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Superantigens Subvert the Neutrophil Response To Promote Abscess Formation and Enhance *Staphylococcus aureus* Survival *In Vivo*

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Staphylococcus aureus **is a versatile bacterial pathogen that produces T cell-activating toxins known as superantigens (SAgs). Although excessive immune activation by SAgs can induce a dysregulated cytokine storm as a component of what is known as toxic shock syndrome (TSS), the contribution of SAgs to the staphylococcal infection process is not well defined. Here, we evaluated the role of the bacterial superantigen staphylococcal enterotoxin A (SEA) in a bacteremia model using humanized transgenic mice expressing SAg-responsive HLA-DR4 molecules. Infection with *S. aureus* Newman induced SEA-dependent Vβ skewing of T cells and enhanced bacterial survival in the liver compared with infection by *sea* knockout strain. SEA-induced gamma interferon, interleukin-12, and chemokine responses resulted in increased infiltration of $CD11b^+$ $Ly6G^+$ neutrophils into the liver, promoting the formation of abscesses that contained large numbers of viable staphylococci. Hepatic abscesses occurred significantly more frequently in *S. aureus* Newman-infected livers than in livers infected with the Newman *sea* knockout strain, promoting the survival of *S. aureus in vivo*. This represents a novel mechanism during infection whereby *S. aureus* utilizes SAgs to form a specialized niche and manipulate the immune system.**

Staphylococcus aureus is a common human commensal equipped with numerous virulence factors that allow this organism to successfully colonize and infect host tissues. Staphylococcal diseases most frequently manifest as skin and soft-tissue infections with a high propensity for abscess formation (1, 2); however, *S. aureus* is also readily capable of disseminating into deeper tissues to cause invasive and life-threatening infections, including endocarditis, osteomyelitis, and sepsis (3). Moreover, *S. aureus* also can induce toxin-driven diseases, such as food poisoning, staphylococcal scalded skin syndrome, and toxic shock syndrome (TSS) (4). The versatility of this bacterium as a successful commensal and pathogen, coupled with the development of resistance to a wide array of antibiotics, has led to the establishment of *S. aureus* as a leading cause of both hospital- and community-associated infections (5, 6).

Many of the specialized *S. aureus* virulence factors have evolved to target innate immune mechanisms, primarily neutrophils and macrophages, which are key cells involved in the clearance of *S. aureus* (7–9). In contrast, *S. aureus* also secretes superantigens (SAgs) that directly target and activate cells of the adaptive immune system (10, 11). The family of SAgs in *S. aureus* now includes over 20 genetically distinct SAg variants that comprise the staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like (SEls) toxins, and toxic shock syndrome toxin-1 (TSST-1) (12). These functionally unique exotoxins circumvent antigen presentation by engaging lateral surfaces of major histocompatibility complex class II (MHC-II) molecules (13–16) and complementarity determining region 2 (CDR2) of the T cell receptor (TCR) β-chain variable region (Vβ) (17–20). Thus, SAgs alter the conventional TCR-peptide-MHC-II activation complex to prevent antigen recognition by the CDR loops (21), leading to the activation and expansion of numerous T cells in a Vβ-restricted manner (22). In cases of severe SAg intoxication, excessive T cell activation can result in a cytokine storm that leads to the development of TSS (11, 23).

In vivo mouse experiments using the injection of purified SAgs have demonstrated many important features of SAg biology, yet these experiments cannot recapitulate the complex interactions between *S. aureus* and the host. Although *S. aureus* has been studied intensively using live *in vivo* infection models, relatively few reports have examined the role of SAgs using genetically controlled SAg knockout strains. Early work by Tarkowski and colleagues has demonstrated a pathogenic role of TSST-1 for the onset of dermatitis, arthritis, and septic mortality in mice (24, 25). In addition, vaccination with SAg toxoids or neutralization of SAgs with monoclonal antibodies has prevented or reduced mortality from experimental *S. aureus* sepsis (26–28). Rabbits are particularly sensitive to the effects of SAgs; using this animal species, deletion of the gene encoding SEl-X from *S. aureus* USA300 demonstrated reduced mortality from necrotizing pneumonia (29), and deletion of the gene encoding staphylococcal enterotoxin C (*sec*) from *S. aureus* MW2 prevented mortality in a rabbit model of sepsis/infective endocarditis (30). Furthermore, engineered high-affinity SAg inhibitors or vaccination with SAg toxoids can protect rabbits from *S. aureus* pneumonia, infective endocarditis, and sepsis (31–33). Collectively, these studies show unequivocally that SAgs enhance the severity and lethality of staphylococcal infection.

The majority of the human population has circulating anti-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. aureus Newman	Early methicillin-sensitive isolate from secondary infection in a patient with tubercular osteomyelitis	90
S. aureus RN4220	Restriction-deficient derivation of NCTC8325-4	91
S. aureus Newman Δ*sea*	*sea*-null *S. aureus* Newman	This study
S. aureus Newman Δ*sea*(pSEA)	*sea*-null *S. aureus* Newman complemented with wild-type *sea*	This study
E. coli DH5α	Cloning strain	Invitrogen
E. coli BL21(DE3)	Protein expression strain	New England Biolabs
Plasmids		
pET28	Protein expression vector	Novagen
pET28::*sea*	Recombinant SEA expression plasmid	This study
pDG1513	Source of *tetR* gene	41
pMAD	Integration plasmid	40
pALC2073	Complementation vector	42

bodies against SAgs that are protective against TSS, which rarely develops (34, 35), indicating that SAg exposure does not usually result in overt disease. Furthermore, at least 80% of clinical strains of *S. aureus* are genetically positive for at least one SAg gene (36), and the recently discovered *selx* has been found in ~95% of all *S. aureus* strains (29). Thus, the high prevalence and widespread distribution of SAgs in *S. aureus* suggests these toxins provide an evolutionary advantage to *S. aureus*. Although SAg-induced virulence has been attributed to the cytokine storm that results in immune cell infiltration, pyrexia, hypotension, endothelial damage (29, 30), and ultimately death, enhanced host mortality may not provide an evolutionarily prudent tactic for bacterial survival and propagation. We reasoned that there are other biologically relevant SAg functions that contribute to *S. aureus* fitness, and given that *S. aureus* is one of the most common sources of bacteremia (37), we set out to study the role of SAgs in this context. Using an isogenic *sea* knockout strain of *S. aureus*, we found that SEA manipulates the immune system and recruits neutrophils to promote formation of hepatic abscesses, forming a protective niche for staphylococcal survival *in vivo*.

MATERIALS AND METHODS

Mice. Six-to-twelve-week-old male and female HLA-DR4-IE (DRB1*0401) humanized transgenic mice lacking endogenous mouse MHC-II on a C57BL/6 (B6) background (here referred to as DR4-B6 mice) (38) were used for all *in vivo* infection experiments. B6 mice were purchased from Charles River. All animal experiments were in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, and the animal protocol was approved by the Animal Use Subcommittee at Western University.

Bacterial strains, media, and growth conditions. *S. aureus* strains listed in Table 1 were grown aerobically at 37°C in tryptic soy broth (TSB) (Difco) with shaking (250 rpm) or on tryptic soy agar (TSA) supplemented with the appropriate antibiotics (Sigma-Aldrich). *Escherichia coli* DH5α was used as a cloning host and was grown in Luria-Bertani (LB) broth (Difco) or LB agar supplemented with appropriate antibiotics at 37°C with shaking (250 rpm). Growth curves were done using a Bioscreen C MBR system (Thermo Labsystems).

Construction of recombinant *S. aureus* strains. Restriction enzymes were purchased from New England BioLabs, and primers were designed using Primer3 software (39) and supplied by Sigma-Aldrich. The gene encoding staphylococcal enterotoxin A (SEA) in *S. aureus* strain Newman was insertionally inactivated with a tetracycline-resistant cassette using the pMAD integration vector by following an established protocol (40). Wild-type *sea* along with its corresponding upstream (Up) and downstream (Down) fragments were PCR amplified from the genome of Newman using seaFP (5′-AACGGGATCCCATGTGCTTGAACTTAGAGAG GAA-3′) and seaRP (5′-TTCGGTCGACCCCAATAGCTTTTGCGATG T-3′) and directionally cloned into pMAD via BamHI and SalI sites. A 262-bp fragment was excised from the middle of *sea* using ClaI and EcoRI and replaced with a tetracycline resistance marker (*tetR*) excised from pDG1513 (41). This construct then was transformed into *S. aureus* Newman after undergoing methylation in *S. aureus* RN4220. Allelic replacement of the wild-type *sea* with *tetR* via homologous recombination was conducted as described previously (40). The *sea*-null Newman Δ*sea* strain was complemented by amplifying the native *sea* promoter and complete *sea* gene from Newman using the primers seaFP and seaRP and cloned into the BamHI and SalI sites of the plasmid pALC2073 (42). This construct (pALC2073::*sea*) was electroporated into the Newman Δ*sea* mutant, generating the *S. aureus* Newman Δ*sea*(pSEA) complementation strain.

Construction and purification of recombinant SEA. Wild-type *sea* lacking the signal peptide was PCR amplified from the genome of *S. aureus* Newman using the primers 5′-GGGCCATGGGCAGCCATCATCATCA TCATCACAGCAGCGGCGAAAACTTGTATTTCCAAAGCGAGAAAA GCGAAGAAAT-3′ and 5′-GGGGGATCCTTAACTTGTATATAAATAT ATATC-3′, introducing nucleotide sequences encoding a His_6 tag and a tobacco etch virus (TEV) protease cleavage site (ENLYFQ ↓ G) onto the N terminus of *sea*. This PCR product was inserted into pET28a (Novagen) via BamHI and NcoI sites to create pET28a::*sea* and transformed into *E. coli* BL21(DE3) for protein purification. Cells were induced with isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) to express His_6-tagged SEA and purified using nickel column chromatography as previously described (43). The His_6 tag was removed with TEV protease and dialyzed in HEPES or PBS before use.

Cellular proliferation and cytokine quantification. The ability of B6 and DR4-B6 mice to respond to SEA was assessed using the incorporation of [^{3}H]thymidine as described previously (44). Mouse spleens were collected and broken into a single-cell suspension, followed by erythrocyte lysis in ammonium-chloride-potassium (ACK) buffer. The remaining cells were suspended in RPMI (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco), 2 mM L-glutamine (Gibco), 1 mM MEM sodium pyruvate (Gibco), 100 μM nonessential amino acid (Gibco), and 25 mM HEPES (pH 7.2) (Gibco) and then seeded into 96-well plates at a density of 1.1×10^6 cells/ml. Various concentrations of recombinant SEA were added to cells and incubated for 72 h at 37°C. Cells then were pulsed with 1 μCi/well of [^{3}H]thymidine for an additional 18 h prior to harvesting on fiberglass filters. Counts were measured using a 1450 Microbeta liquid scintillation counter (Wallac).

Supernatants from the *S. aureus* strains were tested for SAg activity using DR4-B6 splenocytes seeded into 96-well plates as described above. Titrations of recombinant SEA and supernatants from overnight cultures of *S. aureus* Newman and the Newman Δ*sea* mutant diluted 1:10 were added to splenocytes for 18 h at 37°C, and supernatants were assayed for interleukin-2 (IL-2) by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience).

Staphylococcal bacteremia model. Single bacterial colonies were picked from a TSA plate and grown in 5 ml TSB overnight (16 to 18 h), and the optical density at 600 nm (OD_{600}) was adjusted to 1.0. Cells subsequently were subcultured into (2%) TSB and grown to exponential phase (OD_{600} of ~3.0 to 3.5). The bacterial pellet was washed 3× with Hanks balanced salt solution (HBSS) (HyClone) and resuspended in HBSS to an

OD_{600} of 0.15, corresponding to ~5×10^7 CFU/ml. Mice were injected via the tail vein with 5×10^6 CFU of *S. aureus* in a total volume of 100 μl. Mice were weighed and monitored daily. At 8 or 96 h postinfection, mice were sacrificed and the heart, lungs, kidneys, and liver were aseptically harvested. All organs were homogenized, plated on mannitol salt agar (Difco), and incubated at 37°C overnight. *S. aureus* colonies were enumerated the following day with a limit of detection determined to be 3 CFU per 10 μl.

Determination of Vβ populations targeted by SAgs using flow cytometry. Lymph nodes (cervical, axillary, brachial, inguinal, and popliteal) were isolated *in toto* from mice and pushed through a cell strainer to create a single-cell suspension. Cells were stained with allophycocyanin (APC)-conjugated anti-CD3 (clone 145-2C11) (eBioscience) and fluorescein isothiocyanate (FITC)-conjugated anti-Vβ3 (clone KJ25) (BD Pharmingen) or FITC-conjugated anti-Vβ8 (clone KJ16) (eBioscience). Events were acquired using a FACSCanto II (BD Biosciences), and data were analyzed using FlowJo v.8.7 (TreeStar).

Detection of cytokines and chemokines *in vivo*. Eight hours postinfection, serum supernatants and livers were collected. Supernatants were obtained from whole livers by homogenization in HBSS supplemented with the complete protease inhibitor cocktail (Roche). Samples were analyzed using a 32-multiplex array against mouse cytokines and chemokines (Eve Technologies).

Liver leukocyte isolation, staining, and cytofluorometric analysis. Livers were extracted from mice and pushed through a fine mesh. Leukocytes were isolated from livers as previously described using a 33.75% Percoll gradient (GE Healthcare) (45). Cells were stained with FITC-conjugated anti-F4/80 (clone BM8), FITC-conjugated anti-Ly6G (clone RB6-8C5), phycoerythrin (PE)-conjugated anti-CD11b (clone M1/70), or APC-conjugated anti-CD3 (eBioscience). Events were acquired and data analyzed as outlined above.

Histological analysis. Standard histology techniques were used. Briefly, tissues were fixed in 10% formalin, embedded in paraffin, and thin sectioned. Sections were stained with a combination hematoxylin and eosin (H&E)/Gram stain, and images were captured using a BX-61 upright microscope (Olympus).

Statistical analyses. Data were analyzed using unpaired Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest analysis. All statistical analyses were performed using Prism v5.0 (GraphPad), with $P < 0.05$ being considered significant.

RESULTS

Construction of isogenic *sea*-null *S. aureus* Newman mutant. A general feature of most bacterial SAgs is that these toxins do not efficiently bind mouse MHC-II molecules (46, 47). *S. aureus* Newman encodes the SEA SAg (48), so we first tested the ability of recombinant SEA protein to activate splenocytes isolated from both B6 and DR4-B6 transgenic mice. SEA resulted in a dose-dependent proliferative response as low as 1 pg for splenocytes from DR4-B6 mice, while proliferation of B6 splenocytes was not detected (Fig. 1A). Thus, the remaining experiments were conducted in DR4-B6 mice.

We next aimed to evaluate the role of SEA in a model of *S. aureus* bacteremia. To accomplish this, we generated an isogenic SEA-negative *S. aureus* strain. Using an allelic replacement strategy, we integrated a tetracycline resistance marker into a deleted portion of the *sea* gene in *S. aureus* Newman (Fig. 1B). The resulting *sea*-null *S. aureus* strain was confirmed to be tetracycline resistant and erythromycin sensitive, with the *tetR* insertion verified by PCR and DNA sequencing. Growth curve analysis demonstrated no apparent defect for the Newman Δ*sea* mutant compared to wild-type Newman (Fig. 1C). We next tested the supernatants from both *S. aureus* Newman and Newman Δ*sea* strains for SAg activity on DR4-B6 splenocytes using IL-2 production as a measure of T cell activation. Ten-fold-diluted supernatants from wild-type *S. aureus* Newman induced ~50 pg/ml IL-2 from DR4-B6 splenocytes, which extrapolates to secreted SEA concentrations of ~100 ng/ml. In contrast, we did not detect any IL-2 production from Newman Δ*sea* mutant supernatants, confirming both the genetic deletion and that other functional DR4-B6-reactive SAgs, such as the genome-encoded SEl-X, which is the only other known SAg encoded by Newman (29), do not display superantigenic activity under these growth conditions (Fig. 1D).

SEA is produced *in vivo* during staphylococcal bacteremia. *S. aureus* Newman is known to produce SEA during the exponential phase of growth *in vitro* (49); however, the exact environmental triggers *in vivo* are not well defined. We aimed to determine if SEA was produced during *S. aureus* Newman infection by examining the Vβ profiles of infected mice, as SEA is known to target $V\beta3^+$ T cells but not $V\beta8^+$ T cells (50, 51). Vβ-specific T cell subpopulations from lymph nodes were measured using flow cytometry from mice inoculated with *S. aureus* strain Newman, the Newman Δ*sea* mutant, or the Newman Δ*sea*(pSEA) mutant or from vehicle-treated mice. Ninety-six hours postinoculation, mice infected with the *S. aureus* Newman Δ*sea* mutant did not show a difference in $V\beta3^+$ $CD3^+$ lymphocyte populations compared to vehicle-treated mice. Conversely, wild-type *S. aureus* Newman and Newman Δ*sea*(pSEA) mutant infection demonstrated a significant decrease in $V\beta3^+$ $CD3^+$ cells compared to levels for vehicle-treated mice, indicating Vβ-specific targeting by SEA (Fig. 2A). Concurrent analysis of $V\beta8^+$ $CD3^+$ cells was used as an internal control, since it is an irrelevant T cell subpopulation that is not targeted by SEA. Thus, the significant decrease in the ratio of $V\beta3^+$ $CD3^+$ to $V\beta8^+$ $CD3^+$ cells from 0.33 (vehicle) to 0.16 (Newman) during infection with *S. aureus* Newman showed that SEA was specifically targeting the $V\beta3^+$ $CD3^+$ population (Fig. 2B), thereby confirming the production of SEA *in vivo* during infection in our model.

Bacterial survival is enhanced in the livers of mice infected with SEA-producing *S. aureus*. To evaluate a role for SEA in *S. aureus* bacteremia, we injected 5×10^6 CFU of *S. aureus* Newman or the *S. aureus* Newman Δ*sea* mutant into the tail vein of DR4-B6 mice and assessed the bacterial burden in multiple organs at 96 h postinfection. Bacterial loads were highest in the kidneys and livers but also were found in the heart and lungs (Fig. 3A to D). Although the bacterial load was not statistically different in the kidneys or lungs, we observed an ~100-fold decrease in bacterial burden in the livers of mice infected with the *S. aureus* Newman Δ*sea* mutant compared with wild-type *S. aureus* Newman-infected mice. There was also a significant difference between the bacterial loads in the heart (Fig. 3D) between *S. aureus* Newman- and Newman Δ*sea* mutant-infected mice. In order to confirm this pronounced phenotype was SEA dependent on and not due to an inadvertent secondary site mutation in the *S. aureus* Newman Δ*sea* mutant, we restored SEA expression in *trans* using the pSEA plasmid. The Newman Δ*sea*(pSEA) complemented strain restored the virulence phenotype in both the liver and heart, as seen with wild-type *S. aureus* Newman (Fig. 3). These data indicate that expression of SEA by *S. aureus* Newman promotes infection within the liver and heart but apparently does not alter bacterial burden in other organs tested.

SEA induces production of IFN-γ and other inflammatory cytokines and chemokines both locally and systemically during *S. aureus* infection. Since it is well known that SAgs function to

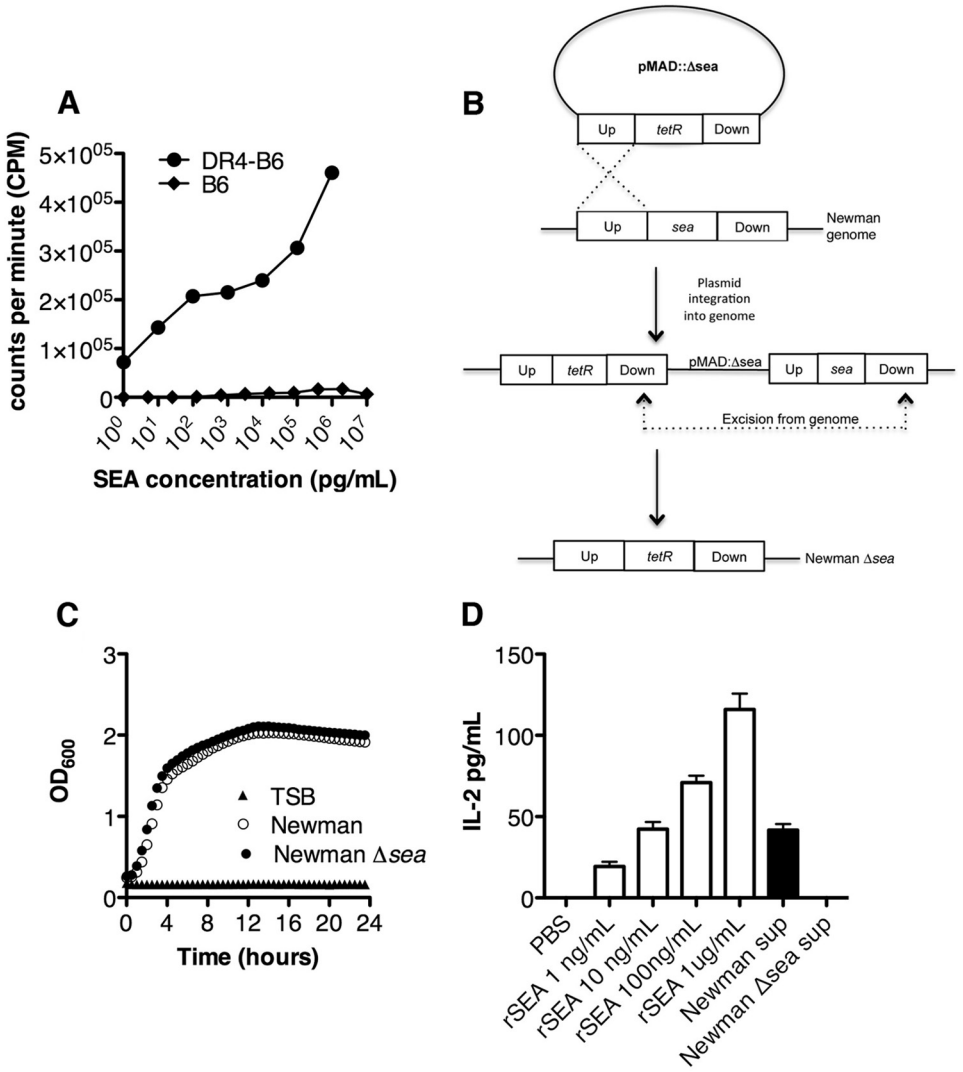

FIG 1 Construction of *S. aureus* Newman Δ*sea* mutant strain that does not display superantigenic activity in SAg-sensitive DR4-B6 mice. (A) Splenocytes isolated from DR4-B6 (black circles) and B6 (black squares) mice were treated with various concentrations of recombinant SEA for 72 h, followed by the addition of [^{3}H]thymidine. Proliferation was recorded as radioactive counts per minute. (B) Schematic diagram of the double homologous recombination method used to generate the *S. aureus* Newman Δ*sea* strain from wild-type *S. aureus* Newman. Up and Down designate the region upstream and downstream, respectively, of *sea* from the genome of *S. aureus* Newman. (C) Growth curve analysis of *S. aureus* Newman (open circles) and the Newman Δ*sea* mutant (black circle) in TSB and TSB only (black triangle). (D) IL-2 production from DR4-B6 splenocytes activated with various concentrations of recombinant SEA (white bars) and bacterial supernatants diluted 1:10 from *S. aureus* Newman and the Newman Δ*sea* mutant (black bars).

induce cytokine production, we reasoned that the survival advantage seen during infection with *S. aureus* Newman was a downstream result of SAg-mediated immune activation. We investigated early cytokine production to assess both local and systemic inflammation of infected mice 8 h postinfection. Liver homogenate supernatants and sera from Newman- and Newman Δ*sea* mutant-infected mice were analyzed for 32 cytokines and chemokines (see Table S1 in the supplemental material). Systemically, gamma interferon (IFN-γ) and IL-12p70 were upregulated in wild-type-infected mice sera compared to Newman Δ*sea* mutant infection, as was the chemokine interferon-induced protein 10 (IP-10) (Fig. 4A). Elevated levels of IFN-γ, tumor necrosis factor alpha (TNF-α), IL-6, and IL-12p40 were detected from mouse livers infected with *S. aureus* Newman compared to Newman Δ*sea* mutant-infected mice (Fig. 4B), which are known to be induced by SAgs (23, 52). Additionally, the chemokines MIP-2 and MCP-1 were upregulated in Newman-infected livers (Fig. 4B). Bacterial burdens in the liver at 8 h postinfection were not significantly different between wild-type and Newman Δ*sea* mutant-infected mice (Fig. 4C), and no bacteria were detected in blood from any mice (data not shown), indicating that the differences in chemokine and cytokine production likely are not due to differences in bacterial load. Overall, these data demonstrate that SEA is an important driver of SAg-induced inflammation during our model of *S. aureus* bacteremia in DR4-B6 mice.

CD11b$^+$ Ly6G$^+$ neutrophils are recruited to the liver during *S. aureus* infection in an SEA-dependent manner. Given the production of the MIP-2 and MCP-1 chemokines in the liver induced by SEA from *S. aureus* Newman-infected mice, we predicted that there also would be a difference in the number of immune cells

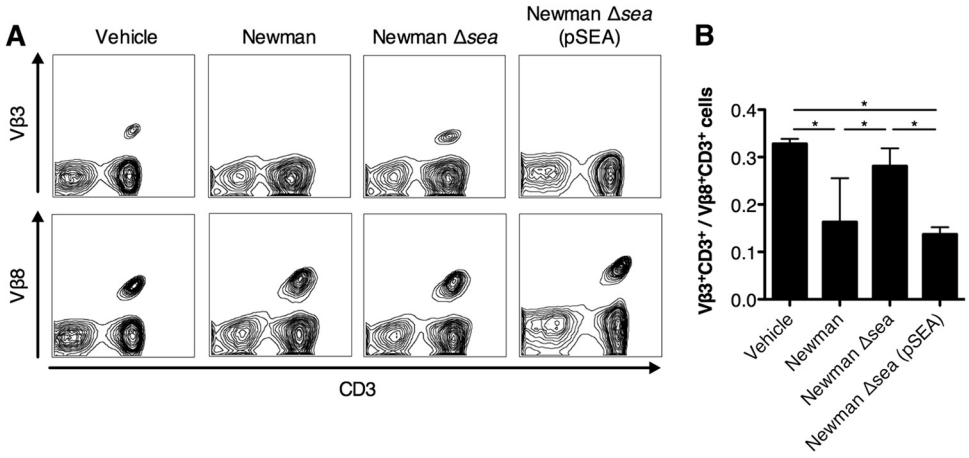

FIG 2 SEA is produced during *S. aureus* bacteremia and interacts specifically with the V$\beta3^+$ subset of T cells. Flow cytometry analysis of lymph node populations 96 h postinfection [vehicle, $n = 3$; Newman strain, $n = 4$; Newman Δ*sea* mutant, $n = 4$; and Newman Δ*sea*(pSEA) mutant, $n = 5$]. (A) Representative fluorescence-activated cell sorter plots from each infection group stained with antibodies against either CD3 and Vβ3 or CD3 and Vβ8. Vβ3 and Vβ8 staining were from the same mouse, with Vβ8 acting as the internal control for each mouse. Each sample was gated for the $CD3^+$ $V\beta^+$ population. (B) Ratio of $CD3^+$ $V\beta3^+$ to $CD3^+$ $V\beta8^+$ cells per mouse for each infection group. Data are shown as means ± standard errors of the means (SEM). Significant differences ($P < 0.05$) as determined by one-way ANOVA with Tukey's posttest are denoted with an asterisk.

trafficking to the liver. Since macrophages and neutrophils are the primary cells responsible for the clearance of *S. aureus*, we examined these populations to evaluate if there was a defect in phagocyte recruitment during staphylococcal infection with SAgs. Additionally, the liver is known to contain large numbers of resident macrophages (Kupffer cells), so we hypothesized that SEA would have an effect on the macrophage population. Leukocytes were isolated from mouse livers 96 h postinfection and stained for various surface markers. Analysis of $F4/80^+$ macrophages showed no significant difference between mice infected with *S. aureus* Newman and the Newman Δ*sea* mutant (Fig. 5A). Similarly, levels of $CD3^+$ T cells were not significantly different (Fig. 5B) despite the decreased number of $V\beta3^+$ T cells detected in lymph nodes (Fig. 3). However, mice infected with *S. aureus* Newman showed an increased frequency of $CD11b^+$ $Ly6G^+$ neutrophils (Fig. 5C), suggesting that SEA-induced chemokines (Fig. 4) resulted in the recruitment of neutrophils to the liver.

SEA promotes the formation of hepatic abscesses that contain viable bacteria at high densities. During organ retrieval following the bacteremia model, we observed numerous white hepatic lesions that commonly formed on the surface of livers of *S. aureus* Newman-infected mice (Fig. 6A). An abscess score was established whereby livers were examined on a lobe-by-lobe basis for visible surface lesions and enumerated. We observed a significant increase in the number of abscesses formed in the livers of *S. aureus* Newman-infected mice compared with mice infected with the *S. aureus* Newman Δ*sea* mutant. The number of abscesses from the *S. aureus* Newman Δ*sea* strain complemented with pSEA was similar to that of wild-type *S. aureus* Newman-infected mice, demonstrating that this phenotype was SEA dependent (Fig. 6B). H&E/Gram staining of thin sections from both groups showed high neutrophilic infiltration into the abscess with polymorphonuclear cells and associated tissue damage. Abscesses contained large numbers of Gram-positive cocci in the center (Fig. 6C, insets i and ii). However, the overall abscess structure appeared to be very similar between wild-type *S. aureus* Newman and Newman Δ*sea* mutant infections (Fig. 6C). Abscesses also were excised from the liver, homogenized, and compared to hepatic immune cells isolated from the surrounding liver tissue. Compared to nonabscessed liver tissue, abscesses contained few live host cells as assessed by trypan blue staining and had lost forward and side scatter when analyzed with flow cytometry (data not shown). Additionally, individually excised abscesses yielded high counts of viable *S. aureus* (10^6 to 10^7 CFU/abscess) (data not shown). We did not detect staphylococci distant from the abscesses within the surrounding liver parenchyma or in sham-infected mice (Fig. 6D). These data indicated that the enhanced fitness phenotype of *S. aureus* Newman we observed (Fig. 3A) is attributed to an increase in abscess formation that confers greater bacterial survival and growth in the liver.

DISCUSSION

In this work, we combined SAg-sensitive humanized transgenic DR4-B6 mice with an isogenic *sea* knockout strain of *S. aureus* to study the role of SAgs during staphylococcal bacteremia. By using a live infection model, we were able to study not only the detrimental effects of SAg intoxication on the host but also the advantageous effects of SAg expression for *S. aureus*. We demonstrated an SEA-specific downstream effect that enhanced the number of abscesses formed in the liver, although individual abscesses appeared similar in both morphology and bacterial counts from both strains. This in turn increased bacterial persistence in the liver overall, since staphylococcal abscess communities are sustained within a fibrin pseudocapsule that is protective against immune cells and permits bacterial survival *in vivo* (53, 54). Abscess formation is an important host immune response during infection for limiting the spread of infection to other tissues. Host immunity against *S. aureus* infection is dependent on abscess formation by neutrophils (55), and suppurative abscesses have long been recognized as a hallmark of *S. aureus* infection (54, 56). However, successful eradication of *S. aureus* by neutrophils exists in a balance, with staphylococci actively subverting neutrophil responses in order to persist *in vivo* (9, 53). The presence of abscesses

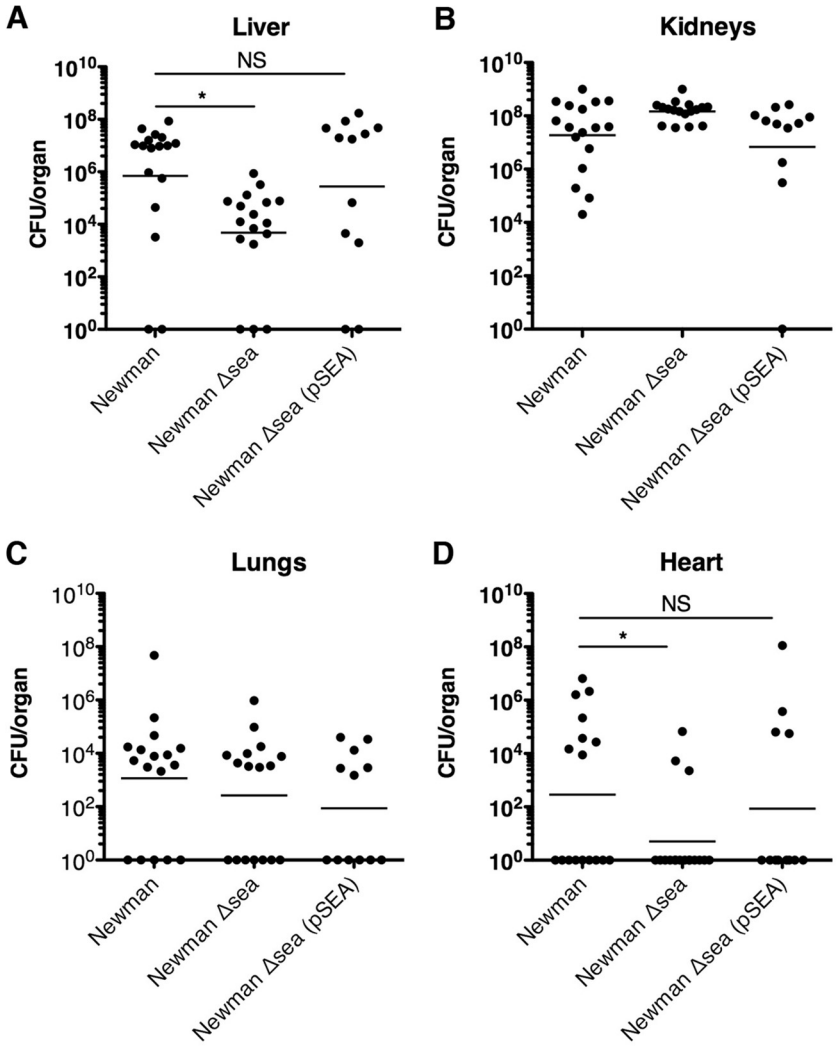

FIG 3 Septic infection with SEA-producing *S. aureus* results in higher bacterial loads in the liver than SEA-deficient *S. aureus*. Bacterial counts of mice infected with *S. aureus* Newman ($n = 17$), the Newman Δ*sea* mutant ($n = 17$), or the Newman Δ*sea*(pSEA) mutant ($n = 12$) from liver (A), kidneys (B), lungs (C), and heart (D) 96 h postinfection. Each point represents data from one mouse. Results reflect 3 independent experiments. The line in each treatment group represents the mean, and counts below the limit of detection are interpreted as being negligible. Significant differences ($P < 0.05$) as determined by unpaired Student's *t* test are denoted with an asterisk. NS, no significance.

during staphylococcal bacteremia is clinically significant, since hematogenous spread from the abscess is well documented (54, 57, 58).

A basal level of abscess formation still could be observed during *S. aureus* Newman Δ*sea* mutant infection, albeit at a lower frequency than that of wild-type infection, since the former still retains essential cell surface proteins required for abscess formation (53). The lower bacterial counts seen in the Newman Δ*sea* mutant likely are not due to an inherent growth defect (Fig. 1C) or an inability to survive within neutrophils, since viable bacteria were observed within both Newman and Newman Δ*sea* mutant abscesses. To our knowledge, this model is the first to describe a liver tropism for *S. aureus* related to SAg expression. Although we also observed renal abscesses in the infected mice, no differences were detected in bacterial counts between SEA-expressing and *sea*-null mutant infections. We speculate that given the paucity of resident T cells in the kidney (59), the initial infection within the kidney remained independent of SEA function. Additionally, high densities of staphylococcal cell walls (such as the loads observed in the kidneys) have been shown to downregulate SAg-mediated T cell activation (60), which may nullify SAg activity locally.

Consistent with our findings, SEC has been shown to increase renal damage during experimental infective endocarditis/sepsis in rabbits, including the formation of kidney abscesses, although this was attributed to the embolization of valve vegetations (30). Similarly, blocking SEC function using a high-affinity SEC binding inhibitor resulted in a drastic reduction of vegetation size (32). Although the Newman Δ*sea* mutant demonstrated decreased counts within the hearts, we did not observe any obvious aortic valve vegetations from wild-type Newman, although our protocol is not an endocarditis model as valve damage is not actively induced. Neutralization of SEB also decreased abscess size using a murine thigh infection model (28). Although it is difficult to aggregate these collective findings, an overall picture is now emerging that SAg-induced inflammation contributes to the formation

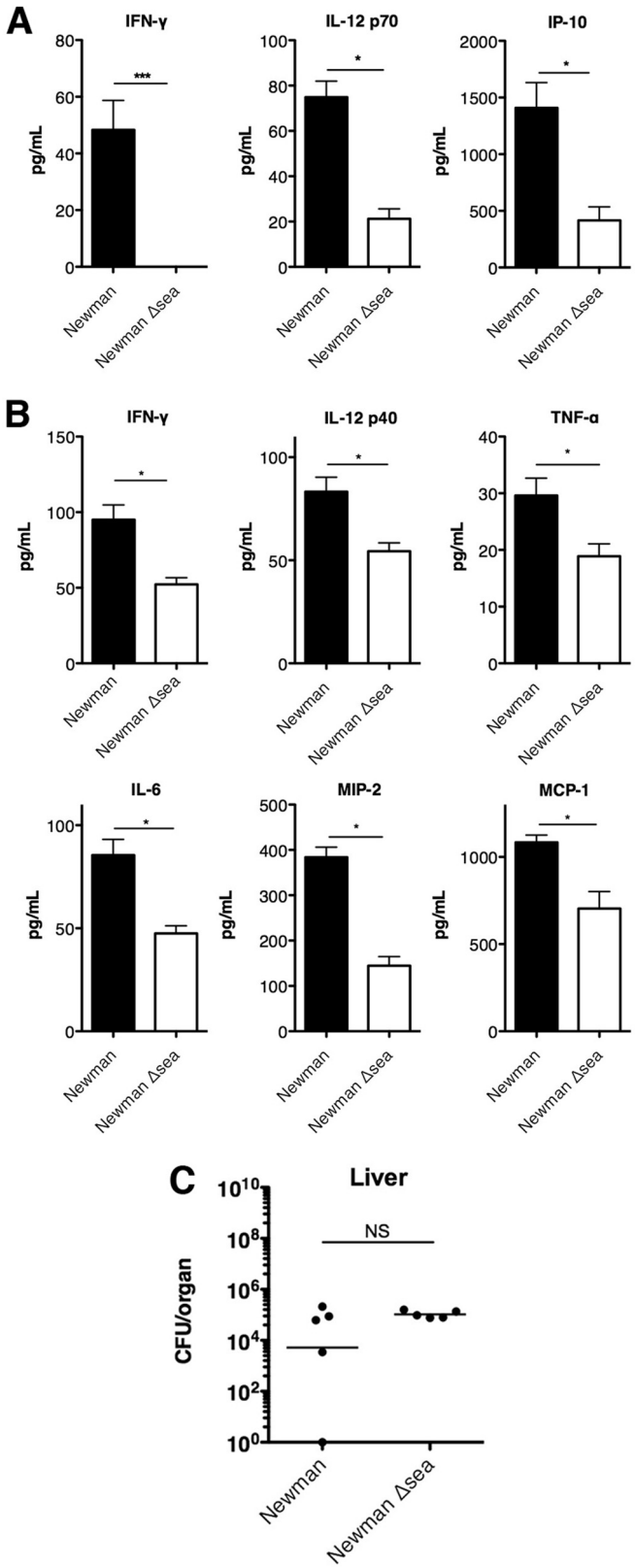

FIG 4 Cytokines and chemokines induced by *S. aureus* Newman and Newman Δ*sea* mutant infection. Serum and liver supernatants were collected from mice 8 h postinfection from *S. aureus* Newman- and Newman Δ*sea* mutant-infected mice. Blinded samples were sent for multiplex cytokine array analysis ($n = 3$ to 4 per experimental group). (A) Serum levels of cytokines and chemokines were significantly different in Newman and Newman Δ*sea* mutant infection. (B) Local production of liver chemokines and cytokines were significantly different during infection with Newman compared to that during infection with the Newman Δ*sea* mutant. (C) Bacterial burdens in the liver at 8 h postinfection ($n = 5$ per group). Data are shown as means ± SEM. Significant differences ($P < 0.05$) as determined by unpaired Student's *t* test are denoted with an asterisk. ***, $P < 0.001$.

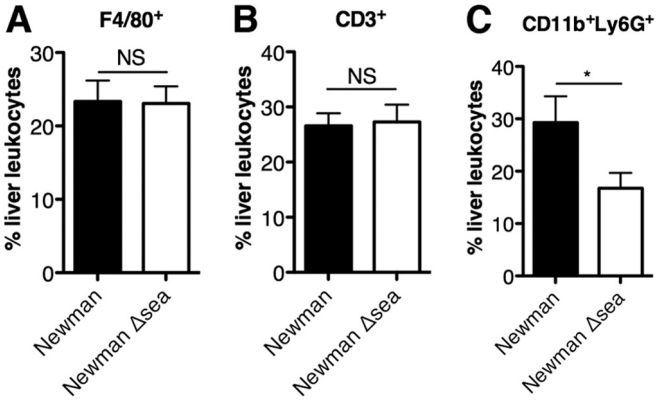

FIG 5 Livers of mice infected with *S. aureus* Newman show an increase in $CD11b^+$ $Ly6G^+$ neutrophils but not $F4/80^+$ macrophages or $CD3^+$ T cells. Livers of infected mice 96 h postinfection were broken into single-cell suspensions, and leukocytes were isolated by Percoll gradient. Samples were stained with antibodies against F4/80, CD3, or CD11b and Ly6G and analyzed by flow cytometry. Samples underwent doublet discrimination with debris gated out. Subsequently, cells were gated on $F4/80^+$ macrophages (A), $CD3^+$ T cells (B), or $CD11b^+$ $Ly6G^+$ neutrophils (C). Data are shown as the means ± SEM from three independent experiments, with $n = 12$ for each group. Significant differences ($P < 0.05$) as determined by unpaired Student's *t* test are denoted with an asterisk. NS, no significance.

and severity of *S. aureus* abscesses in multiple experimental settings.

Compared to Newman Δ*sea* mutant infection, wild-type Newman infection produced significantly higher quantities of cytokines and chemokines that correspond to those induced by SAgs reported in the literature (23, 52, 61, 62). Although IL-2 is a cytokine typically used to measure T cell-dependent superantigenic activity *in vitro*, we did not detect differences in IL-2 production from the *in vivo* liver samples. This finding may be explained by IL-2 levels peaking at 2 to 4 h *in vivo* in response to SAg (63) and by the very short half-life of IL-2 *in vivo* (64, 65). The SEA-driven inflammatory milieu likely mediates the promotion of abscesses and seems to be driven by the early production of both IL-12 and IFN-γ, which are detected in both serum and liver supernatants 8 h postinfection. IL-12 enhances production of IFN-γ after SAg challenge (63), productively boosting the cytokine and chemokine response. McLoughlin et al. have shown that IFN-γ is a master regulator during *S. aureus* infections, mediating chemokine responses that allow for neutrophil recruitment and trafficking (66). This is consistent with our observations in Newman-infected mice where we observed an increase in IFN-γ and chemokines that are chemotactic for neutrophils. Purified SAgs have been shown to recruit neutrophils (but not T cells) mediated by TNF-α and chemokines (67). Notably, our study is the first report showing SEA increases trafficking of $CD11b^+$ $Ly6G^+$ neutrophils during a live infection. Given that abscess formation is largely driven by

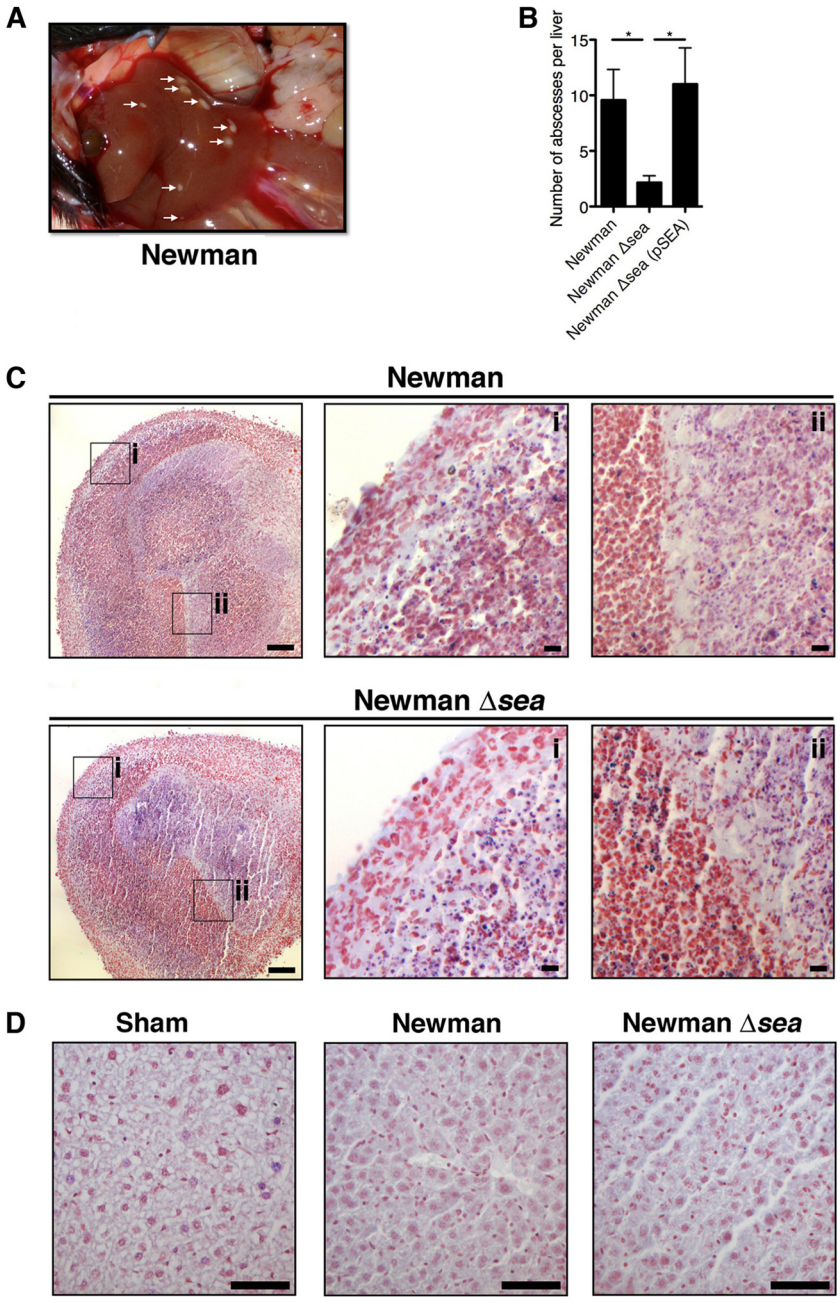

FIG 6 Infection with *S. aureus* Newman results in greater abscess formation than infection with the *S. aureus* Newman Δ*sea* mutant. (A) Visible white lesions (abscesses) on a representative liver of a Newman-infected mouse, indicated by white arrows. (B) Liver abscess score from mice infected with Newman ($n = 14$), the Newman Δ*sea* mutant ($n = 13$), and the Newman Δ*sea*(pSEA) mutant ($n = 5$). Data are shown as means ± SEM from at least three independent experiments. Significant differences ($P < 0.05$) as determined by unpaired Student's *t* test are denoted with an asterisk. (C) Representative H&E/Gram-stained histological sections of abscesses from Newman- and Newman Δ*sea* mutant-infected mice. The black bar indicates 100 μm and 10 μm on insets i and ii, respectively. (D) Representative sections of Gram-stained liver parenchyma surrounding abscesses from sham-, Newman-, and Newman Δ*sea* mutant-infected mice. The black bar indicates 50 μm.

neutrophils, the infiltration into the liver correlates well with the increased incidence of hepatic abscesses.

It appears paradoxical that an increased influx of $CD11b^+$ $Ly6G^+$ neutrophils had an inverse correlation to bacterial survival, considering the important role of neutrophils in staphylococcal clearance. As a successful human pathogen, *S. aureus* has evolved many mechanisms to counteract neutrophils (9). While neutrophils are absolutely necessary for the eradication of staphylococcal infections (68, 69), their presence during infection has also been described as pathogenic (66, 70). IP-10, which we showed to be upregulated systemically by SEA, can promote phagocytosis (71); however, MIP-2, which is also upregulated, is capable of enhancing intracellular bacterial survival within neutrophils (66, 70). The avoidance of neutrophil bactericidal activity

likely contributed to *S. aureus* survival during early abscess formation and, subsequently, the staphylococcal community in the mature abscess. This supports the paradigm that neutrophils can be pathogenic during systemic infection due to SAgs usurping the immune system to form abscesses, thereby conferring staphylococcal fitness and survival *in vivo*. The *sea* gene is located on the same immune evasion cluster (IEC) of β-hemolysin converting phage, which includes staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein of *S. aureus* (CHIPS), and staphylokinase (SAK) (72, 73). It has been proposed that CHIPS and SCIN stall early neutrophil recruitment to successfully establish an infection. This is due to their inhibitory action against complement proteins, an early innate response (73, 74). While this seems counterintuitive to the neutrophil recruiting activities of SEA, it also has been proposed that this early blockade of neutrophils allows later modulation of the immune system by SEA and SAK. This theory fits with our model where we see an accumulation of neutrophils later on at 96 h in response to SEA. SCIN may work in tandem with SEA by inhibiting phagocytosis and bactericidal activity of recruited neutrophils (75), which may help form neutrophilic abscesses that *S. aureus* can survive in. SAK may be involved in dissemination from abscesses due to its ability to cleave fibrin (76), which is characteristic of abscesses. However, it should be noted that the IEC factors, SEA included, are highly human specific (72); thus, they may not be active in our murine model, which is sensitized to SEA only.

SAg function typically has been attributed to crippling the adaptive arm of the immune system by inducing T cell anergy and deletion of T cell-dependent B cell responses (77, 78). Indeed, an inability to form neutralizing antibodies has been linked to many cases of TSS (11, 79); however, SAgs also are highly immunogenic, and the majority of the population is able to form both anti-SAg and anti-staphylococcal antibodies (80). Although purified SAgs have long been shown to induce T cell anergy (81–84), to our knowledge, the role of SAg-mediated T cell anergy has not been demonstrated during a live infection. In our model, SEA-expressing *S. aureus* caused a decrease in the detectable $V\beta3^+$ $CD3^+$ cells, although SAg-activated T cells usually undergo early expansion (85, 86). This decrease may be a result of Vβ-specific TCR internalization (87, 88), T cell deletion (85), or a combination thereof. Injection of mice with purified SEA similarly resulted in Vβ3-specific $CD4^+$ T cell suppression mediated by IFN-γ and myeloid-derived suppressor cells (89), and this may represent an additional role for SAgs to subvert the immune response. The effect of T cell anergy during staphylococcal disease may inhibit numerous T cells in the context of chronic infection; however, SAgs do not target T cells in an antigen-specific manner, so it is unclear how Vβ-specific anergy would contribute to staphylococcal infections. Thus, it will be important to dissect the role of SAg-mediated T cell suppression during live infections in future studies. Given that SAgs have an inherent ability to impact numerous immune cells, it is highly likely that these toxins are multifunctional virulence factors and are able to influence both the adaptive and the innate immune systems. Overall, this work shows that SAgs are used by *S. aureus* during infection to target not only T cells directly but also neutrophils as a result of the SAg-elicited cytokines. While the recruitment of neutrophils appears to be counterintuitive to survival, our work demonstrates that SAg expression by *S. aureus* enables a sophisticated method of *in vivo* survival by subverting the neutrophil response into a protective niche, demonstrating a biologically relevant and highly novel role for SAgs during infection.

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