048.

[20] M. Shi, X. Chen, Y. Sun, H. K. Kim, O. Schneewind, and D. Missiakas, "A protein A based Staphylococcus aureus vaccine with improved safety," *Vaccine*, vol. 39, no. 29, pp. 3907–3915, 2021, doi: 10.1016/j.vaccine.2021.05.072.

[21] P. K. Peterson, J. Verhoef, L. D. Sabath, and P. G. Quie, "Effect of protein A on staphylococcal opsonization," *Infect. Immun.*, vol. 15, no. 3, pp. 760–764, 1977, doi: 10.1128/iai.15.3.760-764.1977.

[22] F. Falugi, H. K. Kim, D. M. Missiakas, and O. Schneewind, "Role of protein a in the evasion of host adaptive immune responses by Staphylococcus aureus," *MBio*, vol. 4, no. 5, 2013, doi: 10.1128/mBio.00575-13.

[23] A. I. Levinson, L. Kozlowski, Y. Zheng, and L. Wheatley, "B-Cell superantigens: Definition and potential impact on the immune response," *J. Clin. Immunol.*, vol. 15, no. 6 Supplement, pp. 26–36, 1995, doi: 10.1007/BF01540891.

[24] G. J. Silverman and C. S. Goodyear, “Confounding B-cell defences: Lessons from a staphylococcal superantigen,” *Nat. Rev. Immunol.*, vol. 6, no. 6, pp. 465–475, 2006, doi: 10.1038/nri1853.

[25] S. D. Kobayashi and F. R. DeLeo, “Staphylococcus aureus protein A promotes immune suppression,” *MBio*, vol. 4, no. 5, pp. 4–6, 2013, doi: 10.1128/mBio.00764-13.

[26] F. R. DeLeo, B. A. Diep, and M. Otto, “Host Defense and Pathogenesis in Staphylococcus aureus Infections,” *Infect. Dis. Clin. North Am.*, vol. 23, no. 1, pp. 17–34, 2009, doi: 10.1016/j.idc.2008.10.003.

[27] T. J. Foster, “Immune evasion by staphylococci,” *Nat. Rev. Microbiol.*, vol. 3, no. 12, pp. 948–958, 2005, doi: 10.1038/nrmicro1289.

[28] R. P. N. Mishra, E. Oviedo-orta, P. Prachi, R. Rappuoli, and F. Bagnoli, “Vaccines and antibiotic resistance,” *Curr. Opin. Microbiol.*, vol. 15, no. 5, pp. 596–602, 2012, doi: 10.1016/j.mib.2012.08.002.

[29] F. Bagnoli, S. Bertholet, and G. Grandi, “Inferring reasons for the failure of Staphylococcus aureus vaccines in clinical trials,” *Front. Cell. Infect. Microbiol.*, vol. 2, no. February, pp. 2–5, 2012, doi: 10.3389/fcimb.2012.00016.

[30] R. S. Daum and B. Spellberg, “Progress Toward a Staphylococcus aureus Vaccine,” *Vaccines*, vol. 54, no. 4, pp. 560–567, 2012, doi: 10.1093/cid/cir828.

[31] L. S. Miller, V. G. Fowler, S. K. Shukla, W. E. Rose, and R. A. Proctor, *Development of a vaccine against Staphylococcus aureus invasive infections: Evidence based on human immunity, genetics and bacterial evasion mechanisms*, vol. 44, no. 1. 2019.

[32] C. Pozzi, R. Olaniyi, L. Liljeroos, I. Galgani, R. Rappuoli, and F. Bagnoli, “Vaccines for Staphylococcus aureus and Target Populations,” in *Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis*, F. Bagnoli, R. Rappuoli, and G. Grandi, Eds. Cham: Springer International Publishing, 2017, pp. 491–528.

[33] H. Shinefield *et al.*, “Use of a Staphylococcus aureus conjugate vaccine in patients receiving hemodialysis,” *N. Engl. J. Med.*, vol. 346, no. 7, pp. 491–496, 2002.

[34] A. Fattom, A. Matalon, J. Buerkert, K. Taylor, S. Damaso, and D. Boutriau, “Efficacy profile of a bivalent Staphylococcus aureus glycoconjugated vaccine in adults on hemodialysis: Phase III randomized study,” *Hum. Vaccines Immunother.*, vol. 11, no. 3, pp. 632–641, 2015, doi: 10.4161/hv.34414.

[35] V. G. Fowler *et al.*, “Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: A randomized trial,” *JAMA - J. Am. Med. Assoc.*, vol. 309, no. 13, pp. 1368–1378, 2013, doi: 10.1001/jama.2013.3010.

[36] C. D. Harro *et al.*, “The immunogenicity and safety of different formulations of a novel Staphylococcus aureus vaccine (V710): Results of two Phase I studies,” *Vaccine*, vol. 30, no. 9, pp. 1729–1736, 2012, doi: 10.1016/j.vaccine.2011.12.045.

[37] C. Harro *et al.*, “Safety and immunogenicity of a novel Staphylococcus aureus vaccine: Results from the first study of the vaccine dose range in humans,” *Clin. Vaccine Immunol.*, vol. 17, no. 12, pp. 1868–1874, 2010, doi: 10.1128/CVI.00356-10.

[38] M. Moustafa *et al.*, “Phase IIa study of the immunogenicity and safety of the novel Staphylococcus aureus vaccine V710 in adults with end-stage renal disease receiving hemodialysis,” *Clin. Vaccine Immunol.*, vol. 19, no. 9, pp. 1509–1516, 2012, doi: 10.1128/CVI.00034-12.

[39] A. I. Fattom, G. Horwith, S. Fuller, M. Propst, and R. Naso, “Development of StaphVAX™, a polysaccharide conjugate vaccine against S. aureus infection: From the lab bench to phase III clinical trials,” *Vaccine*, vol. 22, no. 7, pp. 880–887, 2004, doi: 10.1016/j.vaccine.2003.11.034.

[40] F. Micoli, F. Bagnoli, R. Rappuoli, and D. Serruto, “The role of vaccines in combatting antimicrobial resistance,” *Nat. Rev. Microbiol.*, vol. 19, no. 5, pp. 287–302, 2021, doi: 10.1038/s41579-020-00506-3.

[41] H. K. Kim, F. Falugi, L. Thomer, D. M. Missiakas, and O. Schneewind, “Protein a suppresses immune responses during Staphylococcus aureus bloodstream infection in guinea pigs,” *MBio*, vol. 6, no. 1, pp. 1–11, 2015, doi: 10.1128/mBio.02369-14.

[42] H. K. Kim, A. G. Cheng, H. Y. Kim, D. M. Missiakas, and O. Schneewind, “Nontoxigenic protein A vaccine for methicillin-resistant staphylococcus aureus infections in mice,” *J. Exp. Med.*, vol. 207, no. 9, pp. 1863–1870, 2010, doi: 10.1084/jem.20092514.

[43] H. K. Kim, D. M. Missiakas, and O. Schneewind, “Mouse models for infectious diseases caused by Staphylococcus aureus,” *J. Immunol. Methods*, vol. 410, no. August, pp. 88–99, 2014, doi: doi:10.1016/j.jim.2014.04.007.

[44] A. Abdullahi, S. Amini-Nik, and M. G. Jeschke, “Animal models in burn research,” *Cell Mol Life Sci.*, vol. 71, no. 17, pp. 3241–3255, 2014, doi: 10.1007/s00018-014-1612-5.Animal.

[45] D. Schulz *et al.*, “Laboratory mice are frequently colonized with Staphylococcus aureus and mount a systemic immune response-note of caution for in vivo infection experiments,” *Front. Cell. Infect. Microbiol.*, vol. 7, no. 152, pp. 1–16, 2017, doi: 10.3389/fcimb.2017.00152.

[46] C. Bunce, L. Wheeler, G. Reed, J. Musser, and N. Barg, “Murine Model of Cutaneous Infection with Gram-Positive Cocci,” *Infect. Immun.*, vol. 60, no. 7, pp. 2636–2640, 1992.

[47] N. Malachowa, S. D. Kobayashi, K. R. Braughton, and F. R. DeLeo, “Mouse Model of Staphylococcus aureus Skin Infection,” *Methods Mol. Biol.*, vol. 1031, no. February, pp. 27–35, 2013, doi: 10.1007/978-

1-62703-481-4.

[48] A. D. Kennedy *et al.*, “Targeting of Alpha-Hemolysin by Active or Passive Immunization Decreases Severity of USA300 Skin Infection in a Mouse Model,” *J. Infect. Dis.*, vol. 202, no. 7, pp. 1050–1058, 2010, doi: 10.1086/656043.

[49] C. P. Montgomery, M. Daniels, F. Zhao, M. L. Alegre, A. S. Chong, and R. S. Daum, “Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A,” *Infect. Immun.*, vol. 82, no. 5, pp. 2125–2134, 2014, doi: 10.1128/IAI.01491-14.

[50] Y. Si *et al.*, “Inhibition of protective immunity against Staphylococcus aureus infection by MHC-restricted immunodominance is overcome by vaccination,” *Sci. Adv.*, vol. 6, no. 14, pp. 1–14, 2020, doi: 10.1126/sciadv.aaw7713.

[51] L. Scietti *et al.*, “Exploring host-pathogen interactions through genome wide protein microarray analysis,” *Sci. Rep.*, vol. 6, no. June, pp. 1–14, 2016, doi: 10.1038/srep27996.

[52] F. Bagnoli *et al.*, “Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 12, pp. 3680–3685, 2015, doi: 10.1073/pnas.1424924112.

[53] F. Rigat *et al.*, “Retrospective identification of a broad IgG repertoire differentiating patients with S. aureus skin and soft tissue infections from controls,” *Front. Immunol.*, vol. 10, no. FEB, pp. 1–11, 2019, doi: 10.3389/fimmu.2019.00114.

[54] A. D. Kennedy *et al.*, “Targeting of Alpha-Hemolysin by Active or Passive Immunization Decreases Severity of USA300 Skin Infection in a Mouse Model,” *J. Infect. Dis.*, vol. 202, no. 7, pp. 1050–1058, 2010, doi: 10.1086/656043.

[55] M. Alabdullatif and S. Ramirez-Arcos, “Biofilm-associated accumulation-associated protein (Aap): A contributing factor to the predominant growth of Staphylococcus epidermidis in platelet concentrates,” *Vox Sang.*, vol. 114, no. 1, pp. 28–37, 2019, doi: https://doi.org/10.1111/vox.12729.

[56] N. A. Kuklin *et al.*, “A Novel Staphylococcus aureus Vaccine : Iron Surface Determinant B Induces Rapid Antibody Responses in Rhesus Macaques and Specific Increased Survival in a Murine S . aureus Sepsis Model,” *Infect. Immun.*, vol. 74, no. 4, pp. 2215–2223, 2006, doi: 10.1128/IAI.74.4.2215.

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