Pathogenesis of intradermal staphylococcal infections: rabbit experimental approach to natural *Staphylococcus aureus* skin infections

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Abstract

Despite the enormous efforts made to achieve effective tools that fight against *Staphylococcus aureus*, the results have not been successful. It is likely that such failure is due to the absence of truly representative experimental models. To overcome this deficiency, the present work describes and immunologically characterizes the infection for 28 days, in an experimental low-dose (300 CFU) intradermal model of infection in rabbits, which reproduces the characteristic staphylococcal abscess. Surprisingly, when mutant strains in the genes involved in virulence (JΔ*agr*, JΔ*coa*Δ*vwb*, JΔ*hla* and JΔ*psmα*) were inoculated, no strong effect on the severity of lesions was observed, unlike other models that use high doses of bacteria. The inoculation of a human "rabbitized" (F*dlt*B^{r}) strain demonstrated its capacity to generate a similar inflammatory response to a wild-type rabbit strain and, therefore, validated this model for conducting these experimental studies with human strains. To conclude, this model proved reproducible and may be an option of choice to check both wild-type and mutant strains of different origins.

Keywords: Pathogenesis; Abscess; Experimental infection; Rabbit; *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a widespread bacterium that has adapted well to humans and animals which, if provided with a suitable opportunity, can initiate severe infections at various body sites [1-3]. It can cause a wide range of diseases and syndromes, but one of the most worrying ones, given community-associated infections, are those that affect both skin and soft tissues [4-9]. Skin and soft tissue infections (SSTIs) can be minor and self-limiting [1], but if they become complicated, they can prove life-threatening and be characterized by the formation of large abscesses [10].

After years of study and millions of dollars invested in research, gaps in our understanding of *S. aureus* infections remain, and it is not known in detail how staphylococcal infections evolve in humans. Numerous studies have been conducted on the pathogenesis of this bacterium [1], usually for the ultimate objective of developing therapeutic tools against staphylococcocal infections [11]. However, practical results remain frustrating because even today there are no effective preventive therapies, but resistance to antibiotics is growing and has reached really worrying levels. The problem could lie in the fact that the results experimentally obtained in inadequate animal models have been assumed and almost never questioned.

There are two important aspects to be taken into account in experimental staphylococcal infections: the animal species and the strain used. The murine has been the most frequently studied animal model. However, unlike humans, mice are not natural hosts for *S. aureus*. Conversely, rabbits (the second most frequently used animal species) usually suffer natural infections by *S. aureus.* In fact, numerous strains have been reported in commercial rabbitries [12] ever since a sporadic mutation favored the human-to-rabbit host jump 40 years ago [13]. It has been described that human skin morphological [14,15] and immunological [16] characteristics are more similar to those of rabbit than to those of mice. For this reason, the skin rabbit model is

more appropriate than the murine one if the objective is to simulate *S. aureus* infection in humans.

In order to develop experimental infections using human *S. aureus* strains in mice, and even in rabbits [17], the inoculation of an extraordinary large number of bacteria is necessary as they are strains that have not adapted to these hosts, which places at a distance the results obtained in the laboratory from what actually occurs under natural conditions. So in these cases, animal models can be unreliable predictors of either the potential success of therapeutic or preventive interventions or the roles played by specific determinants of bacterial virulence in infection [2]. This is why it is necessary to develop better animal models of colonization that are more representative of what happens in *in vivo* situations [3].

In this article we present, for the first time, a detailed description of staphylococcal dermal infection in a rabbit model at low doses to mimic natural infection and to approach what should occur in natural infections in humans in the most realistic way possible. The specific objectives of this study are to: (1) describe and characterize a low-dose rabbit *S. aureus* intradermal infection model using a wild-type reference strain and several mutant strains in different key pathogenicity factors that somehow contribute in abscess formation; (2) confirm the utility of this model after infection with a human adapted-to-rabbit ("rabbitized") *S. aureus* strain and compare their response with different rabbit strains of known virulence.

Materials and Methods

General approach

This study was designed in two phases: the first intended to (1a) establish a detailed description of the evolution of lesions and the immune response after infection with low doses of a wild-type *S. aureus* rabbit strain and (1b) study the effect of different genes on the pathogenesis of intradermal staphylococcal infections. The second intended to (2) prove the reproducibility of this experimental model with a human "rabbitized" strain.

Bacterial strains and growth conditions

The *S. aureus* strains used in this study were: Jwt (a rabbit ST121 wild-type strain isolated from a natural case of staphylococcosis) and deletion strains isogenic *coa/vwb* (JΔ*coa*Δ*vwb*), *hla* (JΔ*hla*), *psmα* (JΔ*psmα*) and *agr* (JΔ*agr*); J*rot*$^{+}$ (Jwt with *rot* gen restored); J*dlt*B^{h} (Jwt with the reversion of three identified *dlt*B Single Nucleotide Polymorphism –SNPS-); and F*dlt*B^{r}, a human ST121 strain expressing *dlt*B from rabbit clones [13] (**Table 1**).

Bacteria were grown at 37°C overnight on TSA agar medium supplemented with antibiotics as appropriate. Broth cultures were grown at 37°C in TSB broth with shaking (240 r.p.m.). The procedures for the preparation and analysis of phage lysates, transduction and transformation in *S. aureus* were performed essentially as previously described [20 21].

DNA methods

General DNA manipulations were performed by following standard procedures [22].

To produce the mutant strains, plasmid pMAD was used [23], as previously described [24]. The oligonucleotides used herein are listed in **Table 2**. Briefly, two separate PCR products with overlapping sequences, including the targeted sequence, were combined. A second PCR was run with external primers to obtain a single fragment. Specifically, 1 mL of each of the first

PCRs was mixed with 10 pM of the outside primers and was PCR-amplified. The fusion products were purified and cloned at the appropriate sites of shuttle plasmid pMAD, and the resulting plasmids were transformed into *S. aureus* (RN4220) by electroporation [20]. pMAD contains a temperature-sensitive origin of replication and an erythromycin-resistance gene. The plasmid was integrated into the chromosome through homologous recombination at a non-permissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into 10 mL of TSB and incubated for 24 h at 30°C. Ten-fold serial dilutions of this culture in sterile TSB were plated on TSA containing X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside; 150 mg/mL). White colonies, which no longer contained the pMAD plasmid, were tested to confirm replacement by DNA sequencing. Primers were obtained from Invitrogen Life Technologies (Paisley, UK).

Animals and sampling times

Two hundred and twenty 2-month-old albino hybrid rabbits (*Oryctolagus cuniculus*) (10 animals per sampling time) of either gender were inoculated with the eight above-described *S. aureus* strains. Depending on the inoculated strain, samples were taken at different times (**Table 1**). After a detailed study of lesions induced by the Jwt strain on different days postinfection (dpi) (0, 0.5, 1, 2, 3, 7, 14, 21, 28), it was established that 7 dpi was the optimal time to compare infections with mutants in various toxins (JΔ*coa*Δ*vwb*, JΔ*hla*, JΔ*psm*α) and general regulators (JΔ*agr*) as it was the time of maximum lesion development (abscesses opening and emptying) and when repair phenomena commenced. Then 1, 3 and 7 dpi were selected to compare the behavior of a human "rabbitized" strain (F*dlt*B^{r}) with three rabbit strains (Jwt, J*rot*$^{+}$, J*dlt*B^{h}) of well-known virulence in the acute and early chronic phases of infection evolution because, as before (7dpi), some important milestones were observed: on 1 dpi, externally evident lesions

(papules) and the formation of initial abscesses and on 3 dpi, skin necrosis, multiple Splendore-Hoeppli phenomena and an eosinophilic layer surrounding abscesses.

Animals were housed under conventional environmental conditions with an alternating cycle of 16 h of light and 8 h of darkness in individual cages (600 x 750 x 600 mm) and were fed with a commercial rabbit diet *ad libitum*.

The experimental protocol was approved by the Ethical Committee of the Universidad CEU Cardenal Herrera and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit numbers 2011/010 and 2017/VSC/PEA/00192; date of approval: January 20, 2011). All the animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005).

Intradermal infection model

The experimental procedure was performed as previously described [25], with some modifications. Briefly, rabbits were intradermally-inoculated in their backs with 300 colony forming units (CFU) of each studied strain suspended in 0.1 mL of phosphate-buffered saline (PBS) to inoculate animals with the lowest infective doses of bacteria. The optimal number of bacteria in the inoculum was empirically determined in the preliminary experiments. Previously, animals were sedated with a combination of ketamine (Imalgene®, 100 mg/mL, Merial, Barcelona, Spain) and xylazine (Xilagesic, 200 mg/mL, Calier, Barcelona, Spain) and a 10x10 cm area of the dorsal-lumbar region was shaved and disinfected with chlorhexidine. To avoid interactions among strains, each rabbit was infected with only one strain (except in the phase 1b where Jwt was inoculated as control), which were inoculated in duplicate. The general status, weight and rectal temperature of animals were recorded daily.

178 *Gross, microscopical and microbiological studies*

179 The characteristics of the skin gross lesions (presence of erythema, edema, skin elevation,
180 nodules, dermo-necrosis and/or ulceration) were recorded daily. Abscess dimensions were
181 measured with a calliper. The length (L) and width (W) values were used to calculate abscess
182 areas ($A = \pi$ [L x W]/2) and volumes ($V = 4/3\pi[L/2]^2$ x [W/2]) [26,27]. Macroscopic lesions were
183 evaluated by their abscess area as follows: Healthy (no apparent lesions), Mild (< 0.5 cm^2),
184 Moderate (0.5-5 cm^2) or Severe (>0.5 cm^2 and gross necrosis).

185 Upon sampling, rabbits were sedated with a combination of ketamine (Imalgene®, 100 mg/mL)
186 and xylazine (Xilagesic, 200 mg/mL), and were euthanized by an intravenous injection of
187 barbiturate (T-61, Intervet International GmbH, Unterschleißheim, Germany) and a complete
188 necropsy was carried out. Skin samples from the inoculation place were used for the
189 microscopic and microbiological studies and the cytokine assay. For the histological
190 examination, skin samples were routinely stained with hematoxylin and eosin (H&E), Masson's
191 trichrome and Gram's stains, and were processed by immunohistochemistry for macrophages,
192 T- and B-lymphocytes and plasma cell detection by the avidin-biotin-peroxidase complex
193 method at the dilutions recommended by the manufacturer (**Table 3**). In the
194 immunohistochemical procedure, positive cells were enumerated in the inoculation areas and,
195 when lesions were very severe, in the border of the necrotized tissue of twenty 0.08 mm^2
196 randomly selected fields per slide. The histological and immunohistochemical findings
197 observed on the different skin layers were described and recorded in detail.

198 Prior to fixation, representative samples were taken from skin lesions and kidneys, and were
199 weighed under sterile conditions for the microbiological studies. Then they were immersed in
200 a cold solution of PBS and mechanically homogenized in the presence of ice. One hundred µL
201 of tissue homogenate were cultivated on blood-agar (BioMérieux, Marcy l'Etoile, France) and
202 were incubated aerobically at 37°C for 24 h. This allows the results to be expressed in CFU/g.

203 The colonies that grew were identified by coagulase PCR [12] to prove that the strains isolated
204 from skin lesions were the same as those originally inoculated and to assess the possibility of
205 septicemia in the samples taken from kidneys.
206
207 *Hematology and flow cytometric analyses*
208 Blood samples (1 mL) in EDTA anticoagulant were obtained from the median artery of the ear.
209 White blood cell (WBC) counts and lymphocyte proportions were determined using a
210 hematology analyzer (MEK-6410, Nihon Kohden, Tokyo, Japan). The flow cytometric analysis
211 of the white blood cells (B, T, T CD4+, T CD8+ and activated T-cells, monocytes and
212 granulocytes) was performed using specific primary antibodies (**Table 4**) and secondary
213 antibodies (rat anti-mouse IgG2a+b phycoerythrin conjugate, VMRD; goat anti-mouse IgM
214 phycoerythrin conjugate, AbD Serotec, Kidlington, UK), as previously described [28,29].
215 The common leukocyte antigen CD14 and CD45 expression was used for the "lymphogate"
216 setup, as previously described [28,29]. The calculation of the total lymphocyte and the respective
217 subset counts were performed as the product of the WBC count and specific population
218 percentages, as described elsewhere [29,30].
219
220 *Cytokine assay*
221 The representative interleukins of the immune response (IL-1β, IL-4, IL-17, IL-18 and IFN-
222 gamma) from plasma and the inoculated skin tissues were analyzed by ELISA kits (CUSABIO,
223 Wuhan, Hubei Province, P.R. China) according to the manufacturer's protocol. Previously, the
224 samples taken from skin abscesses were immersed in a cold solution of PBS with a protease
225 inhibitor (Complete Mini, Roche, Basel, Switzerland), and were mechanically homogenized for
226 a 1 min by stopping each 10 s to avoid overheating in a T50 Ultra-Turrax ® (IKA, Staufen,

227 Germany) in the presence of ice. Next they were centrifuged at 13000 g for 10 min. Aliquots
228 of the supernatant were stored at -80°C for further processing.
229
230 *Statistical analysis*
231 Study 1a. Characterization of the model after infection with Jwt.
232 The data obtained in this study were evaluated by five different models depending on the type
233 of data recorded for each trait (**Tables 1 and Supplemental Table 1**). The five models included
234 the postinoculation day (9 levels: 0; 0.5; 1; 2; 3; 7; 14; 21; 28) as the fixed effect. Except for
235 CFU, the data of quantitative traits with one record per animal were analyzed by a linear model
236 M1 (proc GLM, SAS, 9.2). CFU usually presents changes in the order of magnitude as
237 infections evolve. In order to take this situation into account, the CFU data were analyzed by a
238 mixed model M2 and by considering different variances for the random residual errors on each
239 postinoculation day. The data of the quantitative traits with more than one record per animal
240 were analyzed by a mixed model M3 (proc MIXED, SAS, 9.2), which included the effect of
241 the individual upon infection as random effects [90 levels; $N\sim (0, \sigma_p)$]. The data of the
242 categorical traits with two levels (e.g.: yes/no, presence/absence, 0/1, etc.) were analyzed by a
243 generalized linear model M4 (proc GENMOD, SAS, 9.2) after considering that the response
244 variable followed a binomial distribution and by using logistic transformation [$\ln (\mu / (1-\mu))$]
245 as a link function. Finally, the ordinal data of the histology traits had more than two levels
246 because injuries were classified according to a previously described scale (-, - / +, +, + / ++, ++,
247 ++ / +++, +++, +++ / ++++, ++++), where ++ was greater (severer) than +. However, this did
248 not mean that the distance between each class was proportional. To take this situation into
249 account, the data of the histology traits were analyzed by a generalized linear model M5 (proc
250 GENMOD, SAS, 9.2) after considering that the response variable followed a multinomial
251 probability distribution and that the link function followed a cumulative logistic distribution [31].

Study 1b. Effects of the selected genes and the global regulator involved in abscess development.

A pair-wise comparison of the means was performed using Yates correction. The t-student test was performed for the traits with quantitative data and the chi-square test for the traits with categorical data [M6].

Study 2. Comparison of the lesions caused by a human "rabbitized" strain and different rabbit strains of known virulence.

The data obtained in this study were evaluated by four different models depending on the type of data recorded per trait (**Supplemental Table 1**). The four models included day postinoculation (3 levels; 1, 3, 7), the strain (4 levels: Jwt, F*dlt*B^{r}, J*rot*$^{+}$, J*dlt*B^{h}) and their interaction as fixed effects. Except for CFU, the data of the quantitative traits with only one record per animal were analyzed by a linear model M7 (proc GLM, SAS, 9.2). CFU was analyzed by a mixed model M8 after considering the different variances for the random residual errors on each day postinoculation. The data of the quantitative traits with more than one record per individual were analyzed by a mixed model M9 (proc MIXED, SAS, 9.2) and included the effect of the individual at the experimental infection time as random effects [120 levels; N~ (0, σ_p)]. Finally, the data of the ordinal categorical traits with more than two levels (histology variables) were analyzed by a generalized linear model M10 (proc GENMOD, SAS, 9.2) after considering that the response variable followed a multinomial probability distribution and that the link function followed a logistic distribution cumulative [31].

Results

Study 1a. Characterization of the model after infection with a rabbit wild-type strain (Jwt): infection with low doses of bacteria generates a characteristic and identifiable pathological response over time

After evidencing that rabbits can be infected by a low-dose charge of *S. aureus* [13], we set out to make a detailed description, for the first time, of the pathogenesis of staphylococcal infections in the model that best mimicked staphylococcal disease in humans: the rabbit experimental model.

Health parameters

A slight stagnation of animals' weight on the first 3 days after infection was detected, but they grew normally during the rest of the experiment. Although an increase in rectal temperature was observed during the study (38.5 at 0dpi and 39.5 at 28dpi; *P=0.001*), fever was not detected in any animal and the temperature values fell within physiological ranges.

Gross lesions

Lesions of different severities developed in all cases after the inoculation of 300 CFU of *S. aureus* (**Fig. 1A**). Small (< 1 cm in diameter), flat and circumscribed red macules were observed at the point of inoculation at 0.5 dpi in 100% of the animals. These lesions evolved to slightly elevated papules (1dpi) and firm nodules (2dpi) in the 100% of the animals and at 100% of the inoculation points (20 out of 20). The size and intensity of the reddened areas reached its apogee at 2 dpi (2.003±0.51 *vs.* 3.422±0.47 cm^2 on 1 and 2 dpi, respectively; $P<0.001$) and gradually disappeared after 3 dpi. At this time (3 dpi), the volume of nodules was maximum (4.561±1.15 cm^3; *P<0.001*) and 50% of the animals (5 out of 10) at 7 dpi showed epidermal necrosis in the inoculation zone characterized by darkness, and suppurative to dry (parchment) areas that

opened between 7 and 14 dpi in all cases to result in ulceration and discharged a purulent material. Draining abscesses reduced abscess size. From 14 dpi to 28 dpi, a repair of the ulcers occurred, and absence of hair and scar retraction were observed at the end of the study (**Fig. 2**).

Bacteriology

The bacterial counts increased at 0.5 dpi (1.11 x 10^7 CFU/g), with the largest number obtained at 3dpi (50.98 x 10^7 CFU/g) and, coinciding with the opening of abscesses, the number of bacteria lowered until the end of the experiment, when the lesions of only four of ten animals contained bacteria (1.16 x 10^3 CFU/g at 28dpi) (**Fig. 3**). All the cultured and identified bacteria recovered from lesions were the same as those inoculated and no bacteria were isolated from blood or kidneys.

Histological findings

Some animals showed early histopathological modifications at 0.5 dpi (**Table 5**). In these cases, lesions were characterized by moderate edema and minor hemorrhages in the superficial dermis and a by slight increase in the density of heterophils in the deep dermis, which sometimes grouped into small clusters of cells (**Figs. 1B and 4A**). The capillary surrounding these inflammatory foci were hyperemic. The capillaries located near or between cutaneous muscle cells were also dilated and hyperemic, with the presence of heterophils migrating by diapedesis to the interstice. Small hemorrhagic foci between adipocytes near the cutaneous muscle were detected.

Vascular phenomena, such as hyperemia (vascular dilatation and presence of numerous intravascular heterophils), mild to moderate edema and hemorrhages were detected in the superficial dermis on 1 dpi. The deep dermis showed hyperemia and numerous heterophils around dilated blood vessels and inflammatory foci structured as a core of eosinophilic necrosis,

sometimes with coccoid bacteria surrounded by diffusely distributed heterophils (**Fig. 4B**). Cutaneous muscle fibers were infiltrated by heterophils, which provoked a mild degeneration and atrophy of myocytes (**Fig. 1C**).

Two days after infection, lesions were similar to the way there were the previous day, but presented greater severity. It was the first time that clear epidermis thickening (moderate acanthosis) was detected (10% of animals). The presence of mild vascular dilatation, moderate edema and some hemorrhages in the superficial dermis was observed. In the deep dermis, inflammatory foci were better well-defined than they were previously. They were characterized by a center of eosinophilic necrosis, inside which there were coccoid bacteria surrounded by a dense layer of heterophils (**Fig. 4C**). Around these foci there were also heterophils. Cutaneous muscle cells were severely infiltrated by heterophils, which induced their separation and necrosis (**Figs. 1D and 4D**).

On 3 dpi, the percentage of animals with moderate to severe epidermal hyperplasia was higher than on 2 dpi (46% vs. 10%). The presence of more severe vascular phenomena (vascular dilatation, edema and hemorrhages) in the superficial dermis induced skin tumefaction, and also epidermis necrosis when the volume of abscesses was sizeable (**Fig. 4E**). In the deep dermis, the severity of edemas and hyperemia diminished. The latter was observed only around suppurative foci, together with diapedesis phenomena of heterophils. Inflammatory foci were structured by a center with bacterial colonies surrounded by a star-shaped homogeneous eosinophilic material (Splendore-Hoeppli phenomenon) and an external layer of cellular debris and inflammatory cells (mainly lymphocytes, macrophages and heterophils) (**Fig. 4F**). When abscesses were large, this pattern was repeated and they merged with one another to form the abscess. Externally, abscesses were partially or totally surrounded by a thin eosinophilic layer of necrosis (**Fig. 4G**). The myocytes of cutaneous muscle were infiltrated by eosinophils and macrophages, and showed dilatation, degeneration and, sometimes, atrophy (**Figs. 1E and 4H**).

The main characteristic on 7 dpi was the presence of local epithelial necrosis and epidermis ulceration with severe acanthosis in the peripheric epithelium (**Fig. 4I**), together with severe hyperemia, edema and hemorrhages in the superficial dermis. At greater depths, abscesses showed several Splendore-Hoeppli phenomena, whose peripheral inflammatory reactions merged with one another. An external thin eosinophilic layer surrounded abscesses. Around abscesses, heterophils diffusely distributed or a few were seen and some of the capillary vessels were reactive (presence of endothelial hyperplasia and hypertrophy). The cutaneous muscle was interrupted by an inflammatory infiltrate (heterophils and round cells) that caused the atrophy of the affected cells (**Fig. 1F**).

On 14 dpi, the severity of some inflammatory phenomena started to decrease and repair appeared more evident (**Fig. 1G**). In those cases in which the epithelium was lost, epidermis re-epithelization, characterized by the presence of numerous mitotic figures on the basal and spinosum layers at the borders of the ulcer, was observed (**Fig. 4J**) together with the absence of skin adnexa (sweat and sebaceous glands and hair follicles). All the animals showed acanthosis. The vascular phenomena were milder, but still evident in the superficial dermis like inflammatory foci with bacteria and, in some cases, Splendore-Hoeppli phenomena, all surrounded by an eosinophilic layer in the deep dermis. In adjacent areas, granulation tissue (numerous small vessels and abundant fibrous tissue) was observed (**Fig. 4K**). On the muscular layer, focal fibrosis and atrophy of some cells were observed. Other locations presented hypertrophy and hyperplasia of muscular fibers.

On 21 dpi, mitotic cells and hyperplasia in the peripheral epithelium of lesions were observed, even in those few rabbits that still had ulcers in the epithelium. When lesions were completely epithelized, the epidermis was 2-3-fold thicker than in the unaffected areas (**Fig. 5**). In the dermis (mainly in the deep dermis), abundant granulation tissue was observed with numerous small blood vessels and heterophils forming small cellular aggregates or diffusely distributed,

mainly near cutaneous muscle which, in some cases, was still interrupted by inflammatory cells and atrophied (**Fig. 1H**).

Finally, on 28 dpi, the epidermis of all the animals showed hyperplasia, but with a thinner epithelium than on 21 dpi (1.5-fold thicker than normal). Skin adnexa were absent in the central lesion, but the periphery was invaded by hair follicles as a consequence of scar retraction (**Fig. 1I**). The dermis presented mature granulation tissue with abundant fibrous tissue and blood vessels, practically without erythrocytes and with scarce heterophils, as well as some small hemorrhages (**Fig. 4L**). Cutaneous muscle had completely regenerated.

Immunohistochemical studies

Variations in the counts of the studied cells (T-lymphocytes $CD3^+$, plasma cells IgG^+ and macrophages $RAM11^+$) were detected locally in the inoculation area during the experiment. The T-lymphocytes ($CD3^+$) counts increased during the experiment until 21 dpi (17.55±1.90 cells/mm^2) and then abruptly dropped on 28 dpi (**Fig. 6A**). Plasma cells (IgG^+) appeared in small groups, mainly in the deep dermis. They were few in number in lesions in the acute inflammation phase (first 3 days), but significantly increased on 7 dpi and remained high on 14 and 21 dpi (11.03, 11.78 and 11.15±1.93 cells/mm^2, respectively; $P<0.001$), and once again abruptly decreased on 28 dpi (**Fig. 6B**). The macrophages counts ($RAM11^+$) remained with no differences compared to 0 dpi until 3 dpi when they significantly increased until 14 dpi. At this time, the number of macrophages reached the highest level (10.75±1.16 cells/mm^2; $P<0.001$) before lowering on 21 dpi (still higher than on 0 dpi) and 28 dpi (**Fig. 6C**). Macrophages were diffusely located in the inoculation area when no abscess was present, but when abscesses started organizing, macrophages were located around them and formed a palisade on the outer abscess layer. No significant differences were detected in the number of B-lymphocytes $CD79\alpha^+$.

400

401 *Flow cytometer results*

402 Figure 5 shows the results of the flow cytometric analysis done of the peripheral blood cells in
403 the rabbits infected by *S. aureus* Jwt. Intradermal inoculation induced modifications in the
404 counts of most leukocyte populations (granulocytes, monocytes and total B and T $CD25^+$
405 lymphocytes). Total leukocytes (**Fig. 6D**) and granulocytes (**Fig. 6F**) counts showed two peaks
406 during the study: a first sharp increase on 1 dpi and a second greater increase from 7 to 14 dpi,
407 which decreased on 21 dpi (more abruptly for granulocytes). The number of monocytes (**Fig.**
408 **6G**) gradually increased from 3 to 14 dpi, when its highest value was reached (869.5 x 10^6/L;
409 *P <0.001*). The number of total lymphocytes rose from 0 to 2 dpi, when counts were kept
410 elevated until the end of the experiment (**Fig. 6E**). Regarding the lymphocyte subpopulations,
411 statistical differences were observed only in the B (**Fig. 6H**) and $CD25^+$ (**Fig. 6I**) lymphocytes.
412 B-cells had higher values at 0.5, and especially at 21 dpi, compared to 0 dpi (43.29 and 81.66
413 x 10^6/L, respectively; *P<0.001*). The cell counts of the $CD25^+$ lymphocytes abruptly increased
414 on 0.5 dpi and were 11.9-fold higher than on 0 dpi and on later experimental days. No
415 significant differences were observed in the number of T-lymphocytes, T $CD4^+$ lymphocytes
416 and T $CD8^+$ lymphocytes.

417

418 *Plasmatic and tissue cytokines*

419 Statistical differences were detected only in IL-4 and IL-18 in plasma and in IL-4 and IFN-
420 gamma in skin samples (**Table 6**). The plasmatic levels of IL-4 and IL-18 oscillated throughout
421 the experiment. Relative to 0 dpi, statistical differences were only detected for IL-4 on 28 dpi
422 and IL-18 on 14 and 28 dpi, where these cytokines reached their highest plasmatic levels (8.9
423 pg IL-4/mL and 463.4 pg IL-18/mL) on 28 dpi. Similarly to plasma, the IL-4 detected in skin
424 tissue significantly increased on 28 dpi (15.5 pg/mL; *P<0.05*). Conversely, the IFN-gamma

425 level in tissues abruptly increased on 0.5 dpi (14015±1804 pg/ml; *P<0.05*) before returning to
426 previous levels. No significant differences were observed in IL-1β and IL-17 levels.
427
428
429 ***Study 1b.*** **Effects of the selected genes and the global regulator involved in abscess**
430 **development:** ***mutants in different key genes for abscess development cause a delay in***
431 ***lesions, but are still able to infect animals and generate abscesses***
432 Once the lesions and peripheral and local immune responses had been described, the aim of this
433 section was to evaluate the contribution of different isogenic mutants of the Jwt strain (JΔ*agr*,
434 JΔ*coa*Δ*vwb*, JΔ*hla* and JΔ*psm*α) to the development and characteristics of abscesses on 7 dpi
435 using the proposed rabbit skin model. Unexpectedly, all the mutant strains were able to generate
436 lesions like the wild-type in terms degree of severity (**Fig. 7**). Whenever present, lesions were
437 macroscopically indistinguishable from those generated by the wild-type strain.
438 At the histological level, Splendore-Hoeppli phenomena were observed with all the strains, but
439 not with the same incidence. Only statistically differences were observed with JΔ*hla* (85% Jwt
440 *vs.* 68% JΔ*hla*; *P<0.05*) (**Fig. 8A**). The percentage of distributed heterophiles surrounding the
441 abscesses was higher in the mutant strains with respect to Jwt (+20% for JΔ*coa*Δ*vwb*, +50%
442 for JΔ*hla* and +30% for JΔ*psm*α and JΔ*agr*; *P<0.05*) (**Fig. 8B**). All the lesions caused by each
443 studied strain (the wild-type and mutants) presented an eosinophilic layer surrounding
444 abscesses on 7 dpi. However, there were differences in the characteristics of this band. The
445 percentage of complete eosinophilic layers always was lower for mutants than for Jwt (-30%
446 for JΔ*coa*Δ*vwb*, -90% for JΔ*hla*, -45% for JΔ*psm*α and -50% for JΔ*agr*; *P<0.05*) (**Fig. 8C**).
447 These histological differences are related to the degree of maturation of the abscess. Thus, the
448 lesions generated by the mutants at 7 dpi resembled those presented by the wild-type strain in

2-3 dpi, which could be interpreted as the absence of these genes delay the development of the lesions.

Study 2. **Comparison of the lesions caused by a human "rabbitized" strain and different rabbit strains of known virulence: similar, but milder lesions to the rabbit wild-type strain are caused by a human rabbitized strain after intradermal inoculation**

Once the infection model had been characterized and that all the rabbit strains were capable of generating lesions at low doses had been verified, our intention was to test the model with a human strain. The inconvenience here is that human *S. aureus* strains (e.g., F strain) do not cause lesions at low doses (300 CFU) in rabbits [13]. In fact to be able to produce them, it is necessary to use high bacterial doses (more than 10^4 CFU) and, when this occurs, the evolution of lesions (faster) and the produced characteristics (e.g. presence of extensive coagulative necrosis, thrombosis, absence of Splendore-Hoeppli phenomena or peripheral eosinophilic layers on 3 dpi) differ vastly from those observed by rabbit strains at low doses (**JM Corpa, personal communication, Supplemental Figure 1**). Therefore, we had to use a human strain adapted to rabbits (F*dlt*B^r), which we had previously demonstrated was able to infect this species at low doses [13].

The specific aim was to compare the response to infection after inoculating the F*dlt*B^r strain with *S. aureus* strains obtained from rabbits of known virulence, such as Jwt (high virulence), J*rot*$^+$ (medium virulence) and J*dlt*B^h (low virulence), using the previously described model to demonstrate its utility with strains of different origins.

Health parameters

No animal suffered fever and no differences in temperature between the studied groups were observed.

Gross lesions

Differences in both the number of infected animals and the characteristics of their lesions after inoculation with the studied *S. aureus* strains were detected. While 100% of the rabbits were infected by human F*dlt*B^{r} and rabbit Jwt, 76.7% and 60% of the animals inoculated with J*rot*$^{+}$ and J*dlt*B^{h} showed lesions, respectively. The severity of lesions caused by F*dlt*B^{r} was intermediate, between Jwt (severer lesions) and J*rot*$^{+}$ and J*dlt*B^{h} (milder lesions) (**Fig. 9**).

On 1 dpi, the inoculation points showed erythema in 100% of the animals infected by F*dlt*B^{r}, Jwt and J*rot*$^{+}$ and 80% in the rabbits inoculated with F*dlt*B^{r}. On 3 dpi, lesions evolved to nodules in 100% of the animals infected by the F*dlt*B^{r} and Jwt strains, but only in 30% of the rabbits inoculated with J*rot*$^{+}$, and 10% with J*dlt*B^{h}. At the end of the experiment (7 dpi), while all the animals infected with F*dlt*B^{r} and Jwt presented nodules, even some developed dermonecrosis (10% and 50%, respectively), only 50% of the rabbits inoculated with J*rot*$^{+}$ and J*dlt*B^{h} showed lesions. The animals infected with J*rot*$^{+}$ exhibited erythema and nodules (40%), but erythema was observed only in the rabbits infected with J*dlt*B^{h} at this time point.

Bacteriology

The bacterial counts obtained from the animals inoculated with F*dlt*B^{r} and J*rot*$^{+}$ followed the same dynamics as the Jwt strain on 1, 3 and 7 dpi (**Fig. 10**), but the average number of bacteria obtained from their lesions was smaller (6.18±32.21 x 10^{6} and 1.10±47.54 x 10^{6} CFU/g, respectively, $P<0.05$) than from the lesions caused by Jwt (244.36±36.98 x 10^{6} CFU/g). Bacteria were isolated only in one animal infected by J*dlt*B^{h} on 3 dpi (2.26 x 10^{3} CFU/g) at

3dpi. All the cultured and identified bacteria recovered from lesions were the same as those inoculated, and no bacteria were isolated from blood or kidneys.

Histological findings

Different degrees of severity were observed in the histological lesions depending on the type of inoculated strain (**Table 7**). In general, F*dlt*B^{r} caused more similar (but milder) lesions to the Jwt strain than to the other two. Vascular dilatation and edema in the superficial dermis observed in the animals infected by F*dlt*B^{r} were severer than J*rot*$^{+}$ and resembled Jwt.

The abscesses caused by F*dlt*B^{r} were evident on 1 dpi and were more numerous and severer on 3 dpi, similarly to Jwt. Conversely, the severity of the abscesses (heterophils organized, **Table 7**) in the animals infected by J*rot*$^{+}$ and J*dlt*B^{h} reduced on 3 dpi. In this last case, abscesses were classified mainly as "mild" (**Fig. 11A**).

The abscesses produced by the three mutant strains were histologically more immature compared to Jwt as they did not show a complete eosinophilic peri-abscess layer. On 3 dpi, all the Jwt-related abscesses had a well-developed eosinophilic layer, only 55% of F*dlt*B^{r} and 20% of J*rot*$^{+}$ had one, and no band was detected in the J*dlt*B^{h} abscesses. At the end of the experiment (7 dpi), percentages varied, but differences remained (100% Jwt; 36% F*dlt*B^{r}; 30% J*rot*$^{+}$ and 0% J*dlt*B^{h}).

It was noteworthy that Splendore-Hoeppli phenomena were seen only in the abscesses from the rabbits infected by Jwt and J*rot*$^{+}$ on 3 and 7 dpi. There were fewer Splendore-Hoeppli phenomena in J*rot*$^{+}$ (1 on 3 dpi and 2 on 7 dpi) than in Jwt (5 on 3 dpi and 8 on 7 dpi), although the number of abscesses also lowered with J*rot*+ (2 on 3 dpi and 3 on 7 dpi) than with Jwt (10 at both time points).

The degree of dilated blood vessels in the deeper dermis close to cutaneous muscle was higher in the animals inoculated with strains F*dlt*B^{r} and J*dlt*B^{h} for all the studied times, but the incidence of perivascular inflammation was higher with Jwt (P<0.05) (**Table 7**).

The pathological findings (inflammation and degeneration) observed in cutaneous muscle were similar between strains F*dlt*B^{r} and Jwt, and were severer than those caused by strains J*rot*$^{+}$ and J*dlt*B^{h} (P<0.05) (**Table 7**).

Immunohistochemical studies

The number of positive cells was related to lesion severity, with no positive cells in the animals without lesions regardless of the inoculated strain. The number of studied cells in the lesions caused by F*dlt*B^{r} strain infection was lower to those of the wild-type strain (Jwt) and more similar to those of strain J*rot*$^{+}$ and J*dlt*B^{h} (**Fig. 11B-E**). The T-lymphocytes (CD3^{+}) counts were significantly higher during the three moments analyzed in the Jwt compared to F*dlt*B^{r}. The macrophages counts (RAM11^{+}) were significantly higher on 3 and 7 dpi in the animals infected with Jwt regarding to F*dlt*B^{r}, and the counts of B-lymphocytes (CD79α^{+}) and plasma cells (IgG^{+}) were significantly higher on 3 and 7 dpi, respectively. The lesions produced by the inoculation of J*rot*$^{+}$ were characterized mainly by the absence of T-lymphocytes (CD3^{+}) on all the analyzed days and high number of B-lymphocytes on 3 dpi, similar to Jwt. The lesions produced by the inoculation of J*dlt*B^{h} were characterized mainly by the absence of B-lymphocytes (CD79α^{+}) on all the sampling times.

Flow cytometer results

The differences detected in the peripheral blood cell counts in the rabbits infected by *S. aureus* F*dlt*B^{r}, Jwt, J*rot*$^{+}$ and J*dlt*B^{h} are shown in **Fig. 12**. The animals infected by the F*dtl*B^{r} strain had

545 fewer leukocytes (-15.2%) and total lymphocytes (-26.5%) than Jwt, J*rot*$^{+}$ and J*dlt*B^{h}, with
546 similar counts to one another (**Fig. 12A** and **D**).
547 In the B, T, CD4^{+}, and CD8^{+} lymphocyte counts, no significant differences were observed
548 between the rabbits inoculated with the F*dlt*B^{r} strain and Jwt. Both animal groups had more B-
549 lymphocytes and fewer T, CD4^{+} and CD8^{+} lymphocytes than J*rot*$^{+}$ and J*dlt*B^{h} (**Fig. 12E, F, G**
550 and **H**). F*dlt*B^{r} and Jwt strains had less CD4^{+}/CD8^{+} ratio than Jrot^{+} (**Fig. 12J)**.
551 The animals infected by the mutant strains presented fewer granulocytes (-34.5%; $P<0.05$) and
552 monocytes than those inoculated with Jwt (**Fig. 12B** and **C**). For monocytes, the animals
553 inoculated with J*rot*$^{+}$ and J*dlt*B^{h} had an intermediate level (-23.9% J*rot*$^{+}$ and J*dlt*B^{h}, -50.5%
554 F*dlt*B^{r}; $P<0.05$) (**Fig. 12C**). The CD25^{+} lymphocytes showed significantly higher counts in the
555 animals infected by mutant strains F*dlt*B^{r}, J*rot*$^{+}$ and J*dlt*B^{h} (+50.4%, +61.2% and +65%,
556 respectively; $P<0.05$) than the rabbits infected by Jwt (**Fig. 12I**). Finally, strains F*dlt*B^{r} and Jwt
557 had a higher granulocytes/lymphocytes ratio than J*rot*$^{+}$ and J*dlt*B^{h} (**Fig. 12K**).
558
559 **Discussion**
560 Staphylococcal infections have spread worldwide in hospitals and communities in the last
561 decades of the 20th century, are multi-resistant to antibiotic strains (especially methicillin-
562 resistant *S. aureus* – MRSA-) and have become a major health challenge in all industrialized
563 countries today [1]. This problem has also extended to farm animals since 2005, when a livestock-
564 associated MRSA (LA-MRSA) was isolated in pigs [32] and extended to other animal species,
565 including horses, cattle, poultry or rabbits [33-35]. Among the wide range of diseases associated
566 with community infections that can be caused by *S. aureus*, SSTIs are one of the most worrying
567 because they can be a life-threatening process if they are complicated [4-9]. SSTIs are
568 characterized by the formation of bacterial abscesses in dermis, epidermis or subcutaneous
569 tissues [1].

570 In this context, it would be valuable to have a definitive experimental model that allows the
571 pathogenesis of the infection to be studied or new therapeutic tools to be tested. Mice are
572 commonly used for staphylococcal colonization and infection models, mainly for its small size,
573 easy handling and the abundance of research facilities compared to other larger species like
574 rabbits [16,36]. However, these characteristics alone do not justify its use if the results are not truly
575 representative. Traditionally, rabbit models of *S. aureus* infection have been considered optimal
576 to investigate virulence and host-pathogen interactions, mainly due to certain similarities with
577 the human species: (1) similar thickness of their skin [14,15]; (2) similar immune response to
578 infection [16]; (3) both are naturally infected by *S. aureus*. For these reasons, we present a rabbit
579 model because this animal species has its own adapted *S. aureus* strains [12,35,36], which easily
580 allow to naturally study staphylococcal infections.
581 The experimental model was quite respectful of animal health as no animal displayed fever,
582 loss of weight, septicemia or external signs of disease, except for the expected local lesions at
583 inoculation points. The model was reproducible, and previously described characteristic
584 abscesses developed [37], but it also provided more detailed characteristics of lesion development
585 over time than previously described in the bibliography. Although lesions were observed at all
586 the studied time points, staphylococcal infection with a Jwt strain was evaluated with this model
587 at three key time points, 1, 3 and 7 dpi. On 1 dpi it was possible to observe small slightly raised
588 reddish papules that were histologically characterized by the presence of acute vascular
589 changes, small organized abscesses, sometimes with inner bacteria and surrounded by
590 heterophils that were diffusely distributed. One interesting finding was the sharp and one-time
591 increase in the $CD25^+$ lymphocytes 12 h before (0.5 dpi). As these cells are considered T-
592 regulatory cells [38], it would be interesting to verify to what extent the inflammatory response
593 described in this intradermal infection is modulated by this cell population. T-regulatory cells
594 are essential for preventing exacerbation of inflammatory response, but they have been found

595 to lose their activity in the presence of *S. aureus*, as shown in children with atopic skin [39,40]. On
596 3 dpi superficial lesions lost their initial reddish color and became nodular lesions, and it was
597 when they acquired their largest volume and had more bacteria. Microscopically, vascular
598 changes increased in severity, abscesses were surrounded by an eosinophilic necrotic layer in
599 whose core Splendore-Hoeppli phenomena with inner bacteria were observed. Surrounding the
600 abscess there were increasing number of T-lymphocytes ($CD3^+$) and macrophages ($RAM11^+$)
601 compared to previous days, which remained high until 21 dpi., and were related with the
602 increased number of blood monocytes and lymphocytes on 2 dpi, as previously described [41].
603 On 7 dpi, 50% of the nodules suffered epidermal necrosis and opened, which triggered a
604 discharge of pus, and a reduction in both lesion size and the number of isolated bacteria. These
605 epidermal lesions generated a new inflammatory episode, peripherally characterized by an
606 increased number of heterophils in blood, which lasted until 14 dpi. In histological terms,
607 lesions reached their severest point at all the studied locations, with several Splendore-Hoeppli
608 phenomena surrounded by many T-lymphocytes ($CD3^+$), plasma cells (IgG^+) and macrophages
609 ($RAM11^+$).
610 Therefore, by studying on 1 dpi and 3 dpi, it would be possible to evaluate the acute
611 inflammatory response, as well as the subacute inflammatory reaction and initial repair
612 mechanisms on 7 dpi, which could be useful for the majority of experimental studies on this
613 topic. For chronic inflammation studies, other later study times (e.g., 14, 21 or 28 dpi) would
614 be more recommendable, when regenerative modifications were predominant (re-epithelization
615 and acanthosis in the epidermis, granulation tissue and fibrosis in the dermis, and regeneration
616 of the cutaneous muscle), and when blood monocytes (2 to 14 dpi) and tissue lymphocytes and
617 macrophages (3 to 21 dpi) significantly increased, as previously described for chronic
618 inflammatory processes [41].

Several findings in human and animal models have suggested a primarily role for T-cells in immunity to *S. aureus* skin infections by enhancing the recruitment of phagocytes [42-44]. *S. aureus* antigens induce different Th1, Th2 and Th17 cell pathways. Therefore, the role of T-cells in immunity to *S. aureus* skin infections likely involves multiple T-cell effector cytokines. As there is a paucity of reagents available to analyze panels of rabbit immune mediators, in this work it was not possible to accurately characterize the immune response type, but significant differences were observed in some cytokines after the Jwt strain inoculation. Significant changes were found in cytokines INF-gamma and IL-18. Cytokine IFN-gamma is a potent activator of monocytes by increasing their phagocytic activity in tissues. [41]. Despite this cytokine increasing on 21 dpi, it became significant only on 0.5 dpi. IL-18 has been associated with atopic dermatitis expelled by *S. aureus* [45]. Although *S. aureus* is capable of causing the release of IL-18 from keratinocytes [46], in this work an increase was detected only in plasma, but not in skin. An increase in IL-4 in plasma and skin was noted, especially after 21 days. *S. aureus* is able to inhibit the response of T-cell responses and to induce Th2 cell responses by producing IL-4 [47] in relation to chronic inflammations [41].

The difficulty in developing an experimental model that reproduces an immune response under natural conditions might be related to the effect of the origin of both the bacteria and animal species used as hosts on the infective dose [48]. In general, a higher dose is necessary to infect animals with the *S. aureus* strains obtained from a different species (e.g. human bacteria CA-MRSA USA300 in mice: $\geq 5.0 \times 10^8$ CFU [49]). In such cases, animal models can be unreliable predictors of either the potential success of therapeutic or preventive interventions or the roles played by specific determinants of bacterial virulence in infection [2]. Only a few standardized studies about the number of bacteria needed to begin infection can be found [50]. Schmid-Hempel and Frank [50] proposed an infective *S. aureus* dose of 10^5-10^6 bacteria and classified it as a high-infective dose microorganism. In fact it has been reported that inoculation with less than 5×10^8

644 CFU of USA300 does not result in reproducible abscesses in rabbits [17,49]. Our results agree with
645 this observation when we used human strains (not adapted to rabbit), but disagree when rabbit
646 strains were employed, which can generate dermal lesions with only 300 CFU by simulating
647 natural infection. Moreover, when high doses of human strain Fwt (> 10^6 CFU) were used with
648 this model, the generated lesions differed from those observed for low doses of rabbit wild-type
649 strains: lesions rapidly evolved and skin necrotic phenomena (dermonecrosis) predominated
650 and, in histological terms, the presence of thrombosis and the absence of Splendore-Hoeppli
651 phenomena or a peripheral eosinophilic layer were observed on 3 dpi (**JM Corpa, personal**
652 **communication, Supplemental Figure 1**). Similar results have been reported by other authors
653 [17], who recognized that using such high doses would result in rapidly progressive infection,
654 which would vastly differed from that typically encountered in clinical situations, and the
655 results could not be extrapolated to humans. These skin necrotic phenomena are probably
656 caused by the mass production of bacterial toxins, which is far removed from what actually
657 happens in natural infection. This hypothesis would support the results observed in the present
658 study with the mutants (JΔ*coa*Δ*vwb*, JΔ*hla*, JΔ*psm*α, JΔ*agr*) employed to evaluate their
659 contribution to the development and characteristics of abscesses. Studies carried out in rabbits,
660 and mainly in mice, using Δ*hla* mutants have resulted in reduced virulence in animal models of
661 dermonecrotic skin infection. Moreover, immunization with Hla-specific antisera significantly
662 reduces the size of skin lesions and prevents dermonecrosis [11,17,51,52], and similar results have
663 been obtained with mutants in *psm*-α [53]. Surprisingly, when the herein described skin infection
664 model was used, mutants Δ*hla* and Δ*psm*α produced the same percentage of lesions as the wild-
665 type strain, and even became macroscopically severer for Δ*hla* regarding Jwt. Toxins and other
666 virulence factors, such as α-toxin, PSM-α and PSM-β, are produced as a result of increased
667 bacterial population density and environmental conditions, partly under a quorum-sensing
668 control through different regulators [54-56]. Therefore, the effect observed in previous works with

669 either the Δ*hla* or the Δ*psm*α mutant could be due to the use of high doses of bacteria to produce
670 lesions, which would favor the rapid production of numerous toxins, and would make the effect
671 of the toxin more evident. The absence of an effect on the Δ*agr* mutant would follow the same
672 hypothesis. In *S. aureus*, the majority of quorum-sensing components are coded by the
673 accessory gene regulator (Agr) system. This regulation is important for acute disease to develop
674 [37], but we showed that Agr was dispensable in intradermal infections of rabbits. Agr suppresses
675 the expression of the repressor of toxins (*rot*) at a high cell density [57], which could also influence
676 the virulence of the Δ*agr* mutant. However, rabbit strains have a natural loss-of-function
677 mutation in *rot,* which increases the severity and infectivity of the rabbit strain. This could
678 justify the F*dlt*B^{r} strain (with functional *rot*) producing milder lesions than the wild-type strain.
679 A histological characteristic observed from 3 dpi in the lesions produced by the strain wild-type
680 was an eosinophilic necrotic layer in whose core Splendore-Hoeppli phenomena with inner
681 bacteria were observed. These phenomena have been previously described as an electron-dense,
682 granular and amorphous pseudocapsule. This pseudocapsule constitutes a barrier for immune
683 cells by preventing them from penetrating the staphylococcal abscess community (SAC), where
684 bacteria replicate without interference and are partially composed of prothrombin and
685 fibrinogen that separate bacteria from leukocytes [37,58,59]. As the Δ*coa*Δ*vwb* mutant was able to
686 produce lesions like Jwt, the absence of these two clotting factors secreted by *S. aureus* does
687 not seem essential for abscesses to develop in rabbits. Despite all the employed mutants
688 (JΔ*coa*Δ*vwb*, JΔ*hla*, JΔ*psm*α, JΔ*agr*) being able to generate similar lesions to the wild type in
689 size and degree of severity terms, albeit in different percentages, they all triggered a delay in
690 the evolution of histological lesions compared to the wild type. This could indicate that their
691 mutations affected abscess development, but other factors also had to be involved in their
692 development. More studies are needed to learn the specific role of these and other virulence
693 factors in *S. aureus* infection development.

The utility of this model was corroborated when a human strain, modified to infect rabbits ("rabbitized"), was inoculated (F*dlt*B^{r}). This adaptation to the host seemed essential because neither macro- nor microscopic lesions developed when the human Fwt strain was inoculated with 300 CFU [13], while the modified strain (F*dlt*B^{r}) was able to produce similar lesions to the wild strain adapted to rabbit (Jwt).

Finally, although mouse models have been presented as the "gold standard" to study staphylococcal infections in humans, along with their therapy and prevention [60], we propose a low-infective-dose rabbit model as a more representative and realistic model, in which the effect of both homologous and heterologous strains can be proven.

Author contributions

Conceptualization: Laura Selva, Juan M. Corpa, David Viana. Formal analysis: Juan J. Pascual, Alberto Arnau-Bonachera, Juan M. Corpa, David Viana. Funding acquisition: Laura Selva, Juan M. Corpa, David Viana. Methodology: Asunción Muñoz-Silvestre, Mariola Penadés, Laura Selva, Sara Pérez-Fuentes, Elena Moreno, Ana García-Quirós, Juan J. Pascual, Alberto Arnau-Bonachera, Agustín Barragán, Juan M. Corpa, David Viana. Project administration: Juan M. Corpa. Supervision: Juan M. Corpa, David Viana. Writing – original draft: Asunción Muñoz-Silvestre, Alberto Arnau-Bonachera, Juan M. Corpa, David Viana. Writing – review & editing: Alberto Arnau-Bonachera, Juan J. Pascual, Juan M. Corpa, David Viana. Juan M. Corpa and David Viana are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Representation of abscess evolution after experimental infection with *S. aureus* (Jwt strain) with time. **A: 0 days postinoculation (dpi).** Intradermal inoculation of 300 CFU of *S. aureus*. Colored bars and numbers indicate the different histological parts of skin (**1:** epidermis; **2:** superficial dermis; **3:** deep dermis; **4:** cutaneous muscle). **B: 0.5 dpi. 1:** Externally, reddish macule. **2:** Mild vascular phenomena (edema and hemorrhages). **3:** Migration of hererophils mainly from deep vessels (arrows) and grouping at the inoculation point. **4:** Small hemorrhages. **C: 1 dpi. 1:** External slightly elevated reddish papule. **2:** Mild to moderate edema and hemorrhages. **3:** Initial abscess structured as a center of eosinophilic necrosis, with some bacteria surrounded by diffusely distributed heterophils. **4:** Muscular cells moderately infiltrated by heterophils. **D: 2 dpi. 1:** External reddish and elevated nodule and mild epidermal acanthosis, **2:** Moderate edema and hemorrhages. **3:** Well-defined abscess composed of a center of eosinophilic necrosis with bacteria, surrounded by a dense layer of heterophils. **4:** Muscle cells infiltrated by numerous heterophils that induce their separation and necrosis. **E: 3 dpi. 1:** Big dark nodule with epidermal necrosis and acanthosis. **2:** Severe vascular phenomena. **3:** Abscess with the presence of multiple Splendore-Hoeppli phenomena and an external layer of eosinophilic necrosis. **4:** Myocytes severely infiltrated by heterophils causing their degeneration. **F: 7 dpi. 1:** Necrosis and skin ulceration allowing the outflow of purulent content and acanthosis in the periphery to the ulcer. **2:** Very severe vascular phenomena. **3:** Splendore-Hoeppli phenomena merging with one another and the presence of a thin eosinophilic layer of necrosis surrounding the entire abscess. **4:** Cutaneous muscle interrupted by the presence of numerous heterophils and round cells. **G: 14 dpi. 1:** External lack of hair (alopecy), ulcer reepitelization and moderate to severe acanthosis. **2:** Absence of skin adnexa and moderate vascular phenomena. **3:** Presence of immature granulation tissue (numerous small neo-vessels and abundant connective tissue) and some scattered Splendore-Hoeppli phenomena. **4:** Atrophy of myocytes. **H: 21 dpi. 1:** External alopecy, very severe acanthosis. **2 and 3:** Abundant mature granulation tissue. **4:** Interstitial fibrosis and atrophy of myocytes. **I: 28 dpi. 1:** External small area of alopecy and moderate acanthosis. **2:** Invasion of skin appendages to the periphery of the scar. **3:** Mature granulation tissue with abundant fibrous tissue. **4:** Completely regenerated cutaneous muscle.

Figure 2. Correspondence between the gross (external and internally) and histological lesions during the experiment of the wild-type (Jwt) *S. aureus* strain inoculation. A: external aspect; B: sagittal cut of formalin fixed tissue; C: microscopically low magnification view stained with Hematoxilin-Eosin (H&E).

Figure 3. Colony-forming units after intradermal inoculation with Jwt (n = 10 animals per time). [a-c] The means not sharing superscript significantly differ ($P<0.05$). Error bars correspond to the standard error for each least square mean.

Figure 4. Different histological characteristics of lesions throughout infection with the Jwt *S. aureus* strain. **A** (0.5 dpi): Slight increase in the number of heterophils. Deep dermis. H&E. Scale bar = 200 µm. **B** (1dpi): Abscess surrounded by numerous diffusely distributed heterophils. Skin. H&E. Scale bar = 1 mm. **C** (2dpi): Well-defined abscess characterized by a center of eosinophilic necrosis, with numerous coccoid bacteria inside (insert). Skin and bacteria (insert). H&E. Scale bar = 1 mm (skin) and 50 µm (insert). **D** (2pi): Muscular degeneration caused by the intense interstitial infiltration of heterophils. Cutaneous muscle. H&E. Scale bar = 100 µm. **E** (3dpi): Necrosis of epidermis. Epidermis. H&E. Scale bar = 200 µm. **F** (3dpi): A bacterial colony surrounded by star-shaped homogeneous eosinophilic material and an external layer of inflammatory cells. Splendore-Hoeppli phenomenon. H&E. Scale bar = 20 µm. **G** (3dpi): Abscess internally composed of the fusion of several Splendore-Hoeppli phenomena, surrounded by a thin eosinophilic layer of necrosis. Skin. H&E. Scale bar = 1 mm. **H** (3dpi): Severe atrophy of myocytes, infiltrated by connective tissue (left) *versus* normal muscular tissue (right). Cutaneous muscle. Masson's trichrome stain. Scale bar = 200 µm. **I** (7dpi): Significant increase in the number of cells with numerous mitosis (white arrows) on the basal layer of the epidermis (epidermal hyperplasia or acanthosis), compared to normal epidermis (insert). Epidermis. H&E. Scale bars = 100 µm (acanthosis) and 50 µm (insert). **J** (14dpi): Severe inflammatory reaction associated with an ulcer and epidermal hyperplasia on the border. Epidermis. H&E. Scale bar = 200 µm. **K** (14dpi): Repair of the lesion characterized by acanthosis, absence of skin adnexa (left) and presence of abundant granulation tissue composed of numerous vessels and fibrous tissue (right). Skin (left) and deep dermis (right). H&E. Scale bars = 500 µm (left) and 200 µm (right). **L** (28dpi): Epithelial hyperplasia (arrow heads) and dermis with abundant fibrous tissue and blood vessels and scarce heterophils and some small hemorrhages (asterisk). The periphery of the lesion is invaded by hair follicles (arrows). Epidermis and dermis. H&E. Scale bar = 200 µm.

Figure 5. Hyperplasia of the epithelium with the presence of numerous mitosis in the basal layer (white arrows) on 21 days postinfection with the Jwt *S. aureus* strain. Insert: Normal epithelium. H&E. Scale bars: 100 µm.

Figure 6. Evolution of the counts of $CD3^+$(A), IgG^+(B) and $RAM11^+$(C) cells immunochemically marked in the skin samples and total leukocytes (D), total lymphocytes (E), granulocytes (F), monocytes (G), lymphocytes B (H) and lymphocytes T $CD25^+$ (I) (x 10^6/L) in peripheral blood after intradermal inoculation with the Jwt strain (n = 10 animals per day). [a-e] The means that do not share a superscript in the same figure significantly differ (P<0.05) for each postinoculation time. Error bars correspond to the standard error for each least square mean.

Figure 7. Severity assessment of gross lesions on day 7 after intradermal inoculation with different mutants (JΔ*coa*Δ*vwb*, n=16; JΔ*hla*, n=15; JΔ*psm*α n=8; JΔ*agr* n=15) *vs.* Jwt (high virulent) and J*rot*$^+$ (low virulent) strains. [a, b] Within an experiment, the groups not sharing letters above the bar significantly differ (P<0.05).

Figure 8. Presence of the main histological characteristics (Splendore-Hoeppli phenomena, A; heterophils surrounding the abscesses, B; and complete eosinophilic layers, C) on day 7 after intradermal inoculation with different mutant strains (JΔ*coa*Δ*vwb*, JΔ*hla*, JΔ*psm*α and JΔ*agr*). Evaluated as variation in relation to intradermal inoculation with Jwt for: JΔ*coa*Δ*vwb* (n=16), JΔ*hla* (n=15), JΔ*psm*α (n=8), JΔ*agr* (n=15). [*] Significant variation in relation to intradermal inoculation with Jwt (P<0.05).

Figure 9. Mean severity assessment of gross lesions after intradermal inoculation with different strains (F*dlt*B^r, Jwt, J*rot*$^+$ and J*dlt*B^h; n= 30 animals per strain). Assessment is represented as the percentage at four levels: Absence, Mild, Moderate, Severe. [a, b] The groups not sharing letters above the bar significantly differ (P<0.05).

Figure 10. Colony-forming units after intradermal inoculation with different strains of *S. aureus* [F*dlt*B^r, Jwt, J*rot*$^+$; n=10 animals per strain and day]. [a-d] The means not sharing

superscript significantly differ (P<0.05). Error bars correspond to the standard error for each least square mean.

Figure 11. Severity assessment of abscesses (heterophils organized) histologically (A) after infections with strains F*dlt*B^{r}, Jwt, J*rot*$^{+}$ and J*dlt*B^{h} (n=30 animals per strain). Assessment is represented at four levels: Absence, Mild, Moderate, Severe. The groups not sharing letters (a, b, c, d, e) above the bar significantly differ (P<0.05). dpi: days postinfection. Evolution of the counts of T-lymphocytes CD3^{+}(B), B-lymphocytes CD79α^{+} (C), plasma cells IgG^{+}(D) and macrophages RAM11^{+}(E) immunochemically marked in skin samples after infection with strains F*dlt*B^{r}, Jwt, J*rot*$^{+}$ and J*dlt*B^{h}. $^{a\text{-}d}$ The means in a graph not sharing superscript significantly differ (P<0.05). Error bars correspond to the standard error for each least square mean.

Figure 12. Effect of *S. aureus* strains on leukocyte population counts (A-I) in peripheral blood and CD4+/CD8 (J) and granulocytes/lymphocytes (K) ratios after intradermal inoculation with strains F*dlt*B^{r}, Jwt, J*rot*$^{+}$ and J*dlt*B^{h}; n=30 animals per strain. $^{a, b, c}$ The means in a same graph not sharing superscript significantly differ at P<0.05. G/L: Granulocytes/Lymphocytes.

Table 1. Number of rabbits inoculated with different *S. aureus* strains, sampling days, expected outcomes after infection and the study where they were involved.

Strain	No. rabbits	Sampling days	Objective	Study
Jwt	90	0, ½, 1, 2, 3, 7, 14, 21, 28	To describe the model in detail after the intradermal inoculation of a virulent *S. aureus* strain of rabbit origin.	1a
J∆*coa*∆*vwb*	10	7	Both coagulases Coa and vWbp are required for abscesses to form. A reduction in the virulence of J∆*coa*∆*vwb* and the presence of differences in the morphology of abscesses were expected.	1b
J∆*hla*	10	7	α-Hemolysin is a cytolitic toxin that promotes dermonecrosis in animal skin infections [1]. A reduction in abscess size and fewer and milder dermonecrotic lesions were expected.	1b
J∆*psm*α	10	7	PSMα performs cytolytic (lysis of erythrocytes and neutrophils) and immunomodulation (induces tolerogenic phenotypes in dendritic cells and inhibits T_H1 differentiation) activities [3]. Diminished reduction in the severity of dermal lesions was expected.	1b
J∆*agr*	10	7	Agr regulates most *S. aureus* toxins and exoenzymes [18]. A dysfunctional strain in this global virulence regulator should reduce the high toxicity in strongly aggressive *S. aureus* strains.	1b
F*dlt*B^r	30	1, 3, 7	F is a human ST121 strain that does not infect rabbits [13]. After its "rabbitization" (introduction of most 5' nonsynonymous SNP at the *dlt*B* locus from rabbit strains), the virulence of F*dlt*B^r was expected to be similar to that of Jwt.	2
J*rot*$^+$	30	1, 3, 7	*rot* is a global virulence regulator, a repressor of toxins [5], which is altered in rabbits. Its restoral is expected to make J*rot*$^+$ less virulent than Jwt.	2
J*dlt*B^h	30	1, 3, 7	After the reversion of the three identified *dlt*B SNPs (*dlt*B^h) in a rabbit strain J (Jwt), loss of infectivity was expected [13].	2

*The *dlt*B gene is involved in the resistance of *S. aureus* to positively charged antimicrobial peptides, such as defensins and other host defense peptides [19].

1028 **Table 2.** The oligonucleotides used in this study.
1029

Oligonucleotides	Description	Sequence
Coa-1m	Checking the *coa* mutants in Jwt and F*dlt*B^r strains by inside-inside PCR.	5'−ATAGAGATGCTGGTACAGG−3'
Coa-2c	Checking the *coa* mutants in Jwt and F*dlt*B^r strains by inside-inside PCR.	5'−GCTTCCGATTGTTCGATGC−3'
Coa-5mB	Deletion of *coa* (Δ*coa*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−cgcggatccTCATGACGATACTTTCAGAGG−3'
Coa-6c	Deletion of *coa* (Δ*coa*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−CCCAATCTACATTAAAGAAAC−3'
Coa-7m	Deletion of *coa* (Δ*coa*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'−GGTAATTACATTTTGGAGGAAAACTCTATC CATAGACATACAG−3'
Coa-8cE	Deletion of *coa* (Δ*coa*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'−ccggaattcGTACGAATGTGAATGGTGGC−3'
Coa-11m	Checking the *coa* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR.	5'−CATACTTCGATCGTTCTATAAG−3'
Coa-12c	Checking the *coa* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR.	5'−TTGCATCTATTAAAGAAGTAGG−3'
PSMα-1mB	Deletion of *psmα* locus (Δ*psmα*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−cgggatccACATGTTGACCATGAATACC−3'
PSMα-2c	Deletion of *psmα* locus (Δ*psmα*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−gtattcaattcgcttaaattattCATTAAGATTACCTCCT TTGC−3'
PSMα-3m	Deletion of *psmα* locus (Δ*psmα*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'-AATAATTTAAGCGAATTGAATAC−3'
PSMα-4cE	Deletion of *psmα* locus (Δ*psmα*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'−ccggaattcAATTGTGGCTTAATTGTTTGC−3'
PSMα-5m	Checking the *psmα* locus mutants in Jwt and F*dlt*B^r strains by inside-inside PCR.	5'−GCAAAGGAGGTAATCTTAATG−3'
PSMα-6m	Checking the *psmα* locus mutants in Jwt and F*dlt*B^r strains by outside-outside PCR.	5'−TACAAAGCCAGCTAATAACC−3'
PSMα-7c	Checking the *psmα* locus mutants in Jwt and F*dlt*B^r strains by outside-outside PCR.	5'−AATGGCAAATTAGACCAGC−3'
PSMα-8c	Checking the *psmα* locus mutants in Jwt and F*dlt*B^r strains by inside-inside PCR.	5'−CGAATTCCATGTGAATGGC−3'
vwb-1mB	Deletion of *vwb* (Δ*vwb*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−cgcggatccTCAGGTTCTAACAATAATGTAG−3'
vwb-2c	Deletion of *vwb* (Δ*vwb*) in Jwt and F*dlt*B^r strains. 5' flanking region.	5'−GCTCCCAATGATAAAACTAGC−3'
vwb-3m	Deletion of *vwb* (Δ*vwb*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'−gctagttttatcattgggagcAAGCAAATAATGAGTTT GCCG−3'
vwb-4cE	Deletion of *vwb* (Δ*vwb*) in Jwt and F*dlt*B^r strains. 3' flanking region.	5'−ccggaattcTTTGTTGTCAGCTAAACTTCC−3'
vwb-7m	Checking the *vwb* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR. 3' flanking region.	5'−ACCAATTCCAGAGGATTCAG−3'
vwb-8c	Checking the *vwb* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR. 5' flanking region.	5'−TATTCAATTTATCTTCAGAAGC−3'
vwb-11m	Checking the *vwb* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR. 3' flanking region.	5'−GGCTGAAGATGAGGCTTTG−3'
vwb-12c	Checking the *vwb* mutants in Jwt and F*dlt*B^r strains by outside-outside PCR. 5' flanking region.	5'−TAAGTCGCACTTTAATTGC−3'

The sequences recognized by the restriction enzymes used in cloning are denoted lower case letters.
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1032 **Table 3.** The monoclonal antibodies used in the immunohistochemical studies.
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Primary antibodies (dilution)	Isotype	Reactivity	Antigen retrieval	Reference & Company	Secondary antibodies (dilution)	Reference & Company
Mouse anti-canine CD3 (1:20)	IgG1	T-cells	Enzymatic treatment	CA17.2A12, UC Davis, USA	Biotinylated anti-rat IgG (H+L) (1:400)	BA-9400, Vector Laboratories, USA
Mouse anti-human CD79α (1:50)	IgG1	B-cells	Heat treatment	MCA2538H, AbD Serotec, UK	Biotinylated anti-mouse IgG (1 drop into 10mL of diluent)	PK 6102, Vector Laboratories, USA
Mouse anti-rabbit macrophage clone RAM11 (1:50)	IgG1k	Macrophages	Enzymatic treatment	M0633, Dako, USA	Biotinylated anti-mouse IgG (1 drop into 10mL of diluent)	PK 6102, Vector Laboratories, USA
Mouse anti-rabbit light chain, HRP conjugated (1:250)	IgG1k	Plasma cells	Enzymatic treatment	MAB201P, Merck, Germany	None	-

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Table 4. The monoclonal antibodies used in the flow cytometry studies.

Monoclonal antibodies	Isotype	Specificity	Reactivity	Clone	Reference & Company
Mouse anti-rabbit IgM	IgG1	IgM	B-cells	NRBM	MCA812GA, AbD Serotec, UK
Mouse anti-rabbit T-lymphocytes: FITC*	IgG1	CD5	T-cells	KEN-5	MCA800F, AbD Serotec, UK
Mouse anti-rabbit CD4	IgG2a	CD4	T CD4 cells	KEN-4	MCA799, AbD Serotec, UK
Mouse anti-rabbit CD8	IgG2a	CD8	T CD8 cells	ISC27A	WS0796U-100, Kingfisher Biotech, USA
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T-cells	KEI-α1	MCA1119G, AbD Serotec, UK
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK4	MCA1568F, AbD Serotec, UK
Mouse anti-rabbit α-CD45	IgM	CD45	All leucocytes	ISC76A	ISC76A, VMRD Inc., USA

*Clon KEN-5 recognizes rabbit T-lymphocytes and immunoprecipitates. This antibody recognizes rabbit CD5 but does not bind to rabbit CD5 transfectants. Known rabbit CD5 antibodies also show binding to most B-lymphocytes, which are not labeled by this clone (information obtained from datasheet).

Table 5. The histopathological findings observed after the inoculation of a Jwt *S. aureus* strain and their evolution during the experiment (n = 10 animals per day).

	Days postinoculation (dpi)								*P* value
	0.5	1	2	3	7	14	21	28	
Epidermis[*]									
Hyperplasia	100/0/0/0^{a}	100/0/0/0^{a}	30/60/10/0^{b}	27/27/36/10^{b}	0/0/0/100^{d}	0/0/0/100^{d}	0/8/50/42^{c}	0/16/46/38^{c}	0.001
Reepithelization (mitosis)	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	58/8/17/17^{b}	25/25/0/50^{b}	0/0/8/92^{c}	0.001
Superficial dermis									
Vascular dilatation	80/20/0/0^{a}	30/60/10/0^{ab}	30/60/0/0^{ab}	18/55/27/0^{bc}	0/10/40/50^{d}	0/50/50/00^{c}	17/42/33/8^{c}	23/46/31/0^{bc}	0.001
Hemorrhages	89/0/0/11^{a}	10/80/10/0^{bc}	40/50/10/0^{abc}	27/27/36/10^{c}	10/40/30/20^{c}	50/25/17/8^{ab}	67/33/0/0^{a}	31/23/38/8^{c}	0.001
Edema	80/0/20/0^{ab}	70/10/20/0^{ab}	80/0/20/0^{ab}	18/10/36/36^{d}	0/60/20/20^{cd}	33/17/33/17^{bc}	67/25/8/0^{ab}	85/15/0/0^{a}	0.001
Deep dermis									
Vascular dilatation	80/20/0/0^{a}	0/100/0/0^{a}	30/70/0/0^{a}	18/55/27/0^{ab}	0/60/40/0^{b}	0/100/0/0^{ab}	17/42/33/8^{ab}	23/46/23/8^{a}	0.132
Perivascular inflammation	30/50/10/10^{b}	0/20/10/70^{e}	10/10/30/50de	0/36/55/9^{c}	0/20/60/20^{cd}	0/40/30/30^{cd}	0/66/17/17^{bc}	75/15/8/0^{a}	0.001
Heterophiles diffuses	40/50/10/0^{a}	10/40/10/40^{bc}	10/20/50/20^{bc}	0/27/63/0^{bc}	0/30/30/40^{c}	8/33/42/17^{b}	0/41/42/17^{bc}	69/15/16/0^{a}	0.001
Heterophiles organized (abscess)	80/10/0/10^{a}	20/10/10/60^{b}	20/10/10/60^{bc}	0/0/0/100^{c}	0/0/0/100^{c}	20/0/0/80^{bc}	75/0/0/25^{a}	92/8/0/0^{a}	0.001
Eosinophilic peri-abscess layer	100/0/0/0^{a}	50/30/20/0^{b}	40/10/0/50^{cd}	0/0/18/82^{d}	0/0/50/50^{cd}	20/0/40/40^{c}	75/0/17/8^{b}	92/8/0/0^{ab}	0.001
Hemorrhages	100/0/0/0^{a}	40/60/0/0^{cd}	30/70/0/0^{d}	0/100/0/0^{d}	30/70/0/0^{d}	75/17/0/8^{bc}	100/0/0/0^{a}	85/15/0/0^{b}	0.001
Granulation tissue	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	75/8/17/0^{b}	0/9/8/83^{c}	15/0/0/85^{c}	0.001
Regeneration (hair follicles)	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	83/17/0/0^{a}	9/76/15/0^{b}	0.001
Cutaneous muscle									
Interstitial inflammation	40/40/0/20^{b}	10/10/60/20^{cde}	10/50/20/20^{bcd}	0/28/36/36de	0/0/30/70^{e}	0/50/10/40^{cd}	25/33/25/17^{bc}	75/25/0/0^{a}	0.001
Atrophy/degeneration	90/0/10/0^{a}	70/20/10/0^{ab}	70/20/10/0^{ab}	35/55/10/0^{ab}	0/30/70/0^{c}	8/50/8/34^{c}	33/42/8/17^{bc}	75/8/0/15^{a}	0.001
Hypertrophy	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	75/25/0/0	92/8/0/0	69/31/0/0	0.204
Interruption	90/10/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	70/20/10/0^{ab}	40/30/20/10^{b}	50/33/0/17^{ab}	76/8/8/8^{ab}	100/0/0/0^{a}	0.010
Fibrosis	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	100/0/0/0^{a}	40/60/0/0^{a}	25/50/17/8^{c}	58/42/0/0^{b}	92/8/0/0^{b}	0.001

[*] Each histopathological finding presents 4 values w/x/y/z that indicate the percentage of animals with microscopic findings classified as absent (w), mild (x), moderate (y) and severe (z).

For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.

$^{a-e}$ The means in a same row not sharing superscript significantly differ for each inoculation time ($P<0.05$).

Table 6. Evolution of the concentration (pg/mL) of cytokines on plasma and skin tissue after the intradermal inoculation with the Jwt strain (n = 10 animals per day).

	Postinoculation time (days)									SEM	*P*-value
	0	0.5	1	2	3	7	14	21	28		
Plasma											
IL-4	3.587^{ab}	2.317^{a}	3.375^{a}	5.701^{b}	3.780^{b}	3.751^{b}	3.886^{b}	5.552^{b}	8.904^{c}	0.807	<0.001
IL-18	126.8^{ab}	316.4^{bc}	263.0^{bc}	245.9^{b}	328.2^{bc}	87.2^{a}	350.0^{c}	307.2^{bc}	463.4^{d}	39.8	<0.001
Skin											
IL-4	8.998^{a}	6.419^{a}	7.080^{a}	7.780^{a}	9.392^{a}	7.307^{a}	8.774^{a}	9.951^{a}	15.479^{b}	1.554	0.001
IFN-gamma	643^{a}	14015^{b}	412^{a}	401^{a}	547^{a}	558^{a}	619^{a}	3879^{a}	2588^{a}	2282	<0.001

$^{a-d}$ The means in a same row not sharing superscript significantly differ at $P<0.05$ for day postinoculation.
For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.
SEM: standard error of the mean.

Table 7. The histopathological findings observed after the inoculation of different *S. aureus* strains (n = 10 animals per day and strain).

	S. aureus strain				*P value*	
	F*dlt*B^{r}	Jwt	J*rot*$^{+}$	J*dlt*B^{h}	Strain	Time*Strain
Epidermis[*]						
Hyperplasia	74/2/21/3^{b}	42/10/13/35^{c}	83/8/6/3^{a}	90/10/0/0^{a}	0.001	0.001
Reepithelization (mitosis)	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	1.000	1.000
Superficial dermis						
Vascular dilatation	44/52/4/0^{b}	16/42/26/16^{c}	40/57/3/0^{a}	33/67/0/0^{ab}	0.001	0.001
Hemorrhages	58/26/16/0^{a}	16/48/26/10^{b}	50/37/13/0^{a}	50/43/7/0^{a}	0.001	0.001
Edema	23/48/29/0^{b}	29/26/26/19^{b}	40/40/17/3^{a}	50/47/3/0^{a}	0.001	0.005
Deep dermis						
Vascular dilatation	2/46/52/0^{b}	0/84/13/3^{a}	0/77/13/10^{a}	0/41/56/3^{b}	0.001	0.012
Perivascular inflammation	2/46/52/0^{a}	0/26/42/32^{b}	77/7/16/0^{a}	3/47/50/0^{a}	0.001	0.001
Heterophiles diffuses	4/54/34/8^{ab}	0/36/39/25^{b}	0/64/23/13^{a}	0/47/47/6^{a}	0.016	0.060
Heterophiles organized (abscess)	58/4/2/36^{b}	6/4/3/87^{c}	60/7/3/30^{a}	67/17/13/3^{a}	0.001	0.001
Eosinophilic peri-abscess layer	75/17/4/4^{b}	16/10/29/45^{c}	83/4/7/6^{ab}	100/0/0/0^{a}	0.001	0.095
Hemorrhages	100/0/0/0^{a}	23/77/0/0^{b}	100/0/0/0^{a}	100/0/0/0^{a}	0.001	0.845
Granulation tissue	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	1.000	1.000
Cutaneous muscle						
Interstitial inflammation	15/46/27/12^{b}	3/14/42/41^{c}	21/63/3/13^{a}	17/53/27/3^{a}	0.001	0.008
Atrophy/degeneration	71/19/10/0^{b}	35/35/30/0^{b}	86/8/3/3^{a}	100/0/0/0^{a}	0.001	0.029
Hypertrophy	100/0/0/0	100/0/0/0	97/3/0/0	100/0/0/0	0.415	1.000
Interruption	96/2/2/0	68/19/10/3	97/0/0/3	97/3/0/0	0.191	0.525
Fibrosis	100/0/0/0	81/19/0/0	100/0/0/0	100/0/0/0	0.066	0.144

[*] Each histological parameter presents 4 values (w/x/y/z) that indicate the average percentage of animals for the three sampling times (1, 3 and 7 days postinfection). The microscopic findings are classified as absent (w), mild (x), moderate (y), severe (z).
For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.
[a-c] The means in a same row not sharing letters significantly differ (P<0.05).

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Supplemental Figure 1. Severe dermonecrosis (A: external aspect; B: sagittal cut of formalin fixed tissue; C: microscopically low magnification view stained with H&E) characterized by a peripheral halo of inflammatory cells (arrows) and a center of coagulative necrosis (asterisk) at 3 days postinoculation of 10^7 CFU of a human wild-type strain (Fwt).

ONLINE SUPPLEMENTAL MATERIAL

Supplemental Table 1. The statistical models used in this work based on the trait and the study.

Group of traits	Study 1*		Study 2†
Trait	1a. Jwt	1b. Mutants	F*dlt*B^r *vs.* J
Animal weight	M1		
Body temperature	M1		M7
CFU	M2		M8
Gross lesions			
Erythema (area)	M3		M9
Nodule (volume)	M3		M9
Histological findings			
Epidermal thickening	M5		M10
Reepithelization	M5		M10
Vascular dilatation	M5		M10
Perivascular inflammation	M5		M10
Edema	M5		M10
Hemorraghes	M5		M10
Heterophiles diffuses	M5	M6	M10
Heterophiles organized (abscess)	M5		M10
Eosinophilic peri-abscess layer	M5	M6	M10
Granulation tissue	M5		M10
Splendore-Hoeppli phenomena		M6	
Regeneration (mitosis)	M5		M10
Interstitial inflammation	M5	M6	M10
Atrophy/degeneration	M5	M6	M10
Hypertrophy	M5		M10
Interruption	M5		M10
Fibrosis	M5		M10
Immunohistochemistry			
Macrophages	M1		M7
Plasma cells	M1		M7
T-lymphocytes	M1		M7
B-lymphocytes	M4		M7
Hematology and flow cytometry			
Total leukocytes	M1		M7
Granulocytes	M1		M7
Total lymphocytes	M1		M7
B-lymphocytes	M1		M7
T-lymphocytes	M1		M7
T CD4$^+$	M1		M7
T CD8$^+$	M1		M7
T CD25$^+$	M1		M7
Monocytes	M1		M7
Cytokines			
IL-1β	M1		
IL-4	M1		
IL-17	M1		
IL-18	M1		
IFN-gamma	M1		

* Study 1. Characterization of the model after infection with a Jwt (1a. Jwt) and mutant strains (1b. Mutants: JΔ*coa*Δ*vwb*, JΔ*hla*, JΔ*psmα* and JΔ*agr*).

† Study 2. Comparison of lesions caused by a human "rabbitized" strain (F*dlt*B^r) with different-virulence rabbit strains (J: Jwt, J*rot*$^+$ and J*dlt*B^h).