ANIMAL MODELS

Pathogenesis of Intradermal Staphylococcal Infections

Rabbit Experimental Approach to Natural Staphylococcus aureus Skin Infections

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Despite the enormous efforts made to achieve effective tools that fight against *Staphylococcus aureus*, the results have not been successful. This failure may be 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 colony-forming units) intradermal model of infection in rabbits, which reproduces the characteristic staphylococcal abscess. Surprisingly, when mutant strains in the genes involved in virulence (*JΔagr*, *JΔcoaΔvwb*, *JΔhla*, and *JΔpsmA*) 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 (*FdltB'*) 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. (*Am J Pathol* 2020, 190: 1188–1210; <https://doi.org/10.1016/j.ajpath.2020.01.019>)

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 the most worrying ones, given community-associated infections, are those that affect both skin and soft tissues.^{4–9} Skin and soft tissue infections 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 staphylococcal 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.

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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 experience natural infections by *S. aureus*. In fact, numerous strains have been reported in commercial rabbitries¹² ever since a sporadic mutation favored the human-to-rabbit host jump 40 years ago.¹³ It has been described that human skin morphologic^{14,15} and immunologic¹⁶ 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.

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

This study presents, 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: 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; 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*

($\Delta coa\Delta vwb$), *hla* (Δhla), *psma* ($\Delta psma$), and *agr* (Δagr); *Jrot*⁺ (Jwt with *rot* gen restored); *JdltB*^h (Jwt with the reversion of three identified *dltB* single-nucleotide polymorphisms); and *FdltB*^r, a human ST121 strain expressing *dltB* from rabbit clones¹³ (Table 1).

Bacteria were grown at 37°C overnight on trypticase soy agar medium supplemented with antibiotics as appropriate. Broth cultures were grown at 37°C in trypticase soy broth with shaking (240 revolutions per minute). The procedures for the preparation and analysis of phage lysates and 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 pmol/L 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 nonpermissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into 10 mL of trypticase soy broth and incubated for 24 hours at 30°C. Ten-fold serial dilutions of this culture in sterile trypticase soy broth were plated on trypticase soy agar 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

A total of 220 2-month-old albino hybrid rabbits (*Oryctolagus cuniculus*; 10 animals per sampling time) of either sex 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, and 28), it was established that 7 dpi was the optimal time to compare infections with mutants in various toxins ($\Delta coa\Delta vwb$, Δhla , and $\Delta psma$) and general regulators (Δagr) 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

Table 1 Number of Rabbits Inoculated with Different *Staphylococcus aureus* Strains, Sampling Days, Expected Outcomes after Infection, and the Study Where They Were Involved

Strain	Rabbits, <i>n</i>	Sampling days	Objective	Study
Jwt	90	0, 0.5, 1, 2, 3, 7, 14, 21, 28	To describe the model in detail after the intradermal inoculation of a virulent <i>S. aureus</i> strain of rabbit origin.	1a
JΔcoaΔvwb	10	7	Both coagulases Coa and vWbp are required for abscesses to form. A reduction in the virulence of JΔcoaΔvwb and the presence of differences in the morphology of abscesses were expected.	1b
JΔhla	10	7	α-Hemolysin is a cytolytic toxin that promotes dermonecrosis in animal skin infections. ¹ A reduction in abscess size and fewer and milder dermonecrotic lesions were expected.	1b
JΔpsmα	10	7	PSMα performs cytolytic (lysis of erythrocytes and neutrophils) and immunomodulation (induces tolerogenic phenotypes in dendritic cells and inhibits type 1 helper T-cell differentiation) activities. ³ Diminished reduction in the severity of dermal lesions was expected.	1b
JΔagr	10	7	Agr regulates most <i>S. aureus</i> toxins and exoenzymes. ¹⁸ A dysfunctional strain in this global virulence regulator should reduce the high toxicity in strongly aggressive <i>S. aureus</i> strains.	1b
FdltB ^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 SNPs at the <i>dltB</i> * locus from rabbit strains), the virulence of FdltB ^r was expected to be similar to that of Jwt.	2
Jrot ⁺	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 Jrot ⁺ less virulent than Jwt.	2
JdltB ^h	30	1, 3, 7	After the reversion of the three identified <i>dltB</i> SNPs (<i>dltB</i> ^h) in a rabbit strain J (Jwt), loss of infectivity was expected. ¹³	2

*The *dltB* gene is involved in the resistance of *S. aureus* to positively charged antimicrobial peptides, such as defensins and other host defense peptides.¹⁹ Agr, accessory gene regulator; PSMα, phenol-soluble modulín α; SNP, single-nucleotide polymorphism.

behavior of a human rabbitized strain (FdltB^r) with three rabbit strains (Jwt, Jrot⁺, and JdltB^h) of well-known virulence in the acute and early chronic phases of infection evolution because, as before (7 dpi), 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 hours of light and 8 hours of darkness in individual cages (600 × 750 × 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 Alimentacio, 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 (Boletín Oficial del Estado, 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 (CFUs) of each studied strain suspended in 0.1 mL of phosphate-buffered saline 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 10 × 10-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.

Gross, Microscopic, and Microbiological Studies

The characteristics of the skin gross lesions (presence of erythema, edema, skin elevation, nodules, dermonecrosis, and/or ulceration) were recorded daily. Abscess dimensions were measured with a caliper. The length (L) and width (W) values were used to calculate abscess areas ($A = \pi [L \times W]/2$) and volumes ($V = 4/3\pi [L/2]^2 \times [W/2]$).^{26,27} Macroscopic lesions were evaluated by their abscess area as follows: healthy (no apparent lesions), mild (<0.5 cm²),

Table 2 The Oligonucleotides Used in This Study

Oligonucleotide	Description	Sequence
Coa-1m	Checking the <i>coa</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by inside-inside PCR.	F: 5'-ATAGAGATGCTGGTACAGG-3'
Coa-2c	Checking the <i>coa</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by inside-inside PCR.	R: 5'-GCTTCCGATTGTTTCGATGC-3'
Coa-5mB	Deletion of <i>coa</i> (Δ <i>coa</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	F: 5'-cgcggaatccTCATGACGATACTTTCAGAGG-3'
Coa-6c	Deletion of <i>coa</i> (Δ <i>coa</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	R: 5'-CCCAATCTACATTAAAGAAAC-3'
Coa-7m	Deletion of <i>coa</i> (Δ <i>coa</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	F: 5'-GGTAATTACATTTTGGAGGAAAACT CTATCCATAGACATACAG-3'
Coa-8cE	Deletion of <i>coa</i> (Δ <i>coa</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	R: 5'-ccggaattcGTACGAATGTGAATGGTGGC-3'
Coa-11m	Checking the <i>coa</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR.	F: 5'-CATACTTCGATCGTTCTATAAG-3'
Coa-12c	Checking the <i>coa</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR.	R: 5'-TTGCATCTATTAAAGAAGTAGG-3'
PSM α -1mB	Deletion of <i>psmα</i> locus (Δ <i>psmα</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	F: 5'-cgggatccACATGTTGACCATGAATACC-3'
PSM α -2c	Deletion of <i>psmα</i> locus (Δ <i>psmα</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	R: 5'-gtattcaattcgcttaattattCATTAA ATTACCTCCTTTCG-3'
PSM α -3m	Deletion of <i>psmα</i> locus (Δ <i>psmα</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	F: 5'-AATAATTTAAGCGAATTGAATAC-3'
PSM α -4cE	Deletion of <i>psmα</i> locus (Δ <i>psmα</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	R: 5'-ccggaattcAATTGTGGCTTAATTGTTTCG-3'
PSM α -5m	Checking the <i>psmα</i> locus mutants in Jw ^t and <i>FdltB</i> ^r strains by inside-inside PCR.	F: 5'-GCAAAGGAGGTAATCTTAATG-3'
PSM α -8c	Checking the <i>psmα</i> locus mutants in Jw ^t and <i>FdltB</i> ^r strains by inside-inside PCR.	R: 5'-CGAATTCCATGTGAATGGC-3'
PSM α -6m	Checking the <i>psmα</i> locus mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR.	F: 5'-TACAAAGCCAGCTAATAACC-3'
PSM α -7c	Checking the <i>psmα</i> locus mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR.	R: 5'-AATGGCAAATTAGACCAGC-3'
vwb-1mB	Deletion of <i>vwb</i> (Δ <i>vwb</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	F: 5'-cgcggaatccTCAGGTTCTAACAATAATGTAG-3'
vwb-2c	Deletion of <i>vwb</i> (Δ <i>vwb</i>) in Jw ^t and <i>FdltB</i> ^r strains; 5' flanking region.	R: 5'-GCTCCCAATGATAAACTAGC-3'
vwb-3m	Deletion of <i>vwb</i> (Δ <i>vwb</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	F: 5'-gctagttttatcattgggagcAAGCAA ATAATGAGTTTGCCG-3'
vwb-4cE	Deletion of <i>vwb</i> (Δ <i>vwb</i>) in Jw ^t and <i>FdltB</i> ^r strains; 3' flanking region.	R: 5'-ccggaattcTTTGTGTGCTAGCTAAACTTCC-3'
vwb-7m	Checking the <i>vwb</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR; 3' flanking region.	F: 5'-ACCAATTCCAGAGGATTTCAG-3'
vwb-11m	Checking the <i>vwb</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR; 3' flanking region.	F: 5'-GGCTGAAGATGAGGCTTTG-3'
vwb-8c	Checking the <i>vwb</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR; 5' flanking region.	R: 5'-TATTCAATTTATCTTCAGAAGC-3'
vwb-12c	Checking the <i>vwb</i> mutants in Jw ^t and <i>FdltB</i> ^r strains by outside-outside PCR; 5' flanking region.	R: 5'-TAAGTCGCACTTTAATTGC-3'

The sequences recognized by the restriction enzymes used in cloning are denoted by lowercase letters.

F, forward; R, reverse.

moderate (0.5 to 5 cm²), or severe (>0.5 cm² and gross necrosis).

On sampling, rabbits were sedated with a combination of ketamine (Imalgene; 100 mg/mL) and xylazine (Xilagesic; 200 mg/mL), and were euthanized by an i.v. injection of

barbiturate (T-61; Intervet International GmbH, Unterschleißheim, Germany); a complete necropsy was performed. Skin samples from the inoculation place were used for the microscopic and microbiological studies and the cytokine assay. For the histologic examination, skin samples were

Table 3 The Monoclonal Antibodies Used in the Immunohistochemical Studies

Primary antibodies (dilution)	Isotype	Reactivity	Antigen retrieval	Reference (manufacturer)	Secondary antibodies (dilution)	Reference (manufacturer)
Mouse anti-canine CD3 (1:20)	IgG1	T cells	Enzymatic treatment	CA17.2A12 (University of California, Davis, Davis, CA)	Biotinylated anti-rat IgG (H + L) (1:400)	BA-9400 (Vector Laboratories, Burlingame, CA)
Mouse anti-human CD79 α (1:50)	IgG1	B cells	Heat treatment	MCA2538H (AbD Serotec, Kidlington, UK)	Biotinylated anti-mouse IgG (1 drop into 10 mL of diluent)	PK 6102 (Vector Laboratories)
Mouse anti-rabbit macrophage clone RAM11 (1:50)	IgG1k	Macrophages	Enzymatic treatment	M0633 (Dako, Capertina, CA)	Biotinylated anti-mouse IgG (1 drop into 10 mL of diluent)	PK 6102 (Vector Laboratories)
Mouse anti-rabbit light chain, HRP conjugated (1:250)	IgG1k	Plasma cells	Enzymatic treatment	MAB201P (Merck, Darmstadt, Germany)	None	

HRP, horseradish peroxidase.

routinely stained with hematoxylin and eosin, Masson trichrome, and Gram's stains, and were processed by immunohistochemistry for macrophages, T and B lymphocytes, and plasma cell detection by the avidin-biotin-peroxidase complex method at the dilutions recommended by the manufacturer (Table 3). In the immunohistochemical procedure, positive cells were enumerated in the inoculation areas and, when lesions were severe, in the border of the necrotized tissue of 20 0.08-mm² randomly selected fields per slide. The histologic and immunohistochemical findings observed on the different skin layers were described and recorded in detail.

Before fixation, representative samples were taken from skin lesions and kidneys, and were weighed under sterile conditions for the microbiological studies. Then, they were immersed in a cold solution of phosphate-buffered saline

and mechanically homogenized in the presence of ice. Tissue homogenate (100 μ L) was cultivated on blood-agar (BioMérieux, Marcy l'Etoile, France) and was incubated aerobically at 37°C for 24 hours. This allows the results to be expressed in CFUs/g. The colonies that grew were identified by coagulase PCR¹² to prove that the strains isolated from skin lesions were the same as those originally inoculated and to assess the possibility of septicemia in the samples taken from kidneys.

Hematology and Flow Cytometric Analysis

Blood samples (1 mL) in EDTA anticoagulant were obtained from the median artery of the ear. White blood cell counts and lymphocyte proportions were determined using a

Table 4 The Monoclonal Antibodies Used in the Flow Cytometry Studies

Monoclonal antibodies	Isotype	Specificity	Reactivity	Clone	Reference (manufacturer)
Mouse anti-rabbit IgM	IgG1	IgM	B cells	NRBM	MCA812GA (AbD Serotec, Kidlington, UK)
Mouse anti-rabbit T lymphocytes: FITC*	IgG1	CD5	T cells	KEN-5	MCA800F (AbD Serotec)
Mouse anti-rabbit CD4	IgG2a	CD4	T CD4 cells	KEN-4	MCA799 (AbD Serotec)
Mouse anti-rabbit CD8	IgG2a	CD8	T CD8 cells	ISC27A	WS0796U-100 (Kingfisher Biotech, St. Paul, MN)
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T cells	KEI- α 1	MCA1119G (AbD Serotec)
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK4	MCA1568F (AbD Serotec)
Mouse anti-rabbit α -CD45	IgM	CD45	All leukocytes	ISC76A	ISC76A (Veterinary Medical Research & Development, Pullman, WA)

*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 data sheet).

FITC, fluorescein isothiocyanate.

Figure 1 Representation of abscess evolution after experimental infection with *Staphylococcus aureus* (Jwt strain) with time. **A:** At 0 days postinoculation (dpi). Intradermal inoculation of 300 colony-forming units (CFUs) of *S. aureus*. Colored bars and numbers indicate the different histologic parts of skin (1, epidermis; 2, superficial dermis; 3, deep dermis; and 4, cutaneous muscle). **B:** At 0.5 dpi. 1: Externally, reddish macule. 2: Mild vascular phenomena (edema and hemorrhages). 3: Migration of heterophils mainly from deep vessels (arrows) and grouping at the inoculation point. 4: Small hemorrhages. **C:** At 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:** At 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:** At 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:** At 7 dpi. 1: Necrosis and skin ulceration, allowing the outflow of purulent content and acanthosis in the periphery to the ulcer. 2: 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:** At 14 dpi. 1: External lack of hair (alopecia), ulcer re-epithelialization, and moderate to severe acanthosis. 2: Absence of skin adnexa and moderate vascular phenomena. 3: Presence of immature granulation tissue (numerous small neovessels and abundant connective tissue) and some scattered Splendore-Hoeppli phenomena. 4: Atrophy of myocytes. **H:** At 21 dpi. 1: External alopecia, severe acanthosis. 2 and 3: Abundant mature granulation tissue. 4: Interstitial fibrosis and atrophy of myocytes. **I:** At 28 dpi. 1: External small area of alopecia 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 histologic lesions during the experiment of the wild-type (Jwt) *Staphylococcus aureus* strain inoculation. **A:** External aspect. **B:** Sagittal cut of formalin-fixed tissue. **C:** Microscopically low-magnification view stained with hematoxylin-eosin. Dpi, days postinfection.

hematology analyzer (MEK-6410; Nihon Kohden, Tokyo, Japan). The flow cytometric analysis of the white blood cells (B, T, T CD4⁺, T CD8⁺ and activated T cells, monocytes, and granulocytes) was performed using specific primary antibodies (Table 4) and secondary antibodies [rat anti-mouse IgG2a + b phycoerythrin conjugate (Veterinary Medical Research & Development, Pullman, WA) and goat anti-mouse IgM phycoerythrin conjugate (AbD Serotec, Kidlington, UK)], as previously described.^{28,29}

The common leukocyte antigen CD14 and CD45 expression was used for the lymphogate setup, as previously described.^{28,29} The calculation of the total lymphocyte and the respective subset counts were performed as the product of the white blood cell count and specific population percentages, as described elsewhere.^{29,30}

Cytokine Assay

The representative ILs of the immune response [IL-1 β , IL-4, IL-17, IL-18, and interferon (IFN)- γ] from plasma and the inoculated skin tissues were analyzed by enzyme-linked immunosorbent assay kits (CUSABIO, Wuhan, China), according to the manufacturer's protocol. The samples taken from skin abscesses were immersed in a cold solution of phosphate-buffered saline with a protease inhibitor (Complete Mini; Roche, Basel, Switzerland), and were mechanically homogenized for 1 minute by stopping each 10 seconds to avoid overheating in a T50 Ultra-Turrax (IKA, Staufen, Germany) in the presence of ice. Next, they were centrifuged at $13,000 \times g$ for 10 minutes. Aliquots of the supernatant were stored at -80°C for further processing.

Statistical Analysis

Study 1a: Characterization of the Model after Infection with Jwt

The data obtained in this study were evaluated by five different models, depending on the type of data recorded for each trait (Table 1 and Supplemental Table S1). The five models included the postinoculation day (nine levels: 0, 0.5, 1, 2, 3, 7, 14, 21, and 28) as the fixed effect. Except for CFUs, the data of quantitative traits with one record per animal were analyzed by a linear model M1 (proc GLM; SAS version 9.2; SAS Institute, Cary, NC). CFU usually presents changes in the order of magnitude as infections evolve. Considering this situation, the CFU data were analyzed by a mixed model M2 and by considering different variances for the random residual errors on each postinoculation day. The data of the quantitative traits with more than one record per animal were analyzed by a mixed model M3 (proc MIXED, SAS version 9.2), which included the effect of the individual on infection as random effects [90 levels; $N \sim (0, \sigma_p)$]. The data of the categorical traits with two levels (eg, yes/no, presence/absence, 0/1) were analyzed by a generalized linear model M4 (proc GENMOD, SAS version 9.2) after considering that the response variable followed a binomial distribution and by using logistic transformation ($\ln [\mu/(1 - \mu)]$) as a link function. Finally, the ordinal data of the histology traits had more than two levels because injuries were classified according to the following scale (–, –/+, +, +/++, ++, ++/+++, +++, +++/++++, +++++), where ++ was greater (more severe) than +. However, this did not mean that the distance between each class was proportional. To take this situation into account, the data of the histology traits were analyzed by a generalized linear model M5 (proc GENMOD, SAS version 9.2) after considering that the response variable followed a multinomial 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 pairwise comparison of the means was performed using Yates correction. The *t*-test was performed for the traits with

Figure 3 Colony-forming units (CFUs) after intradermal inoculation with Jwt strain of *Staphylococcus aureus*. Error bars correspond to the SEM for each least square mean. $n = 10$ animals per time point. ^{a-c}The means not sharing superscript significantly differ ($P < 0.05$).

quantitative data, and the χ^2 test was performed 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 S1). The four models included day postinoculation (three levels: 1, 3, and 7), the strain (four levels: Jwt, *FdltB*⁺, *Jrot*⁺, and *JdltB*^h), and their interaction as fixed effects. Except for CFUs, the data of the quantitative traits with only one record per animal were analyzed by a linear model M7 (proc GLM, SAS version 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 version 9.2) and included the effect of the individual at the experimental infection time as random effects [120 levels: $N \sim (0, \sigma_p)$]. 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 version 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)

After evidencing that rabbits can be infected by a low-dose charge of *S. aureus*,¹³ this study 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°C at 0 dpi and 39.5°C at 28 dpi; $P = 0.001$), 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 CFUs of *S. aureus* (Figure 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 (1 dpi) and firm nodules (2 dpi) in 100% of the animals and at 100% of the inoculation points (20 of 20). The size and intensity of the reddened areas reached its apogee at 2 dpi (2.003 ± 0.51 versus 3.422 ± 0.47 cm² 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 cm³; $P < 0.001$) and 50% of the animals (5 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 discharge of a purulent material. Draining abscesses reduced abscess size. From 14 to 28 dpi, a repair of the ulcers occurred, and absence of hair and scar retraction were observed at the end of the study (Figure 2).

Bacteriology

The bacterial counts increased at 0.5 dpi (1.11×10^7 CFUs/g), with the largest number obtained at 3 dpi (50.98×10^7 CFUs/g) and, coinciding with the opening of abscesses, the number of bacteria lowered until the end of the experiment, when the lesions of only 4 of the 10 animals contained bacteria (1.16×10^3 CFUs/g at 28 dpi) (Figure 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.

Histologic Findings

Some animals showed early histopathologic modifications at 0.5 dpi (Table 5). In these cases, lesions were characterized by moderate edema and minor hemorrhages in the superficial dermis and by a slight increase in the density of heterophils in the deep dermis, which sometimes grouped into small clusters of cells (Figures 1B and 4A). The capillaries 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 (Figure 4B). Cutaneous muscle fibers were infiltrated by heterophils, which provoked a mild degeneration and atrophy of myocytes (Figure 1C).

Two days after infection, lesions were similar to the way they 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 (Figure 4C). Around these foci, there were also heterophils. Cutaneous muscle cells were severely infiltrated by heterophils, which induced their separation and necrosis (Figures 1D and 4D).

On 3 dpi, the percentage of animals with moderate to severe epidermal hyperplasia was higher than on 2 dpi (46% versus 10%). The presence of more severe vascular phenomena (vascular dilatation, edema, and hemorrhages) in the superficial dermis induced skin tumefaction, and epidermis necrosis when the volume of abscesses was sizable (Figure 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) (Figure 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

Table 5 The Histopathologic Findings Observed after the Inoculation of a Jwt *Staphylococcus aureus* Strain and Their Evolution during the Experiment

Variable	Days postinoculation			
	0.5	1	2	3
Epidermis*				
Hyperplasia	100/0/0/0 ^a	100/0/0/0 ^a	30/60/10/0 ^b	27/27/36/10 ^b
Re-epithelization (mitosis)	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a
Superficial dermis				
Vascular dilatation	80/20/0/0 ^a	30/60/10/0 ^{ab}	30/60/0/0 ^{ab}	18/55/27/0 ^{bc}
Hemorrhages	89/0/0/11 ^a	10/80/10/0 ^{bc}	40/50/10/0 ^{abc}	27/27/36/10 ^c
Edema	80/0/20/0 ^{ab}	70/10/20/0 ^{ab}	80/0/20/0 ^{ab}	18/10/36/36 ^d
Deep dermis				
Vascular dilatation	80/20/0/0 ^a	0/100/0/0 ^a	30/70/0/0 ^a	18/55/27/0 ^{ab}
Perivascular inflammation	30/50/10/10 ^b	0/20/10/70 ^e	10/10/30/50 ^{de}	0/36/55/9 ^c
Heterophiles diffuse	40/50/10/0 ^a	10/40/10/40 ^{bc}	10/20/50/20 ^{bc}	0/27/63/0 ^{bc}
Heterophiles organized (abscess)	80/10/0/10 ^a	20/10/10/60 ^b	20/10/10/60 ^{bc}	0/0/0/100 ^c
Eosinophilic peri-abscess layer	100/0/0/0 ^a	50/30/20/0 ^b	40/10/0/50 ^{cd}	0/0/18/82 ^d
Hemorrhages	100/0/0/0 ^a	40/60/0/0 ^{cd}	30/70/0/0 ^d	0/100/0/0 ^d
Granulation tissue	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a
Regeneration (hair follicles)	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a
Cutaneous muscle				
Interstitial inflammation	40/40/0/20 ^b	10/10/60/20 ^{cde}	10/50/20/20 ^{bcd}	0/28/36/36 ^{de}
Atrophy/degeneration	90/0/10/0 ^a	70/20/10/0 ^{ab}	70/20/10/0 ^{ab}	35/55/10/0 ^{ab}
Hypertrophy	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0
Interruption	90/10/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a	70/20/10/0 ^{ab}
Fibrosis	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a	100/0/0/0 ^a

(table continues)

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. *n* = 10 animals per day.

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

*Each histopathologic finding presents four 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).

necrosis (Figure 4G). The myocytes of cutaneous muscle were infiltrated by eosinophils and macrophages, and showed dilatation, degeneration, and, sometimes, atrophy (Figures 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 (Figure 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 (Figure 1F).

On 14 dpi, the severity of some inflammatory phenomena started to decrease and repair appeared more evident (Figure 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 (Figure 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 (Figure 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 twofold to threefold thicker than in the unaffected areas (Figure 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,

Table 5 (continued)

7	14	21	28	P value
0/0/0/100 ^d	0/0/0/100 ^d	0/8/50/42 ^c	0/16/46/38 ^c	0.001
100/0/0/0 ^a	58/8/17/17 ^b	25/25/0/50 ^b	0/0/8/92 ^c	0.001
0/10/40/50 ^d	0/50/50/00 ^c	17/42/33/8 ^c	23/46/31/0 ^{bc}	0.001
10/40/30/20 ^c	50/25/17/8 ^{ab}	67/33/0/0 ^a	31/23/38/8 ^c	0.001
0/60/20/20 ^{cd}	33/17/33/17 ^{bc}	67/25/8/0 ^{ab}	85/15/0/0 ^a	0.001
0/60/40/0 ^b	0/100/0/0 ^{ab}	17/42/33/8 ^{ab}	23/46/23/8 ^a	0.132
0/20/60/20 ^{cd}	0/40/30/30 ^{cd}	0/66/17/17 ^{bc}	75/15/8/0 ^a	0.001
0/30/30/40 ^c	8/33/42/17 ^b	0/41/42/17 ^{bc}	69/15/16/0 ^a	0.001
0/0/0/100 ^c	20/0/0/80 ^{bc}	75/0/0/25 ^a	92/8/0/0 ^a	0.001
0/0/50/50 ^{cd}	20/0/40/40 ^c	75/0/17/8 ^b	92/8/0/0 ^{ab}	0.001
30/70/0/0 ^d	75/17/0/8 ^{bc}	100/0/0/0 ^a	85/15/0/0 ^b	0.001
100/0/0/0 ^a	75/8/17/0 ^b	0/9/8/83 ^c	15/0/0/85 ^c	0.001
100/0/0/0 ^a	100/0/0/0 ^a	83/17/0/0 ^a	9/76/15/0 ^b	0.001
0/0/30/70 ^e	0/50/10/40 ^{cd}	25/33/25/17 ^{bc}	75/25/0/0 ^a	0.001
0/30/70/0 ^c	8/50/8/34 ^c	33/42/8/17 ^{bc}	75/8/0/15 ^a	0.001
100/0/0/0	75/25/0/0	92/8/0/0	69/31/0/0	0.204
40/30/20/10 ^b	50/33/0/17 ^{ab}	76/8/8/8 ^{ab}	100/0/0/0 ^a	0.010
40/60/0/0 ^a	25/50/17/8 ^c	58/42/0/0 ^b	92/8/0/0 ^b	0.001

Figure 4 Different histologic characteristics of lesions throughout infection with the Jw1 *Staphylococcus aureus* strain. **A:** At 0.5 days postinfection (dpi): Slight increase in the number of heterophils. Deep dermis is shown. Hematoxylin and eosin (H&E) stain was used. **B:** At 1 dpi: Abscess surrounded by numerous diffusely distributed heterophils. Skin is shown. H&E stain was used. **C:** At 2 dpi: Well-defined abscess characterized by a center of eosinophilic necrosis, with numerous coccoid bacteria inside (**inset**). Skin and bacteria (**inset**) are shown. H&E stain was used. **D:** At 2 dpi: Muscular degeneration caused by the intense interstitial infiltration of heterophils. Cutaneous muscle is shown. H&E stain was used. **E:** At 3 dpi: Necrosis of epidermis. Epidermis is shown. H&E stain was used. **F:** At 3 dpi: A bacterial colony surrounded by star-shaped homogeneous eosinophilic material and an external layer of inflammatory cells. Splendore-Hoeppli phenomenon is shown. H&E stain was used. **G:** At 3 dpi: Abscess internally composed of the fusion of several Splendore-Hoeppli phenomena, surrounded by a thin eosinophilic layer of necrosis. Skin is shown. H&E stain was used. **H:** At 3 dpi: Severe atrophy of myocytes, infiltrated by connective tissue (**left panel**) versus normal muscular tissue (**right panel**). Cutaneous muscle is shown. Masson trichrome stain was used. **I:** At 7 dpi: Significant increase in the number of cells with numerous mitosis (**arrows**) on the basal layer of the epidermis (epidermal hyperplasia or acanthosis), compared with normal epidermis (**inset**). Epidermis is shown. H&E stain was used. **J:** At 14 dpi: Severe inflammatory reaction associated with an ulcer and epidermal hyperplasia on the border. Epidermis is shown. H&E stain was used. **K:** At 14 dpi: Repair of the lesion characterized by acanthosis, absence of skin adnexa (**left panel**), and presence of abundant granulation tissue composed of numerous vessels and fibrous tissue (**right panel**). Skin (**left panel**) and deep dermis (**right panel**) are shown. H&E stain was used. **L:** At 28 dpi: Epithelial hyperplasia (**arrowheads**) and dermis with abundant fibrous tissue, blood vessels, scarce heterophils, and some small hemorrhages (**asterisk**). The periphery of the lesion is invaded by hair follicles (**arrows**). Epidermis and dermis are shown. H&E stain was used. Scale bars: 200 μ m (**A**, **E**, **H**, **J**, **K**, **right panel**, and **L**); 1 mm (**B**, **C**, **main image**, and **G**); 50 μ m (**C** and **I**, **insets**); 100 μ m (**D** and **I**, **main image**); 20 μ m (**F**); 500 μ m (**K**, **left panel**).

in some cases, was still interrupted by inflammatory cells and atrophied (**Figure 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 (**Figure 1I**). The dermis presented mature granulation tissue

Figure 5 Hyperplasia of the epithelium with the presence of numerous mitoses in the basal layer (arrows) on 21 days postinfection with the Jwt *Staphylococcus aureus* strain. **Inset:** Normal epithelium. Hematoxylin and eosin stain. Scale bars = 100 μm (main image and inset).

with abundant fibrous tissue and blood vessels, practically without erythrocytes and with scarce heterophils, as well as some small hemorrhages (Figure 4L). Cutaneous muscle had completely regenerated.

Immunohistochemical Studies

Variations in the counts of the studied cells (T-lymphocyte CD3^+ , plasma cell IgG^+ , and macrophage RAM11^+) were detected locally in the inoculation area during the experiment. The T-lymphocyte (CD3^+) counts increased during the experiment until 21 dpi (17.55 ± 1.90 cells/ mm^2) and then abruptly decreased on 28 dpi (Figure 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 (Figure 6B). The macrophages counts (RAM11^+) remained with no differences compared with 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 (Figure 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-lymphocyte $\text{CD79}\alpha^+$.

Flow Cytometer Results

Figure 5 shows the results of the flow cytometric analysis of the peripheral blood cells in the rabbits infected by *S. aureus* Jwt. Intradermal inoculation induced modifications in the counts of most leukocyte populations (granulocytes, monocytes, and total B and T CD25^+ lymphocytes). Total leukocyte (Figure 6D) and granulocyte (Figure 6F) counts showed two peaks during the study: a first sharp increase on

1 dpi and a second greater increase from 7 to 14 dpi, which decreased on 21 dpi (more abruptly for granulocytes). The number of monocytes (Figure 6G) gradually increased from 3 to 14 dpi, when its highest value was reached ($869.5 \times 10^6/\text{L}$; $P < 0.001$). The number of total lymphocytes increased from 0 to 2 dpi, when counts were kept elevated until the end of the experiment (Figure 6E). Regarding the lymphocyte subpopulations, statistical differences were observed only in the B (Figure 6H) and CD25^+ (Figure 6I) lymphocytes. B cells had higher values at 0.5, and especially at 21, dpi compared with 0 dpi (43.29 and $81.66 \times 10^6/\text{L}$, respectively; $P < 0.001$). The cell counts of the CD25^+ lymphocytes abruptly increased on 0.5 dpi and were 11.9-fold higher than on 0 dpi and on later experimental days. No significant differences were observed in the number of T lymphocytes, T CD4^+ lymphocytes, and T CD8^+ lymphocytes.

Plasmatic and Tissue Cytokines

Statistical differences were detected only in IL-4 and IL-18 in plasma and in IL-4 and IFN- γ in skin samples (Table 6). The plasmatic levels of IL-4 and IL-18 oscillated throughout the experiment. Relative to 0 dpi, statistical differences were only detected for IL-4 on 28 dpi and IL-18 on 14 and 28 dpi, where these cytokines reached their highest plasmatic levels (8.9 pg IL-4/mL and 463.4 pg IL-18/mL) on 28 dpi. Similar to plasma, the IL-4 detected in skin tissue significantly increased on 28 dpi (15.5 pg/mL; $P < 0.05$). Conversely, the IFN- γ level in tissues abruptly increased on 0.5 dpi ($14,015 \pm 1804$ pg/mL; $P < 0.05$) before returning to previous levels. No significant differences were observed in IL-1 β and IL-17 levels.

Study 1b: Effects of the Selected Genes and the Global Regulator Involved in Abscess Development

Once the lesions and peripheral and local immune responses had been described, the aim of this section was to evaluate the contribution of different isogenic mutants of the Jwt strain ($\text{J}\Delta\text{agr}$, $\text{J}\Delta\text{coa}\Delta\text{vwb}$, $\text{J}\Delta\text{hla}$, and $\text{J}\Delta\text{psmA}$) to the development and characteristics of abscesses on 7 dpi using the proposed rabbit skin model. Unexpectedly, all of the mutant strains were able to generate lesions like the wild type in terms of degree of severity (Figure 7). Whenever present, lesions were macroscopically indistinguishable from those generated by the wild-type strain.

At the histologic level, Splendore-Hoeppli phenomena were observed with all the strains, but not with the same incidence. Only statistically differences were observed with $\text{J}\Delta\text{hla}$ (85% Jwt versus 68% $\text{J}\Delta\text{hla}$; $P < 0.05$) (Figure 8A). The percentage of distributed heterophiles surrounding the abscesses was higher in the mutant strains with respect to Jwt (20% for $\text{J}\Delta\text{coa}\Delta\text{vwb}$, 50% for $\text{J}\Delta\text{hla}$, and 30% for $\text{J}\Delta\text{psmA}$ and $\text{J}\Delta\text{agr}$; $P < 0.05$) (Figure 8B).

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), lymphocyte B (H), and lymphocyte T CD25⁺ (I) ($\times 10^6/L$) in peripheral blood after intradermal inoculation with the Jwt *Staphylococcus aureus* strain. Error bars correspond to the SEM for each least square mean (A–I). $n = 10$ animals per day (A–I). ^{a–e}The means that do not share a superscript in the same figure significantly differ for each postinoculation time ($P < 0.05$).

All the lesions caused by each studied strain (the wild type and mutants) presented an eosinophilic layer surrounding abscesses on 7 dpi. However, there were differences in the characteristics of this band. The percentage of complete eosinophilic layers always was lower for mutants than for Jwt (–30% for JΔ*coa*Δ*vwb*, –90% for JΔ*hla*, –45% for JΔ*psma*, and –50% for JΔ*agr*; $P < 0.05$) (Figure 8C). These histologic differences are related to the degree of maturation of the abscess. Thus, the lesions generated by the mutants at 7 dpi resembled those presented by the wild-type strain in 2 to 3 dpi, which could be interpreted as the absence of these genes delays the development of the lesions.

Study 2: Comparison of the Lesions Caused by a Human Rabbitized Strain and Different Rabbit Strains of Known Virulence

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 herein is that human *S. aureus* strains (eg, F strain) do not cause lesions at low doses (300 CFUs) in rabbits.¹³ In fact, to be able to produce them, it is necessary to use high bacterial doses ($>10^4$ CFUs) and, when this occurs, the evolution of lesions (faster) and the produced characteristics (eg, presence of extensive

Table 6 Evolution of the Concentration (pg/mL) of Cytokines on Plasma and Skin Tissue after the Intradermal Inoculation with the Jwt *Staphylococcus aureus* Strain ($n = 10$ Animals per Day)

	Postinoculation time, days										
Variable	0	0.5	1	2	3	7	14	21	28	SEM	P value
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-γ	643 ^a	14015 ^b	412 ^a	401 ^a	547 ^a	558 ^a	619 ^a	3879 ^a	2588 ^a	2282	<0.001

For a given trait presented in a row, P value tests hypothesis H_0 : there was no effect of treatment to explain the observed trait variance.

^{a-d}The means in a same row not sharing superscript significantly differ at $P < 0.05$ for day postinoculation.

IFN- γ , interferon- γ .

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 (J.M.C., unpublished data) (Supplemental Figure S1). Therefore, we had to use a human strain adapted to rabbits (*FdltB^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 *FdltB^r* strain with *S. aureus* strains obtained from rabbits of known virulence, such as Jwt (high virulence), *Jrot⁺* (medium virulence), and *JdltB^h* (low virulence), using the model described in Study 1 to demonstrate its utility with strains of different origins.

Health Parameters

No animal experienced 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. Although 100% of the rabbits were infected by human *FdltB^r* and rabbit Jwt,

76.7% and 60% of the animals inoculated with *Jrot⁺* and *JdltB^h* showed lesions, respectively. The severity of lesions caused by *FdltB^r* was intermediate, between Jwt (more severe lesions) and *Jrot⁺*, and *JdltB^h* (milder lesions) (Figure 9).

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

Bacteriology

The bacterial counts obtained from the animals inoculated with *FdltB^r* and *Jrot⁺* followed the same dynamics as the

Figure 7 Severity assessment of gross lesions on day 7 after intradermal inoculation with different mutants (*JΔcoaΔvwb*, *JΔhla*, *JΔpsma*, and *JΔagr*) versus Jwt (high virulent) and *Jrot⁺* (low virulent) strains of *Staphylococcus aureus*. $n = 16$ *JΔcoaΔvwb*; $n = 15$ *JΔhla* and *JΔagr*; $n = 8$ *JΔpsma*. ^{a,b}Within an experiment, the groups not sharing letters above the bar significantly differ ($P < 0.05$).

Figure 8 Presence of the main histologic 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 (JΔcoaΔvwb, JΔhla, JΔpsmA, and JΔagr) of *Staphylococcus aureus*. Evaluated as variation in relation to intradermal inoculation with Jwt for: JΔcoaΔvwb, JΔhla, JΔpsmA, and JΔagr. *n* = 16 JΔcoaΔvwb; *n* = 15 JΔhla and JΔagr; *n* = 8 JΔpsmA. **P* < 0.05 versus intradermal inoculation with Jwt.

Jwt strain on 1, 3, and 7 dpi (Figure 10), but the average number of bacteria obtained from their lesions was smaller ($6.18 \pm 32.21 \times 10^6$ and $1.10 \pm 47.54 \times 10^6$ CFUs/g, respectively; *P* < 0.05) than from the lesions caused by Jwt ($244.36 \pm 36.98 \times 10^6$ CFUs/g). Bacteria were isolated only in one animal infected by JdltB^h on 3 dpi (2.26×10^3 CFUs/g) at 3 dpi. All the cultured and identified bacteria recovered from lesions were the same as those inoculated, and no bacteria were isolated from blood or kidneys.

Histologic Findings

Different degrees of severity were observed in the histologic lesions, depending on the type of inoculated strain (Table 7). In general, FdlitB^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 FdlitB^r were more severe than Jrot⁺ and resembled Jwt.

The abscesses caused by FdlitB^r were evident on 1 dpi and were more numerous and more severe on 3 dpi, similarly to Jwt. Conversely, the severity of the abscesses (heterophils organized) (Table 7) in the animals infected by Jrot⁺ and JdlitB^h reduced on 3 dpi. In this last case, abscesses were classified mainly as mild (Figure 11A).

The abscesses produced by the three mutant strains were histologically more immature compared with 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 FdlitB^r and 20% of Jrot⁺ had one, and no band was detected in the JdlitB^h abscesses. At the end of the

Figure 9 Mean severity assessment of gross lesions after intradermal inoculation with different strains (FdlitB^r, Jwt, Jrot⁺, and JdlitB^h) of *Staphylococcus aureus*. Assessment is represented as the percentage at four levels: absence, mild, moderate, or severe. *n* = 30 animals per strain. ^{a,b}The groups not sharing letters above the bar significantly differ (*P* < 0.05).

Figure 10 Colony-forming units (CFUs) after intradermal inoculation with different strains of *Staphylococcus aureus* (FdlB^r, Jwt, and Jrot⁺). Error bars correspond to the SEM for each least square mean. $n = 10$ animals per strain and day. ^{a–d}The means not sharing superscript significantly differ ($P < 0.05$).

experiment (7 dpi), percentages varied, but differences remained (100% Jwt, 36% FdlB^r, 30% Jrot⁺, and 0% JdlB^h).

Splendore-Hoeppli phenomena were seen only in the abscesses from the rabbits infected by Jwt and Jrot⁺ on 3 and 7 dpi. There were fewer Splendore-Hoeppli phenomena in Jrot⁺

(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 Jrot⁺ (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 FdlB^r and JdlB^h for all of the studied times, but the incidence of perivascular inflammation was higher with Jwt ($P < 0.05$) (Table 7).

The pathologic findings (inflammation and degeneration) observed in cutaneous muscle were similar between strains FdlB^r and Jwt, and were more severe than those caused by strains Jrot⁺ and JdlB^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 FdlB^r strain infection was lower to those of the wild-type strain (Jwt) and more similar to those of strain Jrot⁺ and JdlB^h (Figure 11, B–E). The T-lymphocyte (CD3⁺) counts were significantly higher during the three moments analyzed in the Jwt compared with FdlB^r. The macrophage counts (RAM11⁺) were significantly higher on 3 and 7 dpi in the animals infected with Jwt regarding to FdlB^r, and the counts of B lymphocytes (CD79α⁺) and plasma cells (IgG⁺) were significantly

Table 7 The Histopathologic Findings Observed after the Inoculation of Different *Staphylococcus aureus* Strains

Variable	<i>S. aureus</i> strain				<i>P</i> value	
	FdlB ^r	Jwt	Jrot ⁺	JdlB ^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
Re-epithelization (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 diffuse	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

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. $n = 10$ animals per day and strain.

^{a–c}The means in a same row not sharing letters significantly differ ($P < 0.05$).

*Each histologic parameter presents four 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), and severe (z).

Figure 11 **A:** Severity assessment of abscesses (heterophils organized) histologically after infections with strains *FdltB^r*, *Jwt*, *Jrot⁺*, and *JdltB^h* of *Staphylococcus aureus*. Assessment is represented at four levels: absence, mild, moderate, or severe. The groups not sharing letters (a, b, c, d, e) above the bar significantly differ. **B–E:** Evolution of the counts of T-lymphocyte CD3⁺ (**B**), B-lymphocyte CD79α⁺ (**C**), plasma cells positive for IgG⁺ (**D**), and macrophage RAM11⁺ (**E**) immunohistochemically marked in skin samples after infection with *S. aureus* strains *FdltB^r*, *Jwt*, *Jrot⁺*, and *JdltB^h*. Error bars correspond to the SEM for each least square mean (**B–E**). *n* = 30 animals per strain (**A**). ^{a–d}The means in a graph not sharing superscript significantly differ (*P* < 0.05). Dpi, days postinfection.

Figure 12 Effect of *Staphylococcus aureus* strains on leukocyte population counts (A–I) in peripheral blood and CD4⁺/CD8 (J) and granulocyte/lymphocyte (G/L; K) ratios after intradermal inoculation with strains FdltB^r, Jwt, Jrot⁺, and JdltB^h. *n* = 30 animals per strain (A–K). ^{a–c}The means in a same graph not sharing superscript significantly differ (*P* < 0.05).

higher on 3 and 7 dpi, respectively. The lesions produced by the inoculation of Jrot⁺ were characterized mainly by the absence of T lymphocytes (CD3⁺) on all of the analyzed days and high number of B lymphocytes on 3 dpi, similar to Jwt. The lesions produced by the inoculation of JdltB^h were characterized mainly by the absence of B lymphocytes (CD79α⁺) on all of the sampling times.

Flow Cytometer Results

The differences detected in the peripheral blood cell counts in the rabbits infected by *S. aureus* FdltB^r, Jwt, Jrot⁺, and JdltB^h are shown in Figure 12. The animals infected by the FdltB^r strain had fewer leukocytes (−15.2%) and total lymphocytes (−26.5%) than Jwt, Jrot⁺, and JdltB^h, with similar counts to one another (Figure 12, A and D).

In the B, T, CD4⁺, and CD8⁺ lymphocyte counts, no significant differences were observed between the rabbits inoculated with the *FdltB^r* strain and Jw^t. Both animal groups had more B lymphocytes and fewer T, CD4⁺, and CD8⁺ lymphocytes than *Jrot⁺* and *JdltB^h* (Figure 12, E–H). *FdltB^r* and Jw^t strains had a lower CD4⁺/CD8⁺ ratio than *Jrot⁺* (Figure 12J).

The animals infected by the mutant strains presented fewer granulocytes (–34.5%; $P < 0.05$) and monocytes than those inoculated with Jw^t (Figure 12, B and C). For monocytes, the animals inoculated with *Jrot⁺* and *JdltB^h* had an intermediate level (–23.9% *Jrot⁺* and *JdltB^h* and –50.5% *FdltB^r*; $P < 0.05$) (Figure 12C). The CD25⁺ lymphocytes showed significantly higher counts in the animals infected by mutant strains *FdltB^r*, *Jrot⁺*, and *JdltB^h* (50.4%, 61.2%, and 65%, respectively; $P < 0.05$) than the rabbits infected by Jw^t (Figure 12I). Finally, strains *FdltB^r* and Jw^t had a higher granulocyte/lymphocyte ratio than *Jrot⁺* and *JdltB^h* (Figure 12K).

Discussion

Staphylococcal infections have spread worldwide in hospitals and communities in the last decades of the 20th Century, are multiresistant to antibiotic strains (especially methicillin-resistant *S. aureus*), and have become a major health challenge in all industrialized countries today.¹ This problem has also extended to farm animals since 2005, when a livestock-associated methicillin-resistant *S. aureus* was isolated in pigs³² and extended to other animal species, including horses, cattle, poultry, or rabbits.^{33–35} Among the wide range of diseases associated with community infections that can be caused by *S. aureus*, skin and soft tissue infections are one of the most worrying because they can be a life-threatening process if they are complicated.^{4–9} Skin and soft tissue infections are characterized by the formation of bacterial abscesses in dermis, epidermis, or subcutaneous tissues.¹

In this context, it would be valuable to have a definitive experimental model that allows the pathogenesis of the infection to be studied or new therapeutic tools to be tested. Mice are commonly used for staphylococcal colonization and infection models, mainly for their small size, easy handling, and the abundance of research facilities compared with other larger species, like rabbits.^{16,36} However, these characteristics alone do not justify their use if the results are not truly representative. Traditionally, rabbit models of *S. aureus* infection have been considered optimal to investigate virulence and host-pathogen interactions, mainly because of certain similarities with the human species: i) similar thickness of their skin^{14,15}; ii) similar immune response to infection¹⁶; and iii) both are naturally infected by *S. aureus*. For these reasons, we present a rabbit model because this animal species has its own adapted *S. aureus* strains,^{12,35,36} which easily allow to naturally study staphylococcal infections.

The experimental model was respectful of animal health as no animal displayed fever, loss of weight, septicemia, or external signs of disease, except for the expected local lesions at inoculation points. The model was reproducible, and previously described characteristic abscesses developed,³⁷ but it also provided more detailed characteristics of lesion development over time than previously described. Although lesions were observed at all the studied time points, staphylococcal infection with a Jw^t strain was evaluated with this model at three key time points, 1, 3, and 7 dpi. On 1 dpi, it was possible to observe small slightly raised reddish papules that were histologically characterized by the presence of acute vascular changes, small organized abscesses, sometimes with inner bacteria and surrounded by heterophils, that were diffusely distributed. One interesting finding was the sharp and one-time increase in the CD25⁺ lymphocytes 12 hours before (0.5 dpi). As these cells are considered T-regulatory cells,³⁸ it would be interesting to verify to what extent the inflammatory response described in this intradermal infection is modulated by this cell population. T-regulatory cells are essential for preventing exacerbation of inflammatory response, but they have been found to lose their activity in the presence of *S. aureus*, as shown in children with atopic skin.^{39,40} On 3 dpi, superficial lesions lost their initial reddish color and became nodular lesions, and it was when they acquired their largest volume and had more bacteria. Microscopically, vascular changes increased in severity, and abscesses were surrounded by an eosinophilic necrotic layer in whose core Splendore-Hoeppli phenomena with inner bacteria were observed. Surrounding the abscess, there were increasing numbers of T lymphocytes (CD3⁺) and macrophages (RAM11⁺) compared with previous days, which remained high until 21 dpi, and were related with the increased number of blood monocytes and lymphocytes on 2 dpi, as previously described.⁴¹ On 7 dpi, 50% of the nodules experienced epidermal necrosis and opened, which triggered a discharge of pus and a reduction in both lesion size and the number of isolated bacteria. These epidermal lesions generated a new inflammatory episode, peripherally characterized by an increased number of heterophils in blood, which lasted until 14 dpi. In histologic terms, lesions reached their most severe point at all of the studied locations, with several Splendore-Hoeppli phenomena surrounded by many T lymphocytes (CD3⁺), plasma cells (IgG⁺), and macrophages (RAM11⁺).

Therefore, by studying on 1 and 3 dpi, it would be possible to evaluate the acute inflammatory response, as well as the subacute inflammatory reaction and initial repair mechanisms on 7 dpi, which could be useful for most experimental studies on this topic. For chronic inflammation studies, other later study times (eg, 14, 21, or 28 dpi) would be more recommendable, when regenerative modifications were predominant (re-epithelization and acanthosis in the epidermis, granulation tissue and fibrosis in the dermis, and regeneration of the cutaneous muscle), and when blood monocytes (2 to 14 dpi) and tissue lymphocytes and

macrophages (3 to 21 dpi) significantly increased, as previously described for chronic 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} *Staphylococcus aureus* antigens induce different type 1, 2, and 17 helper T-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 IFN- γ and IL-18. Cytokine IFN- γ 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. *Staphylococcus aureus* is able to inhibit the response of T-cell responses and to induce type 2 helper T-cell responses by producing IL-4⁴⁷ 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 (eg, human bacteria community-acquired methicillin-resistant *S. aureus* USA300 in mice: $\geq 5.0 \times 10^8$ CFUs⁴⁹). 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 Frank⁵⁰ proposed an infective *S. aureus* dose of 10^5 to 10^6 bacteria and classified it as a high-infective dose microorganism. In fact, it has been reported that inoculation with $<5 \times 10^8$ CFUs of USA300 does not result in reproducible abscesses in rabbits.^{17,49} The results from the current study agree with this observation when human strains (not adapted to rabbit) were used, but disagree when rabbit strains, which can generate dermal lesions with only 300 CFUs by simulating natural infection, were employed. Moreover, when high doses of human strain Fwt ($>10^6$ CFUs) were used with this model, the generated lesions differed from those observed for low doses of rabbit wild-type strains: lesions rapidly evolved and skin necrotic phenomena (dermonecrosis) predominated and, in histologic terms, the presence of thrombosis and the absence of Splendore-Hoeppli phenomena or a peripheral eosinophilic layer were observed on

3 dpi (J.M.C., unpublished data) (Supplemental Figure S1). Similar results have been reported by other authors,¹⁷ who recognized that using such high doses would result in rapidly progressive infection, which would vastly differ from that typically encountered in clinical situations, and the results could not be extrapolated to humans. These skin necrotic phenomena are probably caused by the mass production of bacterial toxins, which is far removed from what actually happens in natural infection. This hypothesis would support the results observed in the present study with the mutants ($\Delta coa\Delta vwb$, Δhla , $\Delta psma$, and Δagr) employed to evaluate their contribution to the development and characteristics of abscesses. Studies performed in rabbits, and mainly in mice, using Δhla mutants have resulted in reduced virulence in animal models of dermonecrotic skin infection. Moreover, immunization with alpha-hemolysin-specific antisera significantly reduces the size of skin lesions and prevents dermonecrosis,^{11,17,51,52} and similar results have been obtained with mutants in $psma$.⁵³ Surprisingly, when the herein described skin infection model was used, mutants Δhla and $\Delta psma$ produced the same percentage of lesions as the wild-type strain, and even became macroscopically more severe for Δhla regarding JwT. Toxins and other virulence factors, such as α -toxin, phenol-soluble modulin α (PSM- α), and PSM- β , are produced as a result of increased bacterial population density and environmental conditions, partly under a quorum-sensing control through different regulators.^{54–56} Therefore, the effect observed in previous works with either the Δhla or the $\Delta psma$ mutant could be due to the use of high doses of bacteria to produce lesions, which would favor the rapid production of numerous toxins, and would make the effect of the toxin more evident. The absence of an effect on the Δagr mutant would follow the same hypothesis. In *S. aureus*, most quorum-sensing components are coded by the accessory gene regulator (Agr) system. This regulation is important for acute disease to develop,³⁷ but we showed that Agr was dispensable in intradermal infections of rabbits. Agr suppresses the expression of the repressor of toxins (*rot*) at a high cell density,⁵⁷ which could also influence the virulence of the Δagr mutant. However, rabbit strains have a natural loss-of-function mutation in *rot*, which increases the severity and infectivity of the rabbit strain. This could justify the *FdltB*^r strain (with functional *rot*) producing milder lesions than the wild-type strain.

A histologic characteristic observed from 3 dpi in the lesions produced by the strain wild type was an eosinophilic necrotic layer in whose core Splendore-Hoeppli phenomena with inner bacteria were observed. These phenomena have been previously described as an electron-dense, granular, and amorphous pseudocapsule.^{37,58,59} This pseudocapsule constitutes a barrier for immune cells by preventing them from penetrating the staphylococcal abscess community, where bacteria replicate without interference and are partially composed of prothrombin and fibrinogen that separate bacteria from leukocytes.^{37,58,59} As the $\Delta coa\Delta vwb$

mutant was able to produce lesions like Jw^t, the absence of these two clotting factors secreted by *S. aureus* does not seem essential for abscesses to develop in rabbits. Despite all of the employed mutants (*JΔcoaΔvwb*, *JΔhla*, *JΔpsmα*, and *JΔagr*) being able to generate similar lesions to the wild type in size and degree of severity terms, albeit in different percentages, they all triggered a delay in the evolution of histologic lesions compared with the wild type. This could indicate that their mutations affected abscess development, but other factors also had to be involved in their development. More studies are needed to learn the specific role of these and other virulence factors in *S. aureus* infection development.

The utility of this model was corroborated when a human strain, modified to infect rabbits (rabbitized), was inoculated (*FdltB^r*). This adaptation to the host seemed essential because neither macroscopic nor microscopic lesions developed when the human Fwt strain was inoculated with 300 CFUs,¹³ whereas the modified strain (*FdltB^r*) was able to produce similar lesions to the wild strain adapted to rabbit (Jw^t).

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 proved.

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Author Contributions

L.S., J.M.C., and D.V. conceived the study; J.J.P., A.A.-B., J.M.C., and D.V. analyzed the results; L.S., J.M.C., and D.V. acquired funding; A.M.-S., M.P., L.S., S.P.-F., E.M., A.G.-Q., J.J.P., A.A.-B., A.B., J.M.C., and D.V. performed experiments; J.M.C. provided project administration; J.M.C. and D.V. supervised the study; A.M.-S., A.A.-B., J.M.C., and D.V. wrote the manuscript; A.A.-B., J.J.P., J.M.C., and D.V. reviewed and edited the manuscript; J.M.C. and D.V. 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.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.01.019>.

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