

Staphylococcus aureus Virulence Is Enhanced by Secreted Factors That Block Innate Immune Defenses

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Key Words

Neutrophils · Complement · Fibrinogen · Immune evasion · Staphylococci

Abstract

Staphylococcus aureus is a leading human pathogen that causes a large variety of diseases. In vitro studies have shown that *S. aureus* secretes several small proteins that block specific elements of the host innate immune system, but their role in bacterial pathogenicity is unknown. For instance, the extracellular complement-binding protein (Ecb) impairs complement activation by binding to the C3d domain of C3. Its homolog, the extracellular fibrinogen-binding protein (Efb), is known to block both complement activation and neutrophil adhesion to fibrinogen. Here, we show that targeted inactivation of the genes encoding Ecb and Efb strongly attenuates *S. aureus* virulence in a murine infection model: mice experienced significantly higher mortality rates upon intravenous infection with wild-type bacteria (79%) than with an isogenic Δ Ecb Δ Efb mutant (21%). In addition, Ecb and Efb are both required for staphylococcal persistence in host tissues and abscess formation in the kidneys (27% for

wild-type vs. 7% for the Δ Ecb Δ Efb mutant). During staphylococcal pneumonia, Ecb and Efb together promote bacterial survival in the lungs ($p = 0.03$) and block neutrophil influx into the lungs. Thus, Ecb and Efb are essential to *S. aureus* virulence in vivo and could be attractive targets in future vaccine development efforts. Copyright © 2012 S. Karger AG, Basel

Introduction

Staphylococcus aureus is an important human pathogen associated with high mortality and morbidity in a wide spectrum of hospital- and community-acquired infections [1, 2]. Clinical disease ranges from uncomplicated skin infections to life-threatening bacteremia with metastatic complications such as endocarditis and pneumonia. The increasing resistance of *S. aureus* to antibiotics has created global awareness that additional control strategies are required. However, vaccine strategies have

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been unsuccessful to date [3]. The complex and versatile human adaptation of *S. aureus* is due to its diverse array of virulence factors that enable it to adhere to and invade different body sites [4, 5]. In the last decade, it has become clear that *S. aureus* also produces a large array of small, secreted proteins that interact with various components of the innate immune system, our first line of defense against invading bacteria [6], but their role in bacterial pathogenicity is unproven.

Neutrophils are essential for the innate immune response against bacteria. These specialized leukocytes are recruited from the blood to the site of infection where they engulf bacteria and internalize them via phagocytosis [7]. Inside neutrophils, bacteria are killed by exposure to antimicrobial agents such as antimicrobial peptides, reactive oxygen species and lytic enzymes. Several plasma proteins are important for the proper activation and functioning of neutrophils. The complement system, a family of thirty plasma proteins, labels bacteria with opsonins (C3b, C3bi) to support phagocytosis and generates chemoattractants (C5a) that attract neutrophils to the site of infection [8]. Fibrinogen (Fg), one of the most abundant plasma proteins, also supports neutrophil activation via an interaction with the human leukocyte adhesion glycoprotein α M β 2 integrin (also known as Mac-1 or complement receptor 3) [9]. Fg-deficient mice have delayed inflammatory responses [10, 11]. The complement system has a protective role against *S. aureus* infections by labeling bacteria for phagocytosis and attracting neutrophils via C5a. Young children with deficiencies in the complement recognition molecule mannose-binding lectin are more susceptible to *S. aureus* infections [12], and experimental work has demonstrated a major role for C3 in the recognition and phagocytic removal of *S. aureus* [13]. Recent studies in mice demonstrated the importance of C5a in controlling *S. aureus* mortality following intravenous inoculation [14]. Our group previously reported that *S. aureus* produces a number of small, secreted proteins with the potential to interfere with components of the host complement system and neutrophil activation pathways [6]. Since most of these molecules displayed a strict specificity for the human immune system, their hypothesized role in bacterial pathogenicity could not be verified in animal models. However, we recently described two such staphylococcal proteins whose interaction with the immune system extended across different species: extracellular complement-binding protein (Ecb), also known as Ehp [15, 16], and its homolog extracellular fibrinogen-binding protein (Efb) [16]. Ecb is a 10-kDa protein that binds to the C3d domain of C3 and thereby

blocks C3 convertases of the alternative pathway and C5 convertases via all complement pathways. Because of its potent action against C5 convertases, Ecb effectively blocks formation of the anaphylatoxin C5a in both human and mouse serum [16]. In a mouse model for immune-complex disease, purified Ecb could block C5a-dependent neutrophil influx [16]. The 16-kDa protein Efb has two domains that mediate separate immune evasion functions. The C-terminus of Efb (aa 65–136) is highly homologous to Ecb and blocks complement in a similar fashion as Ecb [16]. The N-terminus of Efb (aa 1–64) binds to fibrinogen and blocks the interaction between α M β 2 on neutrophils and immobilized fibrinogen [17]. Furthermore, Efb inhibits fibrinogen-dependent platelet aggregation [18]. It can bind to both human and mouse complement and fibrinogen [16, 17]. In this study, we combine a targeted mutagenesis approach and mouse infection models to study whether endogenous expression of Ecb and Efb is sufficient to promote staphylococcal immune evasion and virulence in vivo.

Materials and Methods

Ethics Statement

Ethics approval for animal experimentation was obtained from the Animal Care Program of the University of California, San Diego, Calif., USA. Human blood and serum were collected from healthy volunteers after informed consent.

Bacterial Strains and Growth Conditions

S. aureus and *Escherichia coli* strains and plasmids are listed in table 1 [19, 20]. *E. coli* was grown in Luria-Bertani medium or agar at 37°C. *S. aureus* was cultured in Todd-Hewitt medium or agar at 37°C. When needed, antibiotics (Sigma, St. Louis, Mo., USA) were added at the following concentrations: carbenicillin (50 μ g/ml) for *E. coli* and tetracycline (5 μ g/ml), kanamycin (50 μ g/ml), neomycin (50 μ g/ml), erythromycin (5 μ g/ml), lincomycin (25 μ g/ml) and chloramphenicol (10 μ g/ml) for *S. aureus*.

Bacterial Mutants

The genes for *ecb* and *efb* in *S. aureus* Newman [loci NWMN1066 (330bp) and NWMN1069 (498 bp), respectively] were disrupted by insertional inactivation using the suicide plasmid pAZ106 [21] as described previously [22, 24]. We created pAZ106::Ecb::tet, pAZ106::Efb::tet and pAZ106::Efb::kan in *E. coli* using standard cloning methods. To generate pAZ106::Ecb::Tet, we amplified PCR fragments of 731 bp (bp 1–216 of the *ecb* gene plus 515 bp upstream of *ecb*) and 1553 bp (bp 221–330 of *ecb* plus 1444 bp downstream of *ecb*) using primer pairs Ecb_BamHI/Ecb_NotI and Ecb_KpnI/Ecb_EcoRI (primers are listed in table 2). These PCR fragments were digested with BamHI/NotI and KpnI/EcoRI, and simultaneously ligated to a NotI/KpnI-digested tetracycline resistance gene (amplified with primers Tet_NotI/Tet_KpnI) and BamHI/EcoRI-digested pAZ106. To generate pAZ106::Efb::tet and pAZ106::Efb::kan, we amplified PCR frag-

Table 1. Strains and plasmids used in this study

Primer	Sequence 5'-3' (restriction sites underlined)
Ecb_BamHI	<u>CGACGGATCC</u> GAAACAATCAGTCATAC
Ecb_NotI	ATAACTGCGGCCGCGTGTGTTGCAACAGTTCTTG
Ecb_KpnI	<u>CCGGTACCGTAAAGCACAAAGAGCTG</u>
Ecb_EcoRI	<u>ACATGAATTCTATTTGTAACCAAATAGCTC</u>
Tet_NotI	<u>ATAACTGCGGCCGCGGCGGATTTTATGACCGATGAAG</u>
Tet_KpnI	<u>CCGGTACCTTAGAAAATCCCTTTGAGAATGTTT</u>
Efb_BamHI	<u>CGACGGATCC</u> GACACTCTTTATGGGTGTGG
Efb_NotI	<u>ATAACTGCGGCCGCGTGGACGTGCACCATATTCG</u>
Efb_KpnI	<u>CCGGTACCGAATATGGTGCACGTCC</u>
Efb_EcoRI	<u>ACATGAATTCTAGCATCAGCCATTGATACG</u>
Kan_NotI	<u>ATAACTGCGGCCGCGGAAAACCCAGGACAATAACC</u>
Kan_KpnI	<u>ACAGGTACCCTCGGGACCCCTATCTAG</u>
EcbF	<u>CCCAAGCTTGGGTTGATTATTTGGTTAAAA</u>
EcbR	<u>GGAATTCCTACCTTTGGATATAGCAA</u>

Table 2. Primers used in this study

Strain or plasmid	Genotype or description	Reference
<i>E. coli</i>		
Top10F ⁺	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> λ	Invitrogen
<i>S. aureus</i>		
RN4220	restriction-deficient transformation recipient	19
Newman	wild-type <i>S. aureus</i> strain	20
NewmanΔEfb	Newman Efb::tet	this study
NewmanΔEcb	Newman Ecb::tet	this study
NewmanΔEcbΔEfb	Newman Ecb::tet; Efb::kan	this study
NewmanΔEcb+pCU-Ecb	Newman Ecb::tet + pCU1-Ecb	this study
Plasmids		
pAZ106	promotorless <i>lacZ</i> insertion vector; Em ^r	21
pAZ106::Ecb::tet	<i>ecb</i> allelic replacement construct; Tet ^r	this study
pAZ106::Efb::tet	<i>efb</i> allelic replacement construct; Tet ^r	this study
pAZ106::Efb::kan	<i>efb</i> allelic replacement construct; Kan ^r	this study
pDG792	template for PCR amplification of Kan ^r	22
pDG1513	template for PCR amplification of Tet ^r	23
pCU1	shuttle vector (Amp ^R in <i>E. coli</i> and Cm ^R in <i>S. aureus</i>)	24
pCU1-Ecb	pCU1 with <i>ecb</i> gene under its own promoter	this study

ments of 941 bp (bp 1–271 of *efb* plus 670 bp upstream of *efb*) and 684 bp (bp 257–498 of *efb* plus 443 bp downstream of *efb*) using Efb_BamHI/Efb_NotI and Efb_KpnI/Efb_EcoRI. These DNA fragments were simultaneously ligated to appropriately digested amplicons of Tet^r (primers Tet_NotI/Tet_KpnI) or Kan^r (primers Kan_NotI/Kan_KpnI) and BamHI/EcoRI-digested pAZ106. Restriction-deficient host RN4220 was transformed with the resultant vectors and clones containing each Campbell plasmid integration were resolved in Newman by transductional outcross using ø11 [25]. Clones of Newman, which had now lost the plasmid and contained an allelic replacement with a kanamycin or tetra-

cycline resistance gene, were confirmed as mutants by PCR amplification, DNA sequencing and immunoblot analysis. For complementation, the *ecb* gene was amplified using primers EcbF/EcbR, digested with HindIII/EcoRI, and cloned into shuttle vector pCU1 [23]. After verification, it was used to transform strain RN4220 and then transduced into *S. aureus* NewmanΔEcb using ø11. For immunoblot analysis, supernatants were collected from logarithmic staphylococcal cultures (OD₆₆₀ of 0.5) grown in Iscove's modified Dulbecco's medium (IMDM; Lonza, BioWhittaker, Basel, Switzerland). Supernatants were concentrated (30×) using trichloroacetic acid (Sigma) and analyzed by silverstaining

or immunoblotting. Ecb and Efb were detected using rabbit anti-Ecb (Biogenes, Berlin, Germany) and peroxidase-conjugated goat anti-rabbit antibodies (Southern, Birmingham, Ala., USA) or sheep anti-Efb (kindly provided by Jan-Ingmar Flock) and peroxidase-conjugated donkey anti-sheep antibodies (Sigma).

Blood Survival and C5a Generation Assays

Overnight cultures of *S. aureus* strains were diluted into fresh THB and grown to an OD₆₀₀ of 0.8 at 37°C. Bacteria were diluted in Roswell Park Memorial Institute medium (RPMI) to a concentration of 5×10^6 CFU/ml. For blood survival, 50 µl of bacteria were mixed with 50 µl freshly isolated blood (anticoagulated using Repludan (Schering, Kenilworth, N.J., USA) and 100 µl RPMI 1640 (Life Technology, Carlsbad, Calif., USA) containing 0.05% HSA (Sanquin, Amsterdam, The Netherlands). Samples were incubated for 6 h at 37°C with shaking. Blood cells were lysed with 1 ml ice-cold H₂O and bacterial survival was enumerated by plating serial dilutions on TH agar plates. To analyze C5a generation in serum, 50 µl of bacteria were mixed with 150 µl 30% human serum at 37°C shaking. Supernatants were collected by centrifugation and tested for the presence of C5a using a calcium mobilization assay as described previously [26]. Supernatants were added to Fluo-4-AM-labeled U937 cells transfected with the C5a receptor [27] and the increase of intracellular calcium was measured by flow cytometry. Calcium mobilization was calculated by subtracting the 'fluorescence after stimulation' from the 'baseline fluorescence'.

Animal Experiments

Overnight cultures of *S. aureus* Newman and *S. aureus* NewmanΔEcbΔEfb were diluted 1:100 in fresh THB and grown with shaking at 37°C to an OD₆₀₀ of 0.8. Bacterial cultures were centrifuged and resuspended in PBS to the desired concentrations. The inoculum was verified by plating and colony enumeration.

The pneumonia model was performed as described previously [28], with minor modifications. Following anesthesia with ketamine and xylazine, 8-week-old female CD1 mice (Charles River Laboratories) were infected with 10 µl of 2×10^8 CFU *S. aureus* in each nare. Animals were held upright for 1 min and recovery from anesthesia was monitored. Animals were euthanized by CO₂ inhalation at 6 and 24 h after challenge. Blood was collected by cardiac puncture and lungs were lavaged with 1 ml PBS to collect bronchoalveolar lavage fluids. Right lungs and noses were excised and bacterial loads were enumerated by plating homogenized tissues in serial dilutions on THA. The pneumonia model was performed twice using cohorts of 5 mice per group for each experiment. Neutrophil influx was analyzed in two separate experiments using a total of 9 mice per group and 3 PBS-infected mice. Mice were euthanized 6 h after challenge and lungs were inflated with 10% formalin. The trachea was closed and formalin-inflated lungs were excised, fixed in 10% formalin for 24 h and kept in 70% ethanol prior to embedding in paraffin.

For the intravenous infection model [29], 8-week-old female Balb/c mice (Charles River Laboratories) were infected with 1.5×10^7 CFU *S. aureus* by intravenous inoculation via the lateral tail vein. To determine bacterial loads in the bloodstream 3 h after infection, a small incision in the tail was made and a few droplets of blood were collected. To assess mortality, mice were checked daily for clinical signs and body weight measurements were taken. To confirm reproducibility, the survival experiment was per-

formed twice using cohorts of 7 mice per group for each experiment. In a separate experiment, Balb/c mice were infected with 1.5×10^7 CFU *S. aureus* (n = 7 for wild-type bacteria, n = 9 for ΔEcbΔEfb) via intravenous inoculation and sacrificed 2 or 10 days after infection by isofluorane inhalation. Blood was collected from the portal vein; lungs, heart, kidneys, spleen and liver were excised. All organs and the right kidney were homogenized and bacterial loads were enumerated by plating serial dilutions on THA. To assess abscess formation, the left kidney was fixed in 10% formalin and embedded in paraffin. Paraffin-embedded kidneys were sectioned 3 and 6 µm away from the organ center. Sections were stained with hematoxylin and eosin (H&E) and examined by microscopy using a Nikon Eclipse E800M microscope. The abscess lesions were quantified by measuring diameters of both abscess and organ; percentage of abscess lesions was calculated using the formula: [(abscess length × abscess width)/(organ length × organ width)] × 100%.

Immunohistochemistry

Lung sections (5 µm thick) were deparaffinized by successive immersion in 3 changes of xylene (10 min each) and rehydrated by immersion in decreasing concentrations of ethanol (100, 95 and 70%, each twice for 5 min). After washing 3 times with PBS, slides were blocked with 1% BSA in PBS + anti-CD16/32 (BD Bioscience; 1 µg/ml), 0.1% avidin, 0.01% biotin and 0.3% H₂O₂ (each 10 min after washing 3 times with PBS). Antigen retrieval was performed by heating slides in a microwave (2 × 5 min) in citrate buffer (Antigen Retrieval Solution, Dako, Glostrup, Denmark). Slides were left at room temperature for 20 min and washed with PBS. Immunostaining was performed overnight at 4°C with primary rabbit anti-myeloperoxidase antibody (Dako; 10 µg/ml in 1% BSA-PBS) or respective isotype control (rabbit IgG, Jackson, Westgrove, Pa., USA). After washing, slides were incubated with biotinylated goat anti-rabbit IgG (Jackson) and peroxidase-labeled streptavidin (Jackson), each for 30 min at room temperature. Sections were developed with the AEC substrate kit for peroxidase (Vector Labs), counter-stained with Mayer's hematoxylin (Sigma) for 1 min and embedded in Vectamount (Vector Labs, Burlingame, Calif., USA).

Statistics

Statistical significance of bacterial survival in blood and calcium mobilization was analyzed using the unpaired 2-tailed Student t test. Statistical significance of mouse survival data was calculated using the log-rank test. Weight loss and abscess formation was analyzed using the unpaired 2-tailed Student t test. Data for quantification of bacterial burden in tissues were log-transformed, checked for normal distribution and then statistically analyzed using the unpaired 2-tailed Student t test.

Results

Bacterial Mutants

Ecb and Efb are genomically clustered on immune evasion cluster 2, which also harbors other candidate immune evasion proteins [16] (fig. 1a). We created a series of insertional mutants in *S. aureus* Newman strain by al-

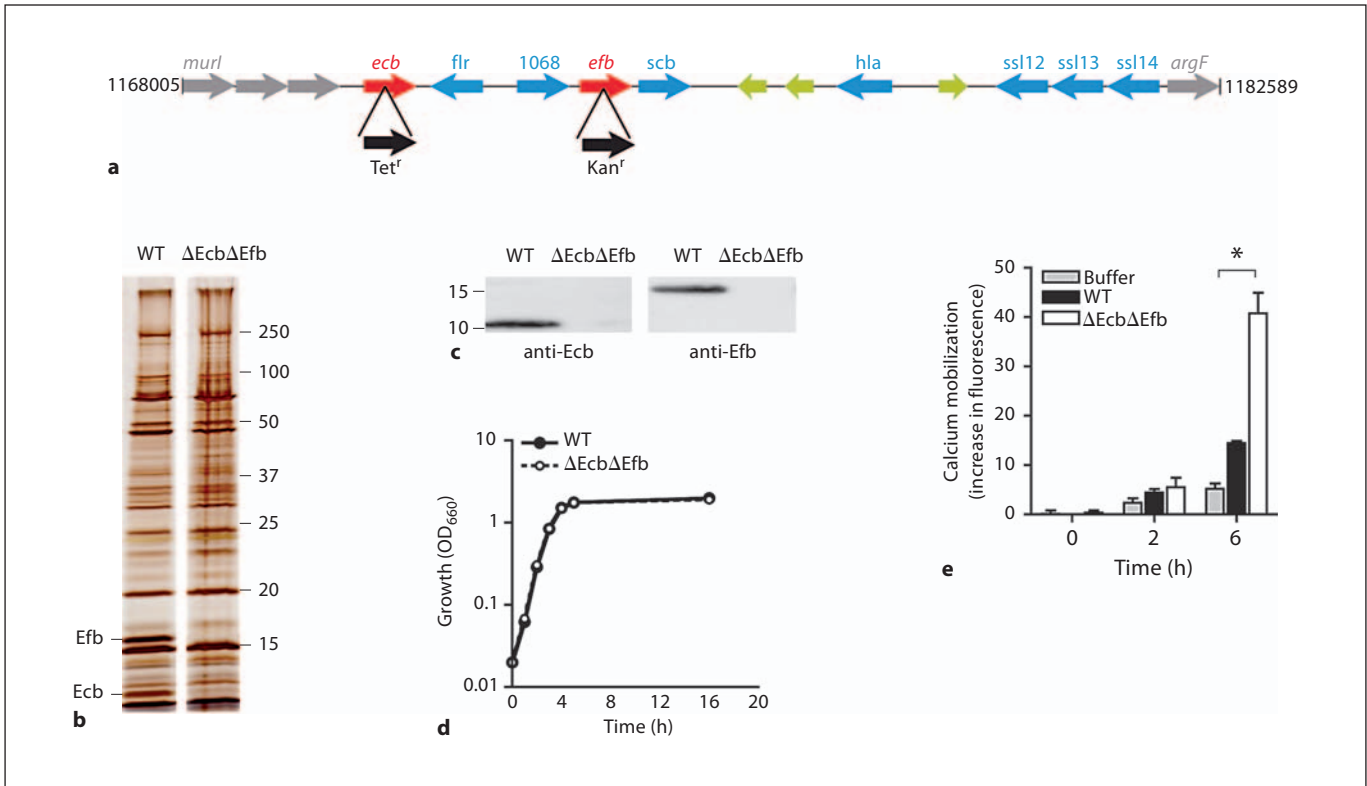


Fig. 1. Generation of *S. aureus* mutants. **a** Genomic location of *ecb* and *efb* on immune evasion cluster 2 in *S. aureus* Newman. The genes for *ecb* and *efb* were inactivated by allelic replacement with tetracycline or kanamycin resistance cassettes (Tet^r and Kan^r). Other immune evasion proteins encoded by IEC2 are FPR-like 1 inhibitory protein (*flr*), NWMN_1068 (unknown function), staphylococcal complement inhibitor B (*scb*), alpha-haemolysin (*hla*) and staphylococcal superantigen-like proteins 12, 13 and 14 (*ssl12*, *ssl13*, *ssl14*). IEC2 is flanked by the household genes glutamate racemase (*murI*) and ornithine carbamoyltransferase (*argF*). **b, c** Supernatants of *S. aureus* Newman (WT) and its isogenic mu-

tant $\Delta Ecb\Delta Efb$ were analyzed by SDS-PAGE and silverstaining (**b**) or immunoblotting (**c**). Representative figures of 3 separate experiments. **d** Growth curves of *S. aureus* Newman strain and the $\Delta Ecb\Delta Efb$ mutant in THB. Graph represents mean \pm SE of 3 experiments. **e** *S. aureus* Newman (WT) and its mutant $\Delta Ecb\Delta Efb$ were incubated with 30% human serum, and the release of complement C5a in supernatants was analyzed via calcium mobilization on U937-C5aR cells. Calcium mobilization was calculated by subtracting the 'fluorescence after stimulation' from the 'baseline fluorescence'. Figure represents mean \pm SE of 3 independent experiments. * $p < 0.05$.

lic replacement: 2 single mutants with antibiotic resistance markers inserted in the genes encoding Ecb (ΔEcb) or Efb (ΔEfb) and 1 double mutant with insertions in both loci ($\Delta Ecb\Delta Efb$) (fig. 1a). Analysis of bacterial supernatants by silverstaining and immunoblotting confirmed the absence of Ecb and Efb in $\Delta Ecb\Delta Efb$ (fig. 1b, c); single mutants also showed no secretion of the corresponding targeted protein (data not shown). In bacterial growth medium (THB), mutants showed comparable growth to the wild-type parent strain (fig. 1d). To further validate our mutant, we analyzed whether Ecb and Efb block C5a formation in the context of the live infectious organism. We incubated *S. aureus* Newman or the isogenic $\Delta Ecb\Delta Efb$ mutant with human serum and quanti-

fied release of C5a into supernatants by a calcium mobilization assay, using U937 cells transfected with the C5aR (U937-C5aR) [27]. The specificity of this assay for C5a is demonstrated in the online supplementary figure 1 (see www.karger.com/doi/10.1159/000334604 for all online suppl. material). Figure 1e shows that wild-type bacteria generate significantly less C5a than the $\Delta Ecb\Delta Efb$ mutant.

Ecb and Efb Contribute to S. aureus Survival in Human Blood

To study the contribution of Ecb and Efb to *S. aureus* virulence, we first analyzed bacterial survival in human blood. Following 6 h of incubation, the single mutants

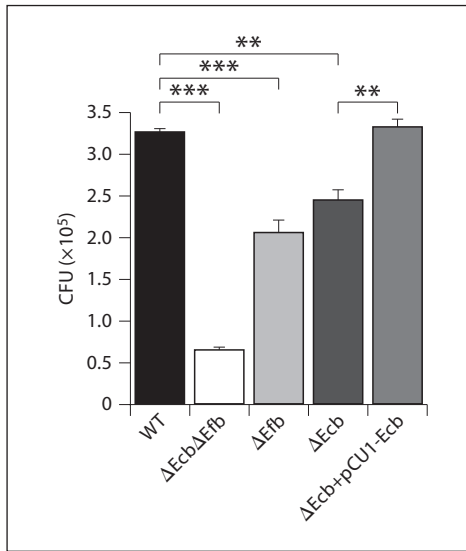


Fig. 2. Contribution of Ecb and Efb to *S. aureus* survival in human blood. *S. aureus* Newman (WT) and its mutants Δ Ecb Δ Efb, Δ Efb, Δ Ecb, or the complemented Δ Ecb+pCU1-Ecb were incubated with 25% human blood for 6 h, and bacterial survival was analyzed by colony enumeration. The figure represents mean \pm SE of 3 independent experiments. ** $p < 0.01$, *** $p < 0.005$.

Δ Ecb and Δ Efb were killed more efficiently than the wild-type strain (fig. 2). The survival defect of the Δ Ecb mutant was reversed by complementation on a plasmid vector. In addition, we observed that the Δ Ecb Δ Efb double mutant was killed more efficiently than the single mutants ($p < 0.0001$). We therefore decided to use this mutant for our subsequent in vivo analyses.

Ecb and Efb Together Block Neutrophil Recruitment during S. aureus Pneumonia

To investigate whether *S. aureus* delays neutrophil recruitment by secretion of Ecb and Efb in vivo, we tested our mutant in a well-established *S. aureus* pneumonia model [28]. Mice were inoculated via the intranasal route with 4×10^8 CFU *S. aureus* Newman or its Δ Ecb Δ Efb mutant. At this dose, animals did not succumb to infection. Animals were sacrificed 6 and 24 h after challenge to quantify bacterial loads in lavage and lung tissue (fig. 3a). All animals appeared to be ill, especially after 24 h when all animals displayed an increased respiratory rate and decreased mobility. We found no differences in bacterial burden 6 h after challenge. However, 24 h after challenge more bacteria were recovered from the lungs of mice infected with wild-type bacteria compared to those infected with mutant bacteria ($p = 0.03$), indicating that

Ecb and Efb together contribute to impairing bacterial clearance from the lungs. The large variability in bacterial loads can be explained by the steep dose-response curve of this model. To determine whether the impaired bacterial clearance could be correlated to alterations in neutrophil recruitment, we infected another cohort of mice and analyzed formalin-fixed lung tissues 6 h after inoculation. H&E staining of lung tissues showed markedly less neutrophils in mice infected with *S. aureus* Newman than those infected with its Δ Ecb Δ Efb mutant (fig. 3b, online suppl. fig. 2). The identity and quantification of neutrophils were confirmed by immunostaining against myeloperoxidase. Our data demonstrate that Ecb and Efb together contribute to *S. aureus* pneumonia by delaying the recruitment of neutrophils to the site of infection.

Ecb and Efb Together Are Required for S. aureus Virulence after Intravenous Infection

Staphylococcal infections in humans are often associated with bacterial persistence in organ tissues. Previously, Cheng et al. [29] developed a mouse model for *S. aureus* persistence and abscess formation. Following intravenous inoculation, pathogenic *S. aureus* quickly spreads into organ tissues and produces abscess lesions that cannot be cleared by the mice. Applying this model to study the role of Ecb and Efb in staphylococcal virulence in vivo, we challenged 8-week-old female Balb/c mice with 1.5×10^7 CFU of *S. aureus* Newman or its Δ Ecb Δ Efb mutant via intravenous inoculation. Survival, clinical signs and body weight were monitored daily for 30 days. We observed that the mice infected with wild-type bacteria (79%) experienced significantly higher mortality rates (fig. 4a) and more weight loss (fig. 4b) than mice infected with the Δ Ecb Δ Efb mutant (21%). These results demonstrate that Ecb and Efb together contribute to staphylococcal virulence after intravenous inoculation.

Ecb and Efb Together Are Required for Bacterial Persistence and Abscess Formation

To study whether Ecb and Efb contribute to bacterial persistence in tissues, we repeated the intravenous infection model and sacrificed mice either 2 or 10 days after challenge. Bacterial burden in several organs was determined from viable counts of homogenized tissues. Bacterial loads in the bloodstream were determined 3 h after infection. Surprisingly, we found significantly more Δ Ecb Δ Efb (1.1×10^5 CFU/ml) than wild-type bacteria (2.7×10^4 CFU/ml) in the bloodstream ($p = 0.02$). Consistent with the original report of this model [29], no

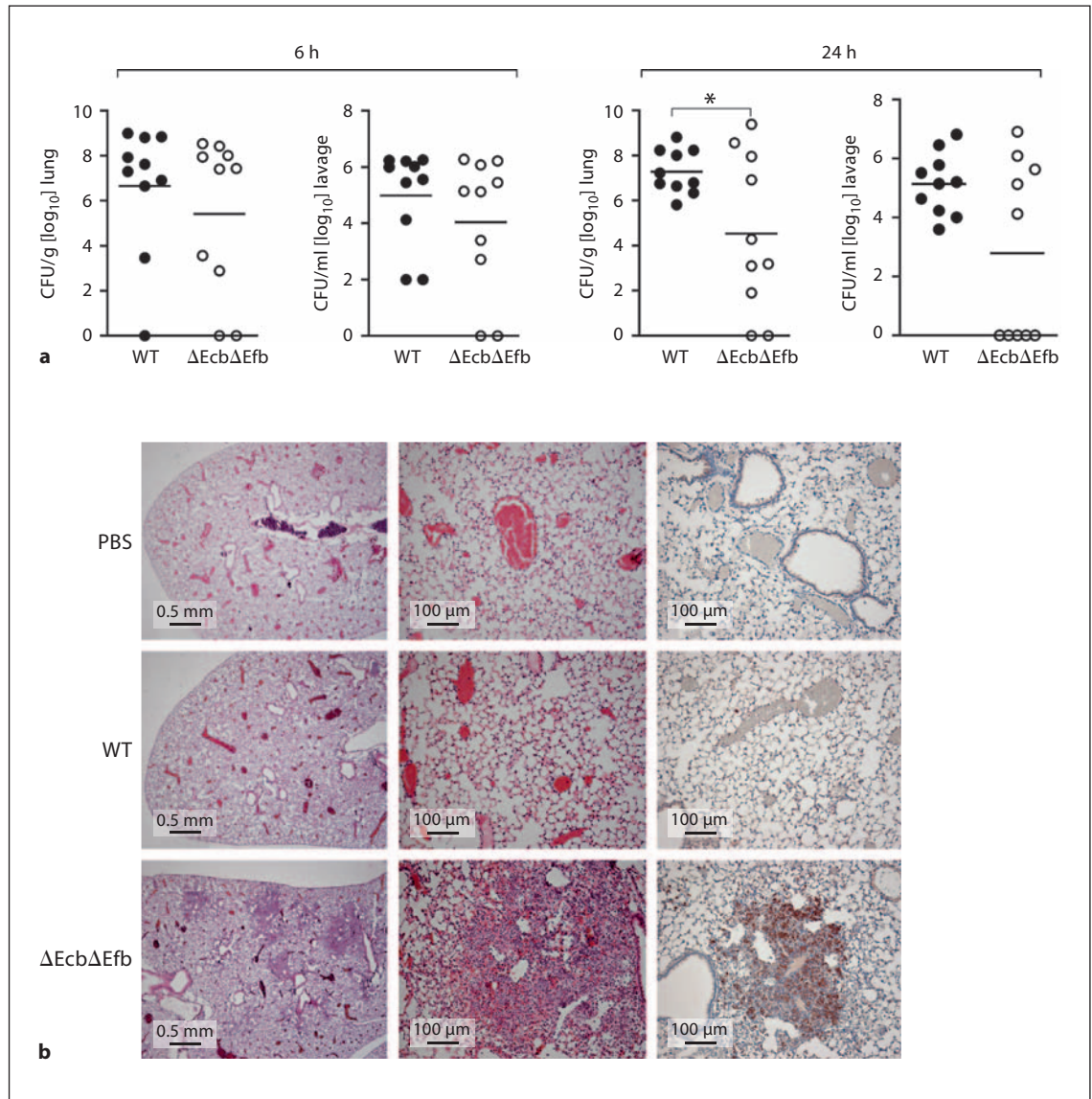


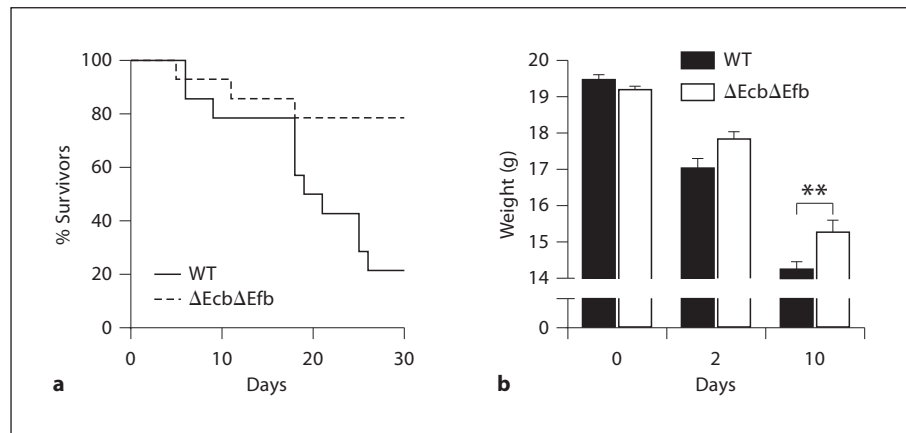
Fig. 3. Ecb and Efb together block neutrophil influx during *S. aureus* pneumonia. **a** Cohorts of 10 mice were infected with 4×10^8 CFU *S. aureus* Newman (WT) or its mutant Δ Ecb Δ Efb via intranasal inoculation. Animals were sacrificed 6 or 24 h after challenge. Bacterial burden in the lungs and lavage was assessed by colony enumeration. * $p < 0.05$. **b** Mice were infected with PBS ($n = 3$), 4×10^8 CFU *S. aureus* Newman (WT) or its mutant

Δ Ecb Δ Efb ($n = 9$ per group) via intranasal inoculation. Animals were sacrificed 6 h after challenge and formalin-fixed lung tissues were stained with H&E (2 left columns) or peroxidase-labeled streptavidin-detection of myeloperoxidase (right column, brownish staining). One representative image per group is shown. See online supplementary figure 2 for more images of lung sections. Data in **a** and **b** are representative of 2 independent experiments.

staphylococci were found in the blood 2 days after challenge, but high bacterial loads were found in the heart, kidneys, lungs and liver. Two days after challenge, we found no differences in bacterial burden between the mice infected with wild-type and Δ Ecb Δ Efb bacteria. However, 10 days after challenge, we observed significantly higher bacterial loads in the heart and kidneys of

mice infected with wild-type bacteria than those infected with Δ Ecb Δ Efb mutants (fig. 5a, b). No significant differences in bacterial loads were found in the lungs, spleen and liver (online suppl. fig. 3). As this model elicits large *S. aureus* abscess communities in the kidney [29], we excised the left kidney for histopathology analysis (10 days after challenge). A gross examination revealed more ab-

Fig. 4. Ecb and Efb together are required for *S. aureus* virulence. Cohorts of 14 mice were infected with 1.5×10^7 CFU of *S. aureus* Newman (WT) or its mutant Δ Ecb Δ Efb via intravenous inoculation. Animal survival and body weight was monitored over time. **a** Higher mortality of mice infected with WT than with Δ Ecb Δ Efb ($p = 0.007$, log-rank test; $n = 14$ mice). **b** Weight of infected mice (mean \pm SE). ** $p < 0.01$. Data are representative of 2 independent experiments.



successes on the outside of the kidneys of mice infected with wild-type bacteria than those infected with mutant bacteria (fig. 5c). Microscopic examination of H&E-stained kidney sections confirmed this result (fig. 5d). We found that the kidney abscesses of mice infected with wild-type *S. aureus* contained central populations of staphylococci that were separated from healthy tissue by amorphous material, likely eosinophils and necrotic neutrophils [29] (fig. 5e). However, kidney abscesses of mice infected with the Δ Ecb Δ Efb mutant showed large zones of viable neutrophils (online suppl. fig. 4), indicating that immune defenses were still active. Altogether, these data demonstrate that Ecb and Efb are staphylococcal virulence factors that collaboratively block neutrophil recruitment to the site of infection and contribute to bacterial persistence and abscess formation.

Discussion

To promote its survival within the human host, *S. aureus* has evolved a wide variety of virulence factors such as adhesins, toxins, superantigens, proteases and immune evasion proteins [6, 30]. Although the in vivo contribution of most virulence factors to staphylococcal pathogenesis and mortality is generally accepted, the role of secreted immune evasion proteins is unclear due to the lack of in vivo data and potential overlapping immune escape strategies targeting similar host defense pathways. In recent years, we and other authors [6] described a large group of small (approx. 10–30 kDa) secreted proteins in *S. aureus* that target different steps in the inflammatory response: staphylococcal superantigen-like 5 (SSL5) and extracellular adherence protein block neutrophil rolling

and adhesion, chemotaxis inhibitory protein of staphylococci prevents neutrophil chemotaxis by blocking chemotactic receptors, staphylokinase and aureolysin neutralize antimicrobial peptides and Ecb, Efb, staphylococcal complement inhibitor (SCIN), SCIN-B/C, *S. aureus* binder of IgG, aureolysin, SSL7 and SSL10 all effectively downmodulate different steps in the complement cascade. The immune evasion proteins are found in almost all human *S. aureus* clinical isolates [31], and increasing evidence suggests they are produced during staphylococcal infections in humans [16, 32–34]. Although the large number of candidate immune escape proteins of *S. aureus* could appear functionally redundant, we show here that removal of only two of these factors already markedly affects bacterial virulence. Apparently, *S. aureus* must attempt to neutralize the host immune system in a multifaceted fashion to maximize its survival in the host. Future research will be needed to establish the in vivo importance of other candidate *S. aureus* immune evasion proteins. An ongoing challenge in these analyses will be to overcome limitations such as host specificity. Also, further development of animal models to study secreted proteins in the context of immune evasion will be needed. The high infective doses required for mouse models are a disadvantage as the innate immune system is instantly activated upon contact with the infectious particle even before the immune evasion proteins are being produced. Still, our studies on Ecb and Efb predict that other secreted immune evasion proteins can be studied in mouse models.

In the pneumonia model, we observed that Ecb and Efb together blocked neutrophil influx to the lungs of mice infected with *S. aureus*. The decreased neutrophil influx corresponded with an impaired capacity to clear

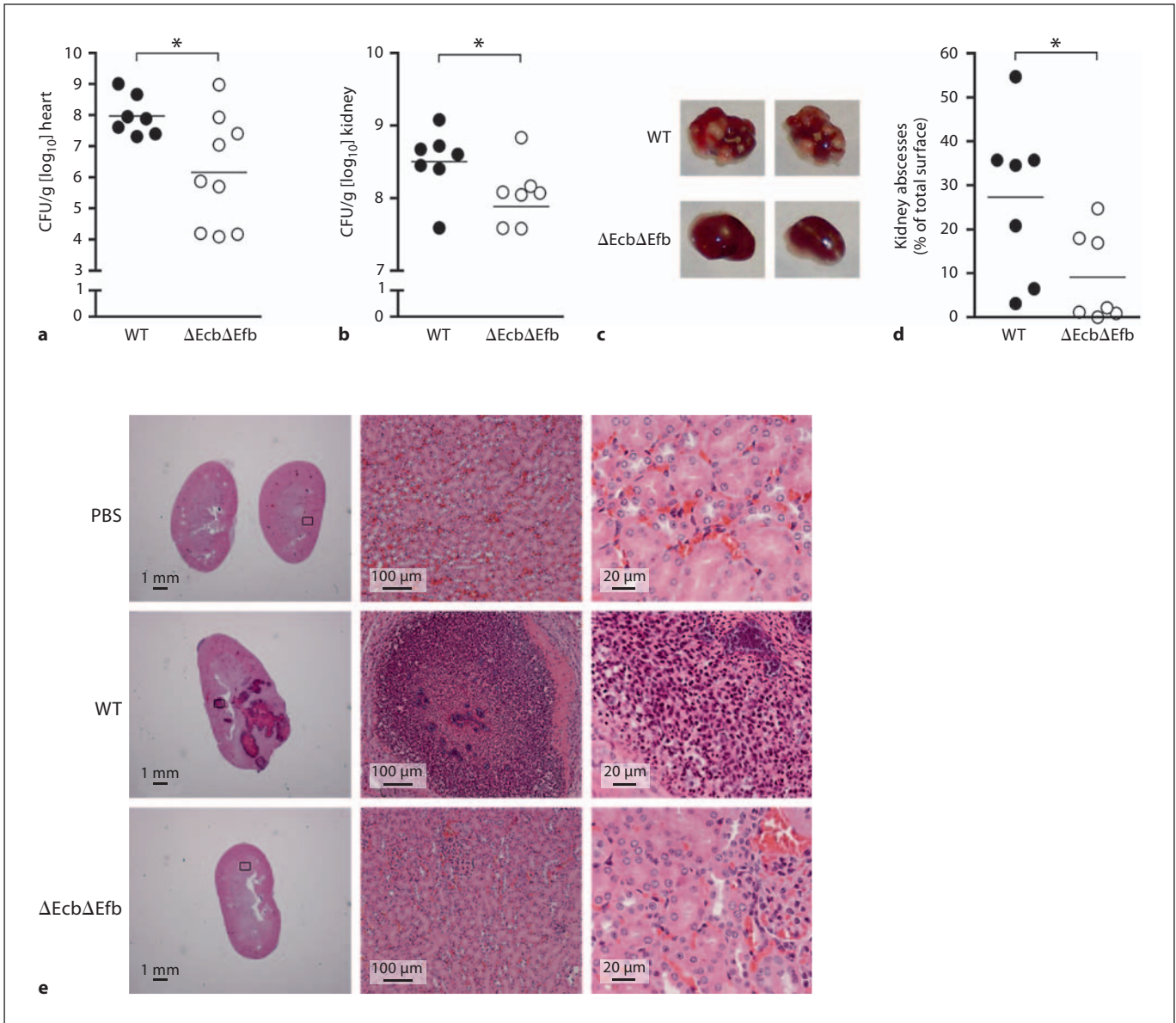


Fig. 5. Ecb and Efb together are required for bacterial persistence and abscess formation. Cohorts of 7 mice were infected with 1.5×10^7 CFU of *S. aureus* Newman WT or its mutant ΔEcbΔEfb via intravenous inoculation. Two mice were injected with PBS. Animals were sacrificed 10 days after challenge. **a** Bacterial loads in heart tissue. **b** Bacterial loads in kidney tissue. **c** Mouse kidneys at day 10 (representative images of 2 mice per group). **d** Quantitative determination of kidney abscesses from H&E-stained kid-

neys at day 10. The abscess lesions were visualized by microscopy and quantified by measuring diameters of both abscess and organ; % of abscess lesions was calculated using the formula: [(abscess length × abscess width)/(organ length × organ width)] × 100%. **e** H&E-stained kidney sections (1 representative image per group, see online suppl. fig. 4 for more images). * p < 0.05. Data represent 1 experiment.

the bacteria later on during infection. These data are in line with previous studies demonstrating a critical role for neutrophils in the clearance of *S. aureus* from the lungs in mice [35]. The importance of neutrophils in *S. aureus* clearance in humans is also well accepted because

people suffering from chronic granulomatous disease encounter recurrent *S. aureus* infections [36]. As Efb can block neutrophil functions via multiple mechanisms, it remains uncertain at this point whether the impaired neutrophil influx for our double mutant was a result of

the inhibition of complement, fibrinogen or both. Complement and fibrinogen are both important for neutrophil recruitment during *S. aureus* infections: (1) complement component C5 is critical in the clearance of *S. aureus* from the lungs in mice [37] and (2) Fg-deficient mice show an impaired clearance of *S. aureus* from the peritoneal cavity [38]. Furthermore, since Efb also interferes with platelet activation [18], this might indirectly impair neutrophil recruitment because platelets produce various chemokines that can chemoattract neutrophils [39]. Although the exact inhibitory mechanism remains unclear, we show that Ecb and Efb, when produced by *S. aureus*, can block neutrophil influx during pneumonia.

The formation of abscesses is a common pathology during *S. aureus* infections; abscesses are comprised of bacteria and recruited neutrophils. In the renal abscess model that we used here, neutrophils seem crucial in abscess development. Previously, Cheng et al. [29] found that during the first days of infection, staphylococci in renal tissues are surrounded by healthy infiltrated neutrophils. However, neutrophils fail to clear the bacteria and are subsequently found as dead cells within the abscess. We found that abscesses are more easily cleared in the absence of Ecb and Efb, suggesting that staphylococcal evasion of neutrophil influx is critical to bacterial survival in abscess communities. It is likely that Ecb and Efb delay neutrophil influx in the early stages of infection and thereby allow bacterial multiplication and the formation of abscesses. As suggested previously, abscess formation seems primarily driven by *S. aureus* rather than the host [29, 40]. In the later stages of disease, abscesses can rupture, leading to a secondary wave of abscesses with a lethal outcome. The fact that wild-type bacteria did not show better survival in mouse blood 3 h after infection

strongly suggests that the increased mortality caused by these bacteria is caused by their improved success in spreading to and surviving in the organs, rather than their survival advantages in the blood.

Increasing evidence is now presented that inactivation of virulence factors can reduce *S. aureus* morbidity and mortality in mice. Therefore, virulence factors are considered essential components for future vaccination strategies. Recent promising data showed that the immunization of mice with (inactivated) forms of virulence proteins confers protection against *S. aureus* disease [41]. Our study indicates that immune evasion proteins should also be considered as targets in *S. aureus* vaccines. This is especially true since bacterial immune evasion also hampers vaccine efficacy: antibacterial antibodies do not work when neutrophils cannot reach the site of infection or when complement is inactivated. The redundant mechanisms that *S. aureus* has evolved to escape innate immune defenses suggest that multivalent vaccines may be required to obtain effective protection against this pathogen in humans.

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