

Contribution of Staphylococcal Enterotoxin B to *Staphylococcus aureus* Systemic Infection

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Staphylococcal enterotoxin B (SEB), which is produced by the major human pathogen, *Staphylococcus aureus*, represents a powerful superantigenic toxin and is considered a bioweapon. However, the contribution of SEB to *S. aureus* pathogenesis has never been directly demonstrated with genetically defined mutants in clinically relevant strains. Many isolates of the predominant Asian community-associated methicillin-resistant *S. aureus* lineage sequence type (ST) 59 harbor *seb*, implying a significant role of SEB in the observed hypervirulence of this lineage. We created an isogenic *seb* mutant in a representative ST59 isolate and assessed its virulence potential in mouse infection models. We detected a significant contribution of *seb* to systemic ST59 infection that was associated with a cytokine storm. Our results directly demonstrate that *seb* contributes to *S. aureus* pathogenesis, suggesting the value of including SEB as a target in multipronged antistaphylococcal drug development strategies. Furthermore, they indicate that *seb* contributes to fatal exacerbation of community-associated methicillin-resistant *S. aureus* infection.

Keywords. *Staphylococcus aureus*; superantigen; staphylococcal enterotoxin B; sepsis; ST59; cytokine storm; CA-MRSA.

The dangerous human pathogen *Staphylococcus aureus* is a producer of many toxins that collectively contribute to its virulence potential [1]. The staphylococcal enterotoxins (SEs) are a subset of the staphylococcal superantigen family, which also includes toxic shock syndrome (TSS) toxin 1 (TSST-1) and enterotoxinlike serotypes [2]. All superantigen toxins are powerful nonspecific stimulators of T cells [2]. They bypass the normal antigen-specific restrictions of immune cell activation by forming a bridge between the major histocompatibility class (MHC) II receptors on antigen-presenting cells with V β chains on T-cell receptors [3]. This nonconventional mechanism of immune cell activation results in rapid T-cell expansion and is accompanied by a massive proinflammatory cytokine release, which is the principal mediator of a number of diseases, such as immunoglobulin E-associated inflammatory diseases, pulmonary, and, most notably, TSS [2]. TSS is a systemic disease characterized by the fast onset of fever, organ failure, and death [4].

There are about 20 different SEs, which are encoded on different staphylococcal pathogenicity islands [5]. Staphylococcal enterotoxin B (SEB), which is encoded together with SEs K, L, and Q on the pathogenicity island SaPI3, has received most attention. It is considered a biological weapon and classified as a select agent in the United States. Therefore, considerable efforts have been dedicated to creating SEB-neutralizing strategies [6]. It has been reported early that there is an epidemiological association of TSS not only with TSST-1 but also with SEB [7]. Furthermore, manifestations of TSS disease have been demonstrated in multiple studies in different animal species that received purified SEB toxin [6] and in animals that were infected with SEB-producing *S. aureus* strains [8]. Moreover, plasmid-based expression of SEB in a laboratory strain devoid of other superantigens resulted in increased fatality in a mouse pneumonia model [9] and anti-SEB antibodies decreased morbidity and mortality rates in several mouse infection models [10]. However, while it has frequently been speculated that SEB may contribute to disease in a natural *S. aureus* strain background, this cannot be concluded from these study findings and has never been directly investigated, which can be done only by comparing an *seb* mutant with an isogenic parental clinical strain in an appropriate animal model.

Because the *seb* gene is encoded on the mobile genetic element SaPI3, SEB production is limited to specific genetic lineages [11–15]. Among clinically important lineages, it has been reported to occur frequently in the predominant Asian community-associated methicillin-resistant *S. aureus*

Received 29 April 2020; editorial decision 4 September 2020; accepted 14 September 2020; published online September 16, 2020.

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The Journal of Infectious Diseases® 2021;223:1766–75

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(CA-MRSA) lineage of sequence type (ST) 59 [16, 17]. Similar to other CA-MRSA lineages such as USA300, ST59 isolates exhibit exceptional virulence that is believed to underlie epidemiological success and an ability to infect otherwise healthy individuals [18]. In the current study, we therefore selected a representative ST59 CA-MRSA strain as a host to create an isogenic *seb* mutant and investigate the contribution of SEB to pathogenesis. We compared the virulence characteristics of this mutant with its wild-type (WT) parent in local and systemic disease, using the HLA transgenic mouse model. This is an established model to determine SEB-induced lethal shock and is based on MHC class II knockout mice that express human MHC class II HLA-DR3 [19–21]. Our data provide previously unavailable direct evidence for a contribution of SEB to *S. aureus* pathogenesis and indicate that SEB plays a significant role in the observed hypervirulence of a clinically important, widespread CA-MRSA lineage.

METHODS

Study Approval

The Animal Care and Use Community at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, reviewed and approved the animal protocol used (Animal Study Protocol LB-1E), according to the animal welfare act of the United States (7 USC 2131 et seq). All mouse experiments were performed at the animal care facilities of the National Institute of Allergy and Infectious Diseases, in accordance with approved guidelines. All animals were euthanized with carbon dioxide (CO₂) at the end of the studies. Human peripheral blood mononuclear cells (PBMCs) were isolated from venous blood samples from healthy volunteers in accordance with a protocol (no. 2019–43) approved by the Shanghai Blood Center, Shanghai, China. Informed written consent was obtained from all volunteers.

Bacterial Strains, Plasmids, and Growth Conditions

MRSA isolates of ST59, ST5, and ST239 were isolated from patients obtained at Renji hospital, Shanghai between 2005 and 2014 [18, 22]. All bacteria were grown in tryptic soy broth or passaged on tryptic soy agar at 37°C. Bacterial cultures were supplemented with chloramphenicol (25 µg/mL) or tetracycline (12.5 µg/mL) when needed, and assays with plasmid-harboring strains were performed with addition of the respective antibiotic. Bacterial culture filtrates were collected at 16 hours, passed through 0.22-µm PES membrane filters (Millipore), and stored at –20 °C until needed. For animal infections, bacteria were prepared as described elsewhere [23].

Basic Molecular Biology Methods

DNA was isolated from 44 randomly selected ST59, ST5, and ST239 clinical isolates, as follows. Bacteria from overnight cultures in tryptic soy broth were lysed with lysostaphin (Sigma), and DNA was extracted using phenol, chloroform, and isoamyl alcohol at a ratio of 25:24:1, respectively. Finally, the DNA was precipitated by adding 2 volumes of 100% ethanol before resuspension in distilled water. The oligonucleotide primer pairs used to detect the presence of SE genes by analytical polymerase chain reaction (PCR) have been described elsewhere [24].

Allelic Gene Replacement by Homologous Recombination and Genetic Complementation

A markerless isogenic *seb* mutant was created in RJ-2, a representative clinical CA-MRSA isolate of the ST59 lineage [18], by homologous recombination using the plasmid, pKOR1, as described elsewhere [25]. For genetic complementation of the *seb* mutation, the *seb* gene was PCR amplified and cloned into the pTX_Δ plasmid via BamH1 and Mlu1 restriction sites. Plasmid pTX_Δ is derived

Table 1. Bacterial Strains and Plasmids Used in the Current Study

Strain or Plasmid	Comment	Source
<i>Staphylococcus aureus</i> strain		
RN4220	Derived from NCTC8325-4; r ^m +	Kreiswirth et al [27]
WT	CA-MRSA ST59 clinical isolate, RJ-2	Li et al [18]
Δ <i>seb</i>	RJ-2 <i>seb</i> mutant	Current study
WT pTX _Δ 16	RJ-2 with pTX _Δ 16	Current study
Δ <i>seb</i> pTX _Δ 16	RJ-2 <i>seb</i> mutant with pTX _Δ 16	Current study
Δ <i>seb</i> pTX _Δ <i>seb</i>	RJ-2 <i>seb</i> mutant with pTX _Δ <i>seb</i>	Current study
Plasmids		
pKOR1	cm ^R and amp ^R , temperature-sensitive vector for allelic replacement via lambda recombination and <i>ccdB</i> selection	Bae et al [25]
pKOR1Δ <i>seb</i>	Temperature-sensitive vector for allelic replacement of <i>seb</i> in ST59	Current study
pTX _Δ 16	Control plasmid for the pTX _Δ plasmid series; lipase gene is deleted.	Wang et al [23]
pTX _Δ <i>seb</i>	pTX _Δ plasmid containing the <i>seb</i> gene of <i>S. aureus</i> ST59; <i>xyIR</i> deleted in that plasmid series for constitutive gene expression	Current study

Abbreviations: amp^R, Ampicillin resistance; CA-MRSA, community-associated; cm^R, Chloramphenicol resistance; *Staphylococcus aureus*; WT, wild type.

from the xylose-inducible plasmid pTX15 [26], in which the inducible *xyIR* repressor gene was deleted to allow constitutive expression of the gene of interest [23]. Strains harboring the empty plasmid pTX_Δ16 were used as controls. Strains and plasmids used in this study are listed in Table 1. Oligonucleotides are listed in Supplementary Table 1. The *seb* gene deletion in RJ-2 was verified by analytical PCR and sequencing of the genomic DNA at the borders of the PCR-derived regions.

PBMC Isolation and T-Cell Proliferation Assay

PBMCs, collected as described above, were purified from heparinized venous blood using lymphocyte separation medium (TBDscience), according to the manufacturer's instructions. Briefly, heparinized blood was mixed with an equal volume of calcium- and magnesium-free Roswell Park Memorial Institute 1640 medium (RPMI-1640). The diluted blood was carefully layered onto lymphocyte separation medium (HBD) and then centrifuged for 15 minutes. The lymphocytes, collected at the interface between PBS and lymphocyte separation medium, were washed once with PBS and any remaining erythrocytes were lysed using Ammonium-Chloride-Potassium (ACK) lysis buffer (TBDscience). The remaining cells were washed again with PBS and then resuspended into RPMI-1640 to the desired cell concentration.

The T-cell proliferation assay was performed with an adenosine triphosphate-based detection method as described elsewhere [28]. Briefly, in 96-well plates, equal volumes of sterile-filtered culture filtrates were added to PBMCs at a concentration of 5×10^4 cells per well and at a final dilution of 1:1000. Plates with stimulated cells were incubated in a humidified incubator at 37°C with 5% CO₂ for approximately 48 hours. After incubation, the plates were centrifuged, and supernatants were collected and assessed for lactate dehydrogenase release, according to the manufacturer's instructions (Promega). No significant cell lysis was observed compared with wells containing cells only. T-cell proliferation was determined from the pelleted cells using the ViaLight Plus kit (Promega), according to the manufacturer's instructions.

Protein Analyses

Production of SEB protein in 16-hour culture filtrates was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining, and Western blotting with a rabbit anti-SEB specific antibody (Sigma-Aldrich) to detect a 28.4-kDa band. Band intensities were determined using ImageJ software (version 1.52k). Overall protein expression in 8-hour and 16-hour culture filtrates was determined using SDS-PAGE followed by Coomassie blue staining.

Mouse Experiments

For animal studies, female, age-matched 8–12 week-old, C57BL-6NCrl (Charles-River) or HLA-DR3 mice were used, as

indicated. HLA-DR3 mice, which do not express any endogenous mouse MHC class II molecules, exhibit enhanced sensitivity to SEB owing to transgenic expression of HLA-DRA*0101 and HLA-DRB*0301 [19–21].

For systemic infections, mice were injected in the tail vein with 100 μL of phosphate-buffered saline (PBS) containing 2×10^8 colony-forming units (CFUs) of live *S. aureus* and deaths in mice were recorded over 8–10 days. In other experiments, mice were euthanized 12 hours after infection. Blood, collected by cardiac puncture, was distributed between serum, heparin, or ethylenediaminetetraacetic acid tubes (Sarstedt) for cytokine, CFU, or complete blood cell count analyses, respectively. Blood samples in ethylenediaminetetraacetic acid tubes were analyzed using a ProCyt Dx analyzer (IDEXX Laboratories) within 3 hours of collection for complete blood cell analysis. Serial dilutions of homogenates from kidneys, livers, and spleens in PBS and blood collected in heparin tubes were plated onto tryptic soy broth agar for CFU determination. Sera were stored at –20°C until needed.

For the skin infection model, the dorsa of mice were shaved, and fine hair was removed with depilatory cream 24 hours before infection. One injection of approximately 5×10^7 CFUs of live *S. aureus* in 50 μL was delivered subcutaneously into the left and right flanks of anesthetized mice. ImageJ software (version 1.52k) was used to calculate abscess areas from images taken daily with a digital camera. To determine CFU loads and cytokine production, mice were euthanized at 1, 2, 3 or 7 days after infection, and the abscess skin tissues were harvested. Skin samples were homogenized in PBS and serially diluted for bacterial CFUs, as described elsewhere [29]. Supernatants from the skin homogenates were collected for the subsequent detection of cytokines, as described below.

The pneumonia model was performed by introducing 30 μL of a bacterial suspension (approximately 2×10^9 CFUs) in PBS equally into both nares of anesthetized mice, and mouse survival was monitored for 7 days. All animals were euthanized with CO₂ at the end of the studies.

Enzyme-Linked Immunosorbent Assays

The concentrations of interferon (IFN) γ, interleukin 10 (IL-10), and tumor necrosis factor (TNF) α in mouse serum samples were measured using Quantikine enzyme-linked immunosorbent assay kits (R&D Systems), according to the manufacturer's instructions.

Statistical Analyses

Statistical analysis was performed with GraphPad Prism software, version 8.3.0, using unpaired, 2-tailed Student *t* tests or Mann-Whitney tests when comparing 2 groups and 1-way analysis of variance (ANOVA) or Kruskal-Wallis tests when comparing >2 groups. Tukey posttests were used in ANOVAs, and Dunn posttests in Kruskal-Wallis tests. Student *t* tests and ANOVAs were used only when data passed all the normality

tests used (Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov). Otherwise, nonparametric Mann-Whitney or Kruskal-Wallis tests were used. Kaplan-Meier survival curves were analyzed by means of log-rank (Mantel-Cox) tests. The number of animals and replicates for each experiment are indicated in the figure legends. All error bars represent standard deviations, and lines represent means.

RESULTS

Presence of the *seb* Gene Distinguishing ST59 CA-MRSA From Geographically Matched Predominant HA-MRSA Isolates

We investigated the distribution of SE genes in the CA-MRSA ST59 lineage in 44 randomly selected clinical isolates obtained between 2005 and 2014 [18] from Renji Hospital, Shanghai, a large teaching hospital that admits >10 000 patients from the whole Shanghai metropolitan area each day. In addition, we compared the SE gene profiles with geographically matched ST5 ($n = 44$) and ST239 ($n = 44$) clinical isolates that represent the 2 major hospital-associated MRSA (HA-MRSA) lineages in Asia [30]. The *sea* gene was the only SE common to all lineages (Figure 1). However, ST239 isolates carried *sea* more frequently than ST5 and ST59 isolates. The *sed* and *sej* genes were not found in any of the genetic lineages. When considering only SE genes unique to specific lineages, we found that all ST5 isolates harbored *seg*, *sei*, *sem*, *sen*, and *seo*, which are found exclusively in a large operon called the enterotoxin gene cluster [31] (Figure 1). The *sec*, *see*, *seh*, *sel*, and *sep* genes, and the *tst* gene encoding TSST-1 were found exclusively in ST5 isolates. Of note, this excludes an impact of TSST-1 on ST59 pathogenesis. In contrast, *sek* and *seq* were present only in ST239 and ST59 isolates (Figure 1). Most importantly, the presence of *seb* was 100% exclusive to ST59 isolates (Figure 1). This association

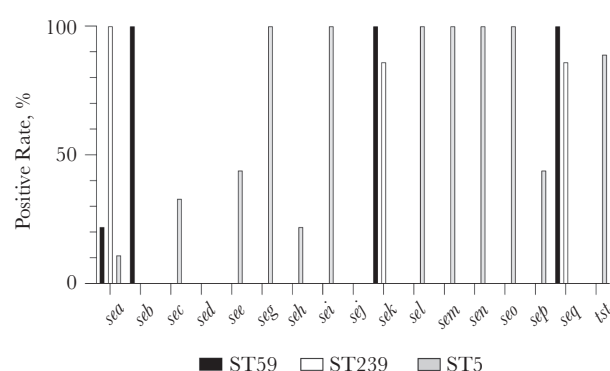


Figure 1. Presence of the *seb* gene distinguishes sequence type (ST) 59 community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) from geographically matched predominant hospital-associated (HA) MRSA isolates. The distribution of staphylococcal enterotoxin genes and the *tst* gene encoding toxic shock syndrome toxin 1 in CA-MRSA ST59, HA-MRSA ST239, and HA-MRSA ST5 clinical isolates from patients obtained at Renji hospital, Shanghai, between 2005 and 2014 was determined by means of analytical polymerase chain reaction. Forty-four isolates were randomly selected for each ST.

with the characteristically more virulent CA-MRSA, as opposed to less virulent HA-MRSA [24], suggested a contribution of *seb* to virulence.

SEB Contribution to Superantigenicity in CA-MRSA ST59

To directly investigate a possible role of SEB in CA-MRSA pathogenesis, we constructed an isogenic *seb* deletion mutant (Δseb) in RJ-2, a representative isolate of the CA-MRSA ST59 lineage [18]. Sterile culture filtrates from the Δseb mutant lacked SEB production (Figure 2A), which was correlated with a significantly decreased capacity to stimulate the proliferation of human PBMCs in vitro (Figure 2B). Complementation of the Δseb mutant with a plasmid constitutively expressing *seb* (pTX $_{\Delta seb}$) restored SEB production (Figure 2A) and superantigenic activity in vitro (Figure 2B). We noted increased expression of SEB in the Δseb pTX $_{\Delta seb}$ strain compared with the WT control harboring the control plasmid (pTX $_{\Delta 16}$), which is likely due to a multicopy plasmid effect. The Δseb mutant of RJ-2 showed no growth deficiency compared with WT, and all complemented strains grew similarly (Supplementary Figure 1), indicating that SEB expression was not affected by growth differences between the strains.

SEB Contribution to Systemic CA-MRSA Infection

To investigate the contribution of SEB to CA-MRSA ST59 pathogenesis in vivo, we examined the capacity of the WT and Δseb mutant strains to cause disease in murine lung, skin and blood infection models. We found no impact of SEB on survival in mouse models of pneumonia or skin infection (Supplementary Figures 2 and 3). However, we found that mice that were systemically infected with the Δseb mutant strain succumbed to infection significantly more slowly than mice infected with the WT strain (Figure 3). To investigate whether this phenotype was due to differences in bacterial burden or leukocyte numbers, we collected blood and prepared homogenates of organs harvested 12 hours after infection, before any fatalities occurred. We did not find any differences in bacterial CFU counts (Figure 4A) or changes in the number of white blood cells, neutrophils, monocytes, or lymphocytes between the 2 groups (Figure 4B).

Expression of the superantigen TSST-1 has been reported to strongly change overall expression of other secreted *S. aureus* proteins, and a similar role has also been suggested for SEB based on experiments in which the *seb* gene was expressed on a plasmid [32]. To investigate whether gene regulatory effects contribute to *seb*-mediated phenotypes in a natural strain background, we determined (1) the protein expression of the ST59 WT strain in comparison with its isogenic *seb* mutant and (2) the contribution of *seb* to systemic infection of WT (C57BL/6NcrJ) mice. If there were significant, general *seb*-mediated regulatory effects on other proteins, one would expect differences in pathogenicity also in WT mice, because most of the affected *S. aureus* toxins are not human specific. We did not detect any impact on general protein expression (Figure 5A), nor any

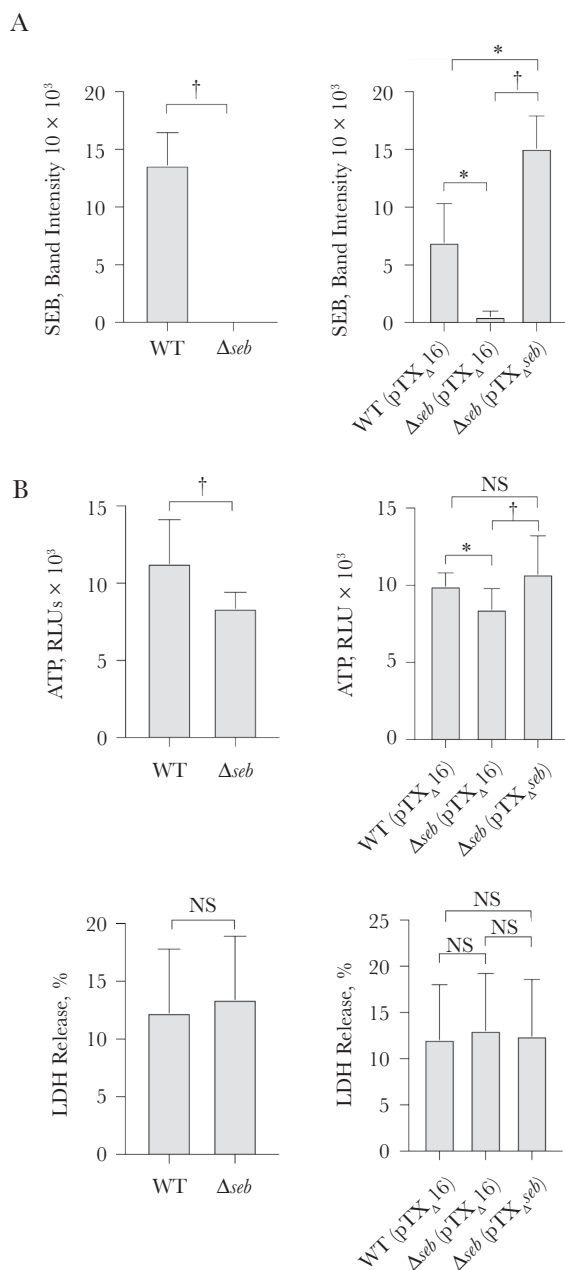


Figure 2. Staphylococcal enterotoxin B (SEB) production from sequence type 59 culture filtrates induces proliferation of human T cells in vitro. **A**, Culture filtrates were collected from 16-hour cultures grown in tryptic soy broth and probed with anti-SEB antibody. Signal intensities were determined by means of densitometry using ImageJ software. Error bars represent means with standard deviations. Statistical analysis was performed using unpaired Student *t* tests or 1-way analysis of variance (ANOVA). **P* < .05; †*P* < .01. **B**, Peripheral blood mononuclear cells from human whole-blood samples were plated at 5×10^4 cells per well and incubated with a 1:1000 dilution of sterile culture filtrates for approximately 48 hours. Adenosine triphosphate (ATP) levels were measured as a function of T-cell proliferation and release of lactate dehydrogenase (LDH) to verify that there were no cytolytic effects. Error bars represent means with standard deviations. Statistical analysis was performed using unpaired Student *t* tests or 1-way ANOVA, or the respective nonparametric tests (Mann-Whitney and Kruskal-Wallis) if data did not pass normality distribution tests. **P* < .05; †*P* < .01. Abbreviations: NS, not significant; RLUs, relative light units; WT, wild type.

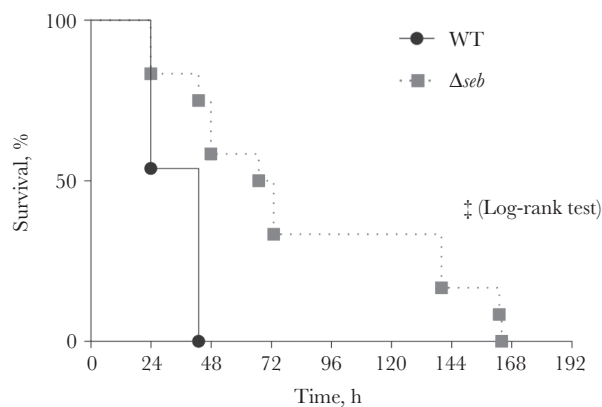


Figure 3. Staphylococcal enterotoxin B (SEB) contributes to virulence of sequence type (ST) 59 in a mouse systemic infection model. Survival of HLA-DR3 female mice was determined after intravenous challenge with 2×10^8 colony-forming units of *Staphylococcus aureus* ST59 wild type (WT) (*n* = 13) or its isogenic *seb* deletion strain (*n* = 12). Animals were monitored for up to 8 days (192 hours) after infection. Statistical analysis was performed using the log-rank (Mantel-Cox) test. Data are representative of 2 independent experiments. †*P* < .001.

significant *seb*-mediated effects on virulence in our systemic infection model using WT mice (Figure 5B). Furthermore, as expected from the absence of SEB superantigenicity-mediated virulence, the median survival time was considerably shorter in ST59-infected HLA-DR3 than in WT mice (43 vs 160 hours, respectively; compare Figures 3B and 5). These results support the notion that the phenotypes observed in the systemic infection model are due to the superantigenic activity of SEB rather than previously alleged regulatory effects.

Next, because cytokine storms have been associated with the administration of SEB and are usually regarded as the main hallmark of TSS, we determined the concentrations of proinflammatory and anti-inflammatory cytokines in organ homogenates and serum samples collected 12 hours after infection. Significant differences in cytokine production were barely evident in the livers or kidneys (Supplementary Figure 4). However, we observed a significant increase in the proinflammatory cytokines IFN- γ , IL-10, and TNF- α in spleens of mice infected with the WT compared with those infected with the Δ *seb* mutant strain (Figure 6A). The most striking difference was seen with IFN- γ ; mice infected with the WT strain produced 5-fold more of that cytokine in the spleen than mice infected with the Δ *seb* mutant strain (Figure 6A). Accordingly, in the serum, we also observed a very pronounced difference in IFN- γ (approximately 20-fold), while differences in IL-10 and TNF- α levels, similar to those observed in the spleen, did not reach statistical significance (Figure 6B).

DISCUSSION

The contribution of SEB to *S. aureus* pathogenesis has not been previously directly analyzed. We selected the ST59 lineage for

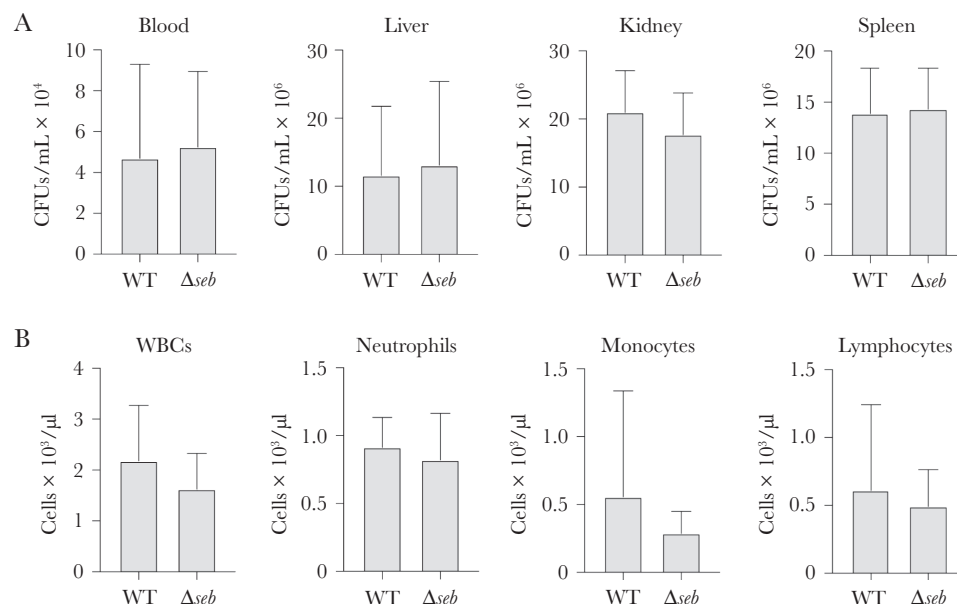


Figure 4. Decreased survival in mouse systemic infection is not due to differences in bacterial burden in organs or complete blood cell counts. *A*, Blood, kidneys, livers, and spleens were collected 12 hours after intravenous challenge with 2×10^6 colony-forming units (CFUs) of *Staphylococcus aureus* ($n = 10/\text{group}$). Blood and organ homogenates were assessed for CFUs. *B*, Complete blood cell counts of white blood cells (WBCs), neutrophils, monocytes, and lymphocytes. *A*, *B*, Error bars represent means with standard deviations. Statistical analysis was performed using unpaired Student *t* tests or Mann-Whitney tests if data did not pass normality distribution tests. No significant differences were observed. Abbreviation: WT, wild type.

that purpose because it is highly clinically important, representing the most widespread CA-MRSA lineage in East Asia [33]. Given that this region is populated by >1.4 billion people, ST59 is presumably the most frequent CA-MRSA lineage globally, despite research efforts having been focused almost entirely on the North American lineage USA300 [34]. Notably, the virulence of ST59 isolates is as high as that of USA300 isolates [18]. Furthermore, ST59 has previously been reported to frequently contain the *seb* gene [35, 36]. In the present study, we confirmed these reports and showed that this feature distinguishes

ST59 from the most frequent HA-MRSA isolates in China, which are of ST5 or ST239, suggesting a contribution to the hypervirulence that distinguishes CA-MRSA from HA-MRSA generally [24] and specifically ST59 from ST5/ST239 [18].

To test the hypothesis that the *seb* gene in ST59 contributes to pathogenesis, we created an *seb* mutant in the representative ST59 isolate RJ-2 and analyzed this mutant in comparison with the parental strain in animal infection models. Our results show that systemic disease caused by ST59 is dependent on the presence of the *seb* gene. Furthermore, SEB-dependent pathogenesis was correlated with high cytokine expression levels, particularly IFN- γ levels. SEB activity appeared to be most active in the spleen, where differences in cytokine levels between WT and *seb* mutant were most pronounced. This finding is in accordance with the close proximity of HLA class II-expressing and T-cell receptor-expressing cells in the spleen [37] and previous reports on splenic T cells being the major source of TNF- α and IFN- γ in the acute cytokine response to SEB [38, 39]. Moreover, we established that the SEB phenotype we describe is not due to a previously reported general effect of SEB on the expression of other *S. aureus* proteins [32]. Together, these observations underscore that the generally established mechanism of SEB-dependent systemic disease, particularly regarding strongly elevated cytokine concentrations during sepsis [40, 41], occurs in ST59 CA-MRSA infection, and they firmly establish a direct link between SEB expression and *S. aureus* systemic disease.

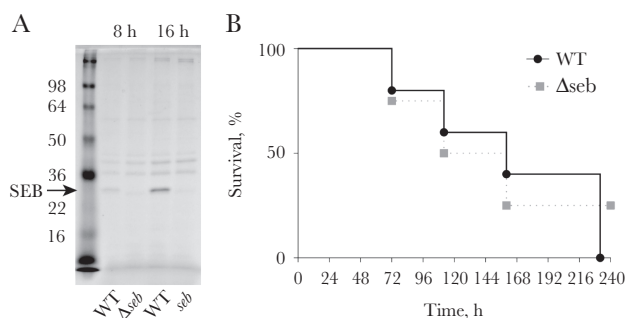


Figure 5. Contribution of staphylococcal enterotoxin B (SEB) to pathogenesis in mouse systemic infection is not due to a gene regulatory effect. *A*, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis with Coomassie stain of filtrates from ST59 wild-type (WT) or Δseb cultures grown for 8 or 16 hours. *B*, Systemic infection model in WT (C57BL/6NCrI) mice. The infection model was performed as described for Figure 3, except that mice were monitored longer owing to expected lower virulence in WT mice ($n = 5$ per group).

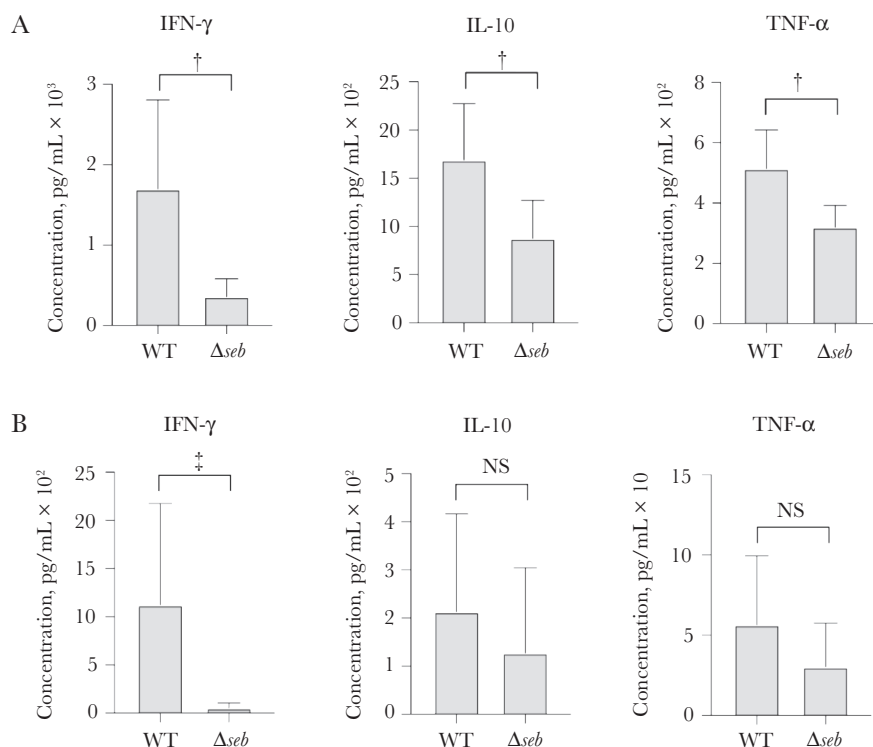


Figure 6. Decreased survival in mouse systemic infection is linked to a cytokine storm. Spleen homogenates (A) and serum samples (B) were assessed for concentrations of the cytokines interferon (IFN) γ , interleukin 10 (IL-10), and tumor necrosis factor (TNF) α , 12 hours after infection with 2×10^8 colony-forming units of *Staphylococcus aureus* wild-type (WT) or Δseb mutant ($n = 10$ per group). Error bars represent means with standard deviations. Statistical analysis was performed using unpaired Student t tests or Mann-Whitney tests if data did not pass normality distribution tests. $\dagger P < .01$; $\ddagger P < .001$. Abbreviation: NS, not significant.

Our findings are also of interest for the understanding of CA-MRSA virulence. While controversially debated since the initial finding that genes encoding Pantone-Valentine leukocidin are present in the first characterized CA-MRSA lineages, the current model of heightened virulence in CA-MRSA compared with HA-MRSA is multifactorial, with assumed contribution of leukocidins such as Pantone-Valentine leukocidin and other mobile genetic-element-encoded factors and increased expression of widely conserved toxins, such as α -toxin and phenol-soluble modulins [42].

Although our study showed a distinct contribution of *seb* to ST59 systemic infection, it did not reveal a role in ST59 lung or skin infection, the main disease types associated with CA-MRSA [34]. This may be due to the fact that there is high expression in ST59 of the above-mentioned toxins [18], overshadowing the contribution of SEB in these disease types. The lack of a significant contribution to lung or skin infection that we observed contradicts results from previous studies comparing nonisogenic *seb*⁺ and *seb*[−] *S. aureus* clinical isolates [8] and plasmid-driven overexpression of *seb* in a genetically altered *S. aureus* laboratory strain [9], emphasizing the need to establish a virulence factor's role in a given background by analysis of isogenic mutants. Importantly, our finding that *seb* contributes to systemic infection in the widespread CA-MRSA

lineage ST59 indicates that it has a significant role in the exacerbation of SEB⁺ CA-MRSA-associated infections and may be a cause of the increased fatalities seen with ST59 infections in China [22].

The direct role of SEB in CA-MRSA ST59 pathogenesis that we established herein further supports the development of therapeutics directed toward SEB. Indeed, there is ample evidence from animal studies that the prophylactic administration of anti-SEB antibodies can reduce the severity of disease in animals caused by SEB-expressing *S. aureus* strains [10, 43–46]. However, no passive therapy has entered clinical trials. The highly immunogenic nature of SEB toxoids has also prompted development of vaccine candidates, such as the recombinant Staphylococcal Enterotoxin B vaccine, STEBvax [47]. The monovalent nature of STEBvax limits widespread applicability as an *S. aureus* vaccine, given the limited presence of SEB among *S. aureus* clinical isolates and the increasing recognition that a multitargeted approach is necessary to neutralize *S. aureus* virulence [48]. Therefore, there has been a shift toward vaccines comprising multiple antigens [49]. The SEB toxoid is included in a multicomponent vaccine (Olymvax), which also contains α -toxin, and the 3 surface proteins staphylococcal protein A, iron surface determinant B N2 domain, and manganese transport protein C [50].

In conclusion, in the current study we present evidence that directly establishes a contribution of SEB expression to *S. aureus* pathogenesis. Our results indicate that SEB plays a role in the exceptional virulence of *seb*⁺ CA-MRSA that leads to exacerbation of systemic disease. Furthermore, our findings substantiate the value of SEB-targeting therapeutic strategies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank David Chella, PhD, Mayo Clinic, Rochester, Minnesota, for the gift of HLA-DR3 breeding pairs.

Author contributions. J. S. B., F. D., R. L., L. H., E. L. F., and G. Y. C. C. performed research. J. S. B., F. D., M. L., G. Y. C. C., and M. O. conceived and designed the experiments. J. S. B., L. H., G. Y. C. C., and M. O. analyzed the data. G. R. and M. L. contributed reagents/materials. J. S. B., G. Y. C. C., and M. O. prepared the figures. J. S. B., G. Y. C. C., and M. O. wrote the article.

Financial support. This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Intramural Research Program project ZIA AI000904 to M.O. and grant 5R21AI142243 to G. R.), and the National Natural Science Foundation of China (grants 81873957 to M.L. and 81974311 to L. H.).

Potential conflicts of interest. All authors: No potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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