Limitations of Murine Models for Assessment of Antibody-Mediated Therapies or Vaccine Candidates against *Staphylococcus epidermidis* Bloodstream Infection

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*Staphylococcus epidermidis* is normally a commensal colonizer of human skin and mucus membranes, but, due to its ability to form biofilms on indwelling medical devices, it has emerged as a leading cause of nosocomial infections. Bacteremia or bloodstream infection is a frequent and costly complication resulting from biofilm fouling of medical devices. Our goal was to develop a murine model of *S. epidermidis* infection to identify potential vaccine targets for the prevention of *S. epidermidis* bacteremia. However, assessing the contribution of adaptive immunity to protection against *S. epidermidis* challenge was complicated by a highly efficacious innate immune response in mice. Naive mice rapidly cleared *S. epidermidis* infections from blood and solid organs, even when the animals were immunocompromised. Cyclophosphamide-mediated leukopenia reduced the size of the bacterial challenge dose required to cause lethality but did not impair clearance after a nonlethal challenge. Non-specific innate immune stimulation, such as treatment with a Toll-like receptor 4 (TLR4) agonist, enhanced bacterial clearance. TLR2 signaling was confirmed to accelerate the clearance of *S. epidermidis* bacteremia, but TLR2−/− mice could still resolve a bloodstream infection. Furthermore, TLR2 signaling played no role in the clearance of bacteria from the spleen. In conclusion, these data suggest that *S. epidermidis* bloodstream infection is cleared in a highly efficient manner that is mediated by both TLR2-dependent and -independent innate immune mechanisms. The inability to establish a persistent infection in mice, even in immunocompromised animals, rendered these murine models unsuitable for meaningful assessment of antibody-mediated therapies or vaccine candidates.
of \textit{S. epidermidis} infections begs for additional solutions. To this end, development of an efficacious vaccine or antibody therapy to prevent or eliminate \textit{S. epidermidis} bloodstream infections would have a significant and beneficial impact on public health.

An important step in vaccine development is the establishment of relevant models to screen and prioritize candidate antigens. Our work focused on the development of a murine model of \textit{S. epidermidis} bloodstream infection with the ultimate goal of identifying candidate vaccine antigens capable of enhancing the clearance of bacteremia. These efforts revealed two important findings about the role of innate immunity in clearance of \textit{S. epidermidis} from the blood: (i) both TLR2-dependent and -independent pathways contribute to this process, and (ii) nonspecific innate immune stimulation alone is able to enhance bacterial clearance. Further, we demonstrate an important limitation of using mice to model \textit{S. epidermidis} bacteremia: challenge either leads to rapid clearance or overwhems the system and culminates in mortality within 24 h. There is no middle ground in which challenge leads to illness or a persistent bacterial burden. Cyclophosphamide-induced leukopenia reduces the size of the bacterial challenge required to cause lethality but does not impact clearance after a nonlethal challenge. Taken together, these data show that \textit{S. epidermidis} infection in the mouse is handled efficiently by innate immunity, which limits the utility of murine models for studies of vaccine or antibody efficacy against this opportunistic pathogen.

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains, media, and growth conditions.} \textit{S. epidermidis} strain RP62A was originally isolated from a patient with catheter sepsis, and we obtained it from the ATCC. Its complete genome sequence is available, and its capacity to grow as a biofilm is well documented. For our studies, biofilm-grown bacteria were produced as follows. Two days prior to challenge, 5 ml of tryptic soy broth (TSB) was inoculated with a single bacterial colony of RP62A from an overnight TSB agar plate and then incubated at 37°C with shaking at 250 rpm. This 5-h biofilm starter culture was transferred into 5 ml fresh TSB. This culture was grown at 37°C with shaking at 250 rpm for exactly 5 h. This 5-h biofilm starter culture was diluted to an optical density at 600 nm (OD600) of 2.0. Next, 37°C with shaking at 250 rpm for exactly 5 h. This 5-h biofilm starter culture was transferred into 5 ml fresh TSB. This culture was grown at 37°C with shaking at 250 rpm for exactly 5 h. This 5-h biofilm starter culture was diluted to an optical density at 600 nm (OD600) of 2.0. Next, 2.5 ml of this biofilm starter culture was combined with 22.5 ml of fresh TSB in an empty, sterile 10-cm-diameter petri dish and incubated overnight at 37°C without shaking. After exactly 20 h of growth, bacteria were harvested by gently removing TSB and then scraping the biofilm layer into phosphate-buffered saline (PBS). The challenge mixture was pipetted and sonicated to disperse the bacteria prior to storage on ice until use. The dispersal of biofilm clumps following sonication was confirmed by visual inspection using phase-contrast microscopy. The challenge material was serially diluted in PBS and enumerated on TSB agar to verify the exact challenge dose—this also confirmed that bacteria remained completely viable following sonication. Planktonic bacteria were produced as follows. At 1 day prior to challenge, 5 ml of TSB was inoculated with 8 to 10 individual colonies from a TSB agar overnight plate, and the culture was grown at 37°C with shaking at 250 rpm for exactly 18 h to allow the bacteria to enter the stationary phase. On the day of the challenge, the 18-h culture was diluted 1:100 into fresh TSB and then grown at 37°C with shaking at 250 rpm for 5 h. The volume of the mixture was then adjusted such that the OD600 was between 2 and 3 as determined using a standard spectrophotometer with a 1-cm-path-length cuvette. Cells were collected by centrifugation at 8,000 \times g for 2 min and resuspended in PBS. This planktonic bacterial suspension was maintained on ice prior to use. The bacterial suspension was serially diluted in PBS and grown on TSB agar to verify the exact challenge dose.

\subsection*{Animals.} Female specific-pathogen-free BALB/c mice (Charles River) as well as C.129(B6)-Tlr2tm1Kir/J (TLR2\(^{-/}\)) and C57BL/6J mice (The Jackson Laboratory) were used. All procedures that involved animals were conducted under protocols approved by the Institutional Animal Care and Use Committee and were performed at an AAALAC-accredited facility.

\subsection*{Cyclophosphamide induction of leukopenia and counting of white blood cells.} Cyclophosphamide monohydrate (Sigma-Aldrich) was dissolved in water to a concentration of 20 mg/ml. Three days prior to challenge, mice were given an intraperitoneal (i.p.) injection at a dosage of 200 mg/kg of body weight. To determine the number of white blood cells present before and after depletion, ACK (ammonium-chloride-potassium) buffer was used to lyse red blood cells in EDTA-treated whole-blood samples. White blood cell counts were performed manually with a hemocytometer.

\subsection*{Bacteremia challenge model.} Bacterial challenges were administered in a 0.2-ml volume by intravenous (i.v.; [tail vein]) or i.p. injection using a 1-ml syringe and 27-gauge 0.5-in. needle. Mice were euthanized at the indicated times, and then blood was collected via cardiac bleed and stored in BD Microtainer MAP Microtubes containing K\(_3\)EDTA to prevent coagulation. Blood was serially diluted in PBS and plated on TSB agar plates with 5 \mu g/ml kanamycin (TSA-Kan) (Teknova) prior to incubation (37°C, 24 h); \textit{S. epidermidis} colonies were counted manually, in a blinded fashion. Spleens were harvested at the indicated times and stored on ice in sterile 1.2-ml polypropylene tubes until ready for processing. All tubes were centrifuged for 10 s to pack spleens into the bottom of the tube. Next, approximately 0.3 g of autoclaved zirconium oxide beads (1.0-mm diameter) was loaded on top of the spleens followed by 300 \mu l sterile PBS (CellGro, Mediatech). Spleens were homogenized using a NextAdvance Bullet Blender Blue tissue homogenizer at speed setting 8 for 3 min. Tubes were visually inspected to ensure complete homogenization. Spleen homogenates were serially diluted and counted as described above. For experiments involving administration of TLR4 agonist, 3 days prior to bacterial challenge, mice were given subcutaneous (s.c.) injections of a 0.2-ml volume in the scapular region containing 2 \mu g of E6020 (Eisai, Tokyo, Japan) or an equivalent volume of PBS vehicle.

\subsection*{Statistical analysis.} All statistical analyses were performed using GraphPad Prism version 6.01.

\section*{RESULTS}

\subsection*{Immunocompetent mouse model of \textit{S. epidermidis} infection.} Numerous murine models of \textit{S. epidermidis} bacteremia have been described (32–37). Experimental variables tested in these models include animal age (pups or adults), route of bacterial challenge (i.v. or i.p.), and readouts (CFU in blood or solid organs). While different groups used different bacterial strains, we performed all studies with \textit{S. epidermidis} clinical isolate RP62A and chose the model described by Sellman and coworkers as our starting point since it was used to screen and identify potential vaccine candidates (34). In this model, female BALB/c mice were immunized with recombinant \textit{S. epidermidis} vaccine antigens and challenged via i.p. injection with planktonic \textit{S. epidermidis} 1 week after the final immunization. Vaccine efficacy was assessed in the spleen and bloodstream after 24 h, since the bacteria were naturally cleared by immunized mice at 48 h (34). In an effort to better model the scenario for bloodstream infections arising from biofilms on indwelling devices, we challenged animals with biofilm-grown bacteria. To assess the impact of the route of bacterial delivery, levels of splenic bacterial recovery were compared in naive mice after i.p. and i.v. delivery of biofilm-grown bacteria. While there was no statistically significant difference in the levels of recovery of bacteria from the spleen after 24 h (\(P = 0.7243\) [Mann-Whitney]), i.v. delivery led to less variability (Fig. 1) and was therefore selected for subsequent experiments. We performed a longitudinal study on the splenic bacterial burden from 1 to 24 h.
postchallenge to determine the best time to assess the contribution of antigen-specific antibody to bacterial clearance (Fig. 2). We hoped to identify a window of time during which the bacterial burden was constant but persistent infection was not observed at any time point. Of note, >90% of bacteria in the spleen were eliminated between hours 1 and 4 postchallenge, and this rose to 99.5% elimination by 24 h.

Since the innate immune response was very effective at mediating rapid clearance of *S. epidermidis*, we considered the possibility that any treatment that stimulated innate immunity would enhance bacterial clearance. To test this hypothesis, naive mice were injected 3 days prior to challenge with either PBS or a TLR4 agonist (E6020). A TLR4 agonist was selected because TLR4 signaling has been shown not to be involved in cytokine production induced by *S. epidermidis* (32); human embryonic kidney cells transfected with TLR2 (but not TLR4/MD-2) dramatically increase interleukin-8 (IL-8) production in response to *S. epidermidis*, and preincubation of whole human blood with neutralizing anti-TLR2 (but not anti-TLR4) antibodies inhibits *S. epidermidis*-induced IL-6 production (32). Despite TLR4 having no identified role in *S. epidermidis* signaling, injection of a TLR4 agonist led to statistically significant 5.8- and 4.9-fold reductions in the splenic bacterial burden at 18 and 24 h, respectively (Fig. 3).

To test whether innate immune stimulation such as might occur during a mouse vaccine study could enhance bacterial clearance, we “immunized” naive mice three times with 10 μg of *E. coli* lipopolysaccharide (LPS) on days 0, 28, and 35 and then challenged them with *S. epidermidis* 1 week after the final immunization. *E. coli* LPS was selected because recombinant protein antigens are often produced in *E. coli* and may contain residual LPS. This immunization with *E. coli* LPS alone was able to reduce the bacterial burden of *S. epidermidis* in the spleen after 24 h (data not shown).

Taken together, these data demonstrate that mice mount a highly effective innate immune response that naturally clears *S. epidermidis* challenge. These data also suggest that any innate immune stimulation, whether specific to *S. epidermidis* or not, can enhance *S. epidermidis* clearance. Therefore, to properly assess the contribution of antigen-specific adaptive immunity, we sought to develop a model in which innate immunity was impaired.

**Leukopenic model of *S. epidermidis* infection.** Cyclophosphamide-induced neutropenia impairs the ability of mice to control *S. epidermidis* infection, and challenge with 10^9 CFU can be lethal within 24 h (33). We hypothesized that challenging leukopenic mice with a lower dose would lead to sustained infection without progression to death, thereby creating an appropriate window of opportunity to evaluate vaccine efficacy. Treatment of mice with 200 mg/kg cyclophosphamide reduced the circulating white blood cell (WBC) counts by >90% on days 3 and 4 postinjection (data not shown). We therefore challenged mice 3 days after treatment so that the entire experiment would fall within the leukopenic window. Cyclophosphamide- or PBS-treated mice were challenged i.v. with 2 × 10^6 CFU of biofilm-grown bacteria, and the splenic bacterial burden was then assessed from 1 to 24 h postchallenge (Fig. 4). Both leukopenic and immunocompetent animals were able to clear the bacterial challenge, and there...
penic mice, were challenged i.v. with 2 × 10^8 CFU of biofilm-grown *S. epidermidis*. The bacterial burden was assessed in spleens at the indicated time points. Each data point represents mean splenic bacterial burden in the group at the indicated time point, and error bars display the standard deviations.

was no statistically significant impact of cyclophosphamide treatment on the CFU recovered from spleens (*P* = 0.2936 [two-way analysis of variance (ANOVA)]). As challenge of leukopenic mice with doses of biofilm-grown bacteria equal to or greater than 8 × 10^8 CFU per 0.2 ml could rapidly lead to a moribund state requiring immediate euthanasia (data not shown), all subsequent experiments were done with only planktonic bacteria.

**FIG 4** Cyclophosphamide-induced leukopenia does not impair clearance of a nonlethal *S. epidermidis* challenge. Groups of 5 mice, including either PBS-pretreated immunocompetent mice or cyclophosphamide-pretreated leukopenic mice, were challenged i.v. with 2 × 10^8 CFU of biofilm-grown *S. epidermidis*. The bacterial burden was assessed in spleens at the indicated time points. Each data point represents mean splenic bacterial burden in the group at the indicated time point, and error bars display the standard deviations.

**TABLE 1** Outcomes of challenge of groups of 5 or 6 immunocompetent or leukopenic mice with various doses of planktonic *S. epidermidis*

| Immune status     | Challenge dose (CFU) | Challenge outcome          |
|-------------------|----------------------|-----------------------------|
| Immunocompetent   | 2.9 × 10^9            | Clearance                   |
|                   | 6.6 × 10^8            | Clearance                   |
|                   | 1 × 10^8              | Clearance                   |
|                   | 2.3 × 10^9            | No clearance                |
| Leukopenic        | 1.8 × 10^8            | Clearance                   |
|                   | 3.5 × 10^8            | Clearance                   |
|                   | 6.6 × 10^8            | Clearance (3 animals) and no clearance (2 animals) |
|                   | 7 × 10^8              | No clearance                |
|                   | 1 × 10^9              | No clearance                |
|                   | 2.3 × 10^9            | No clearance                |

**FIG 5** Loss of TLR2 leads to delayed clearance of *S. epidermidis* from the bloodstream but has no impact on clearance of bacteria from spleen. Groups of 5 animals per time point of WT (black circles) and TLR2^−/−^ (gray squares) mice were challenged i.v. with 2.6 × 10^8 CFU of *S. epidermidis*. Bacterial burden was assessed in blood (A) and in spleens (B) at the indicated time points. Each data point represents a single animal. For both panel A and panel B, the solid bar represents the mean of the results from the WT group and the dashed bar represents the mean of the results from the TLR2^−/−^ group.
that while TLR2 may be involved in the clearance of *S. epidermidis* bacteremia, other innate immune pathways are also at play.

The transient increase that we observed in bloodstream bacterial burden at 24 h followed by a rapid decrease at 48 h led us to ask whether non-TLR2-dependent clearance processes were triggered only upon reaching a threshold concentration of bacteria. If this was the case, challenging animals with a lower dose of *S. epidermidis* might postpone clearance by delaying the onset of the non-TLR2-mediated clearance mechanisms. To test this hypothesis, we challenged TLR2−/− mice with 2.3 × 10^6, 2.3 × 10^7, or 2.3 × 10^8 CFU per mouse, and the bloodstream burden was assessed at 20, 48, and 72 h postchallenge (Fig. 6). Bacteria were cleared from the bloodstream at all doses tested, suggesting that any non-TLR2-mediated clearance mechanism that was present was still active at lower concentrations of bacteria.

**DISCUSSION**

*S. epidermidis* is a ubiquitous commensal that is part of a healthy skin flora, but if this bacterium breaches the epithelial barrier it can act as an opportunistic pathogen in preterm infants, immunocompromised individuals, and those with indwelling medical devices (reviewed in references 26 and 38). Our goal was to develop a murine model of *S. epidermidis* infection to test the efficacy of candidate vaccines against *S. epidermidis* bacteremia. Unfortunately, developing a model of persistent *S. epidermidis* bloodstream infection proved challenging. For both immunocompetent and leukopenic mice, *S. epidermidis* challenge with clinical isolate RP62A led to one of two disparate outcomes: either the infection was very rapidly cleared or there was uncontrolled bacterial growth that culminated in death within 24 h. This problem could not be overcome by optimizing the challenge dose. For immunocompetent mice, the tipping point was somewhere between 1.0 × 10^9 and 2.3 × 10^9 CFU for a challenge with planktonic bacteria. Cyclophosphamide-induced leukopenia reduced the overall number of bacteria required to overwhelm the immune system to approximately 6.6 × 10^8 CFU but still did not lead to persistent infection. The ability of cyclophosphamide-treated leukopenic animals to clear infection raises questions about the specific immune cells mediating bacterial clearance. Cyclophosphamide is a chemotherapeutic and immunosuppressant, alkylating, cytotoxic drug and is most toxic to rapidly proliferating cells and tissues. While some immune cells, such as neutrophils, are more susceptible than others, studies have demonstrated broad-based effects on multiple white blood cells in mice (33, 39). In the future, characterization of the immune cell population remaining after cyclophosphamide treatment, combined with bacterial challenge studies in mice depleted of specific immune cell populations, could provide additional insight into the mechanism of innate clearance of *S. epidermidis* infection.

The absence of a middle ground in which challenge leads to a persistent bacterial burden is problematic, since neither rapid clearance nor rapid death resembles the chronic human infection we are attempting to model. Using a nonlethal *S. epidermidis* challenge, we would be assessing the acceleration of an ongoing efficacious innate immune system-mediated process. Therefore, any attempt to differentiate vaccine candidates is limited by the noise imposed by innate clearance mechanisms. However, a lethal challenge model is also untenable because of the tipping point phenomenon described above. Schaeffer et al. (40) recently showed that cyclophosphamide immunosuppression alone was not sufficient to establish *S. epidermidis* bloodstream infection in rats. However, they were successful in establishing infection when the animals were further weakened by implantation of a foreign body (catheter). Unfortunately, their “two-hit” approach to rendering animals susceptible is highly challenging from a technical perspective, and it is unclear whether infection persists or is continually reseeded from a protected biofilm nidus on the implant.

TLR2 has previously been shown to mediate recognition of live *S. epidermidis* and clearance of bacteremia (32). We found that the loss of TLR2 had no impact on bacterial clearance from the spleen and led to only a transient delay in bloodstream clearance at 24 h postchallenge (Fig. 6). Unfortunately, such a small window of increased susceptibility and the noted variability at that time point rendered TLR2−/− mice an unsuitable model for assessing vaccine candidate efficacy. Our findings with respect to bloodstream clearance of *S. epidermidis* are in agreement with those of Bi and coworkers (36) but differ from those reported by Strunk et al. (32). All these data suggest that TLR2 accelerates the clearance of *S. epidermidis* are in agreement with those of Bi and coworkers (36) but differ from those reported by Strunk et al. (32). All these data suggest that TLR2 accelerates the clearance of bacteremia; however, our data and the work of Bi and coworkers (36) suggest that TLR2 is not necessary for the clearance of bloodstream bacteria. Therefore, while it is clear that TLR2 does play a role in the recognition and clearance of *S. epidermidis* from the blood, our data demonstrate that additional innate immune pathways are also involved. Indeed, the involvement of other innate signaling pathways in the recognition of *S. epidermidis* has been described. For example, the presence of *S. epidermidis* peptidoglycan has been shown to lead to activation of monocyte-like THP-1 cells by both TLR2 and NOD2 (41). TNF production by murine peritoneal macrophages in response to high concentrations of *S. epidermidis* has been shown to be primarily TLR2 independent (32), and production of IL-17A by live CD45+ skin cells has been shown to be independent of TLR2 (4). Therefore, multiple lines of
inquiry suggest that various innate immune signaling pathways function in the recognition and elimination of *S. epidermidis*.

As part of our model optimization efforts, we assessed the ability of potential vaccine antigens to reduce splenic bacterial burden at 24 h postchallenge. Immunization with either SERP0207 (SdrG) or SERP0237 (lipoteic protein ligase A family protein) led to a consistent statistically significant reduction in splenic bacterial burden at 24 h, but our initial preparations of these antigens also led to reactivity that manifested as injection site lumps and liver lesions. Further purification yielded protein preparations that were no longer reactive, but they also lost the efficacy observed with the earlier batches (unpublished data). Since the impact of TLR4 agonists on systemic clearance of *S. epidermidis* is likely due to TLR4 expression on splenocytes, this highlights the need for highly purified materials and suggests that routine testing for residual LPS would be prudent for such studies.

Relevant and translational infection models are a crucial step in the development of new vaccines. In conclusion, our work presents valuable information about the innate immune clearance mechanisms that control *S. epidermidis* bloodstream infections and demonstrates that these innate immunocompetent and immunocompromised mouse models appear to be unsuitable tools for the assessment of candidate vaccines against *S. epidermidis* bacteremia.

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