Superantigens promote *Staphylococcus aureus* bloodstream infection by eliciting pathogenic interferon-gamma production

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**Staphylococcus aureus** is a foremost bacterial pathogen responsible for a vast array of human diseases. Staphylococcal superantigens (SAgs) constitute a family of exotoxins from *S. aureus* that bind directly to major histocompatibility complex (MHC) class II and T cell receptors to drive extensive T cell activation and cytokine release. Although these toxins have been implicated in serious disease, including toxic shock syndrome, the specific pathological mechanisms remain unclear. Herein, we aimed to elucidate how SAgs contribute to pathogenesis during bloodstream infections and utilized transgenic mice encoding human MHC class II to render mice susceptible to SAg activity. We demonstrate that SAgs contribute to *S. aureus* bacteremia by massively increasing bacterial burden in the liver, and this was mediated by CD4+ T cells that produced interferon gamma (IFN-γ) to high levels in a SAg-dependent manner. Bacterial burdens were reduced by blocking IFN-γ, pheno-copying SAg-deletion mutant strains, and inhibiting a proinflammatory response. Infection kinetics and flow cytometry analyses suggested that this was a macrophage-driven mechanism, which was confirmed through macrophage-depletion experiments. Experiments in human cells demonstrated that excessive IFN-γ allowed *S. aureus* to replicate efficiently within macrophages. This indicates that SAgs promote bacterial survival by manipulating the immune response to inhibit effective clearing of *S. aureus*. Altogether, this work implicates SAg toxins as critical therapeutic targets for preventing persistent or severe *S. aureus* disease.

**Significance**

Since their discovery over 30 y ago, it has become clear that the superantigens (SAgs) are important virulence factors produced during severe *Staphylococcus aureus*-mediated disease including bacteremia. However, until the current study, it was unclear how these toxins manipulated the immune system to promote infection. Here, we have demonstrated that the SAgs can target a critical immune signaling molecule (interferon gamma), inducing overproduction that promotes bacterial survival by subverting the ability of macrophages to be able to kill the pathogen. This highlights SAg activity as a critical target for antistaphylococcal therapy to mitigate the impact of severe *S. aureus* disease.

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The authors declare no competing interest.

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In the current study, we deployed targeted antibody depletion protocols that demonstrated, during bloodstream infection, that SAgs target CD4+ T cells to produce pathogenic levels of the key cytokine interferon gamma (IFN-γ). IFN-γ promoted enhanced disease severity and bacterial burden in the liver, and excess IFN-γ levels during infection appeared to perturb liver macrophage activity to promote the survival of S. aureus.

Results

Transgenic HLA-DR4 C57BL/6 Mice Are Sensitive to SAgs Staphylococcal Enterotoxin B and Staphylococcal Enterotoxin C Activity and Can Be Used to Model S. aureus Bacteremia. Previously, we demonstrated that S. aureus burden is promoted during murine bloodstream infections by the SAgs staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin-like W (SElW); however, the mechanism remained uncharacterized (15, 17). In the current study, we first utilized strain COL, a well-studied MRSA isolate from clonal complex (CC) 8 that produces the SAg staphylococcal enterotoxin B (SEB) (18). Splenocyte analysis from C57BL/6 (B6) or transgenic HLA-DR4 C57BL/6 (DR4-B6) animals identified that T cell activation (measured by the production of IL-2) to titrating doses of SEB was orders of magnitude more active on spleen cells from the DR4-B6 animals compared to conventional B6 mice (Fig. 1A). In addition, analysis of stimulated splenocytes using flow cytometry analysis demonstrated a massive expansion of Vβ8+ T cells, the major T cell targets of SEB in mice (19) (Fig. 1B). These cells represent ~20% of the T cell repertoire in the DR4-B6 animals, and the majority of these were activated by SEB as measured by the up-regulation of CD25 (Fig. 1C). In addition, depletion of CD4+ T cells resulted in a markedly reduced bacterial burden, which phenocopied the deletion of S. aureus COL during bloodstream infection.

To determine if SEB contributed to pathogenicity in murine bacteremia, we infected B6 and DR4-B6 animals with S. aureus COL via tail-vein injection. We found that wild-type S. aureus COL was significantly more virulent in DR4-B6 mice with a much higher bacterial burden found in the liver and kidneys when compared to the B6 background (Fig. 1C). This was due to SEB activity, as the bacterial burden of the SEB-null mutant (COL Δseb) essentially phenocopied the data obtained from the B6 animals. Importantly, this phenotype could be complemented with COL Δseb containing pCM29::seb (Fig. 1 C and D). These data clearly demonstrate that SEB contributes to the pathogenicity of S. aureus COL during bloodstream infection.

To determine if additional SAgs other than SEB could also contribute to bacteremia, we expanded our analysis to include S. aureus MW2, a CC1 MRSA isolate that produces staphylococcal enterotoxin C (SEC) (20, 21). SEC is phylogenetically different from SEC from MW2 and was able to complement the gene in trans (SI Appendix, Fig. S1). Like S. aureus COL, we found a significant increase in bacterial burden in the liver and kidneys in DR4-B6 animals compared to the B6 mice when infected with MW2 (Fig. 1E). Furthermore, deletion of sec from MW2 resulted in a significant reduction in bacterial burden and liver pathology (Fig. 1 E and F). These data indicate that both SEB and SEC produced from different S. aureus backgrounds can contribute to the pathogenesis of experimental bloodstream infection and that SAg-sensitive DR4-B6 mice are suitable for modeling SAg activity in the context of live S. aureus infection.

Depletion of CD4+ T Cells Results in Reduced Bacterial Burden in the Liver of S. aureus-Infected DR4-B6 Mice. It has been well established that SAgs can target and activate different T cell subsets that express the appropriate TCR Vβ (10). For efficient nasopharyngeal infection, Streptococcus pyogenes required the expression of the SAg streptococcal pyrogenic exotoxin A (SpeA) (22), and depletion of T cells resulted in a markedly reduced bacterial burden, which phenocopied the deletion of

Fig. 1. SAgs SEB and SEC promote pathogenesis of S. aureus bacteremia in transgenic HLA-DR4 B6 animals. (A) IL-2 production of isolated splenocytes from conventional B6 (open dots) and transgenic DR4-B6 (solid dots) mice following stimulation with a titration of SEB protein. Data shown are from a representative experiment. (B) Activation of Vβ8+ T cells in DR4-B6 mice stimulated by SEB compared to an untreated control as determined by CD25 expression (quartile values represent total cell population). (C–F) B6 and DR4-B6 animals were inoculated i.v. with 5 × 10^8 CFUs and then killed at 96 hpi for S. aureus COL (C and D) and 72 hpi for S. aureus MW2 (E and F). Liver and kidney bacterial burden (C and E) was assessed in conventional B6 mice (open dots) or in transgenic DR4-B6 mice (solid dots). Each dot represents an individual mouse, and the bar indicates the geometric mean. Significant differences were determined using the Kruskal–Wallis test with the uncorrected Dunn’s test for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Representative livers from the infected mice from S. aureus COL and mutants (D) and S. aureus MW2 and mutants (F) with white arrows indicate the presence of liver lesions.
We found that blocking IFN-γ activity is required for pathology to become evident (Fig. 4C). This demonstrates that the stimulation of pathogenic IFN-γ activity is a key function of SEB during bloodstream infection and, taken together with the previous data, suggests that a functional SAg must be present to elicit pathogenic production of IFN-γ.

Blocking of IFN-γ Activity during Systemic S. aureus Infection Results in Reduced Disease Severity and Bacterial Burden. Previous analyses have demonstrated that CD4+ T cells can be targeted by staphylococcal SAgS to result in the release of numerous cytokines including the key cytokines IFN-γ (also known as type II interferon), interleukin-10 (IL-10), and interleukin-17A (IL-17A) (10). These cytokines have antagonistic activity toward each other (25, 26) and, in the case of IL-17A and IFN-γ, have been shown to contribute to SEB-mediated morbidity during toxemia models in HLA-transgenic mice (27, 28).

Together, this suggests that these key cytokines may contribute to SAg-mediated pathogenesis. To test this hypothesis, we used antibody depletion to block cytokine activity during bloodstream infection by both S. aureus COL and MW2 (Fig. 3A). We found that blocking IFN-γ resulted in a significant reduction in bacterial liver burden and liver pathology that pheno-ocopied the deletion of seb or sec in S. aureus COL and MW2, respectively (Fig. 3 B, D, E, and G). However, depletion of either IL-10 or IL-17A had limited impact on the liver burden, suggesting that these cytokines do not promote bacterial burden in this model. Depletion of IFN-γ also resulted in lower bacterial burden in the kidneys, suggesting that the blocking of this cytokine reduced the overall severity of the infection (Fig. 3 C and F). It was also noted that bacterial burden in the kidneys increased once IL-17A was depleted but only for S. aureus COL. This suggests that IL-17A contributes to protection against kidney damage during bloodstream infection by S. aureus in the HLA-transgenic mouse model. Overall, these data indicate that, of the three cytokines tested, only IFN-γ promoted S. aureus burden during a bloodstream infection.

SAGs Drive Pathogenic Production of IFN-γ during S. aureus Bloodstream Infection. Several approaches were taken to determine if the promotion of bacterial burden by IFN-γ was mediated by the SAg toxins. First, we performed cytokine analysis on liver homogenates and serum from animals infected with the wild type or the SAg-deletion mutants at 24 h postinfection (hpi). Compared to wild-type S. aureus COL-infected mice, IFN-γ levels were ~10-fold lower in livers from animals infected with S. aureus COL Δseb (Fig. 4A). There was also a clear trend for more IFN-γ in the serum for animals that were infected with the wild type, although this did not reach statistical significance (Fig. 4D).

Following from the cytokine analysis, we modified our infection model to characterize IFN-γ depletion under circumstances in which the SEB SAg from S. aureus COL was either absent or unable to function. In the SAg-insensitive B6 background, bacte-rial recovery from infected mice was comparatively low regardless of IFN-γ-depletion treatment, suggesting that this phenotype could only be observed in a SAg-sensitive environment (Fig. 4B). Indeed, when we repeated the IFN-γ depletion in the DR4-B6 background and included the COL Δseb construct, bacteria recovered from organs of the isotype or IFN-γ-depleted groups were similarly low, whereas wild-type infections treated with the isotype control antibody produced visible liver lesions and higher bacterial counts in both the liver and kidneys (Fig. 4B). These data demonstrate that a functioning seb gene is required to promote pathogenic IFN-γ activity.

Finally, to establish the link between SEB and IFN-γ, we aimed to determine if the addition of exogenous IFN-γ could functionally complement the deletion of seb in S. aureus COL. In this experiment, animals were intravenously administered, therefore, of recombinant IFN-γ 2 h prior to infection and 1 h after. Treat-ment with exogenous IFN-γ resulted in a ~2-log increase in bac-te-rial burden in the liver when compared to the vehicle control (Fig. 4C). Curiously, very few lesions formed on the surface of the liver with this approach, suggesting that sustained SAg/IFN-γ activity is required for pathology to become evident (Fig. 4C). This demonstrates that the stimulation of pathogenic IFN-γ activity is a key function of SEB during bloodstream infection and, taken together with the previous data, suggests that a functional SAg must be present to elicit pathogenic production of IFN-γ.

IFN-γ Promotes Early Bacterial Survival during S. aureus Infection. With it established that SAgS could drive the production of patho-logic levels of IFN-γ, we next wanted to determine when IFN-γ had the most impact during the disease course. Therefore, we determined bacterial burden at shorter time points (i.e., 2 to 36 hpi) in animals treated with αIFN-γ antibodies (monoclonal antibodies specific for IFN-γ) or an isotype control antibody (Fig. 5A). From these experiments, we determined that much of the infectious dose became trapped within the liver following tail-vein injection with ~2 × 10⁸ colony-forming units (CFU) at 2 hpi (~40% of the dose), and this was followed by rapid clearance between 2 and 8 hpi in both groups. At 12 hpi, the rate of bacte-rial clearance was reduced in the αIFN-γ-treated animals but continued steadily with almost complete clearance of the bacteria by 96 hpi. Conversely, after 24 hpi in the livers of isotype
antibody–treated mice, bacterial burdens rapidly expanded, reaching a level ∼3-logs higher by 96 hpi (Fig. 5A). We performed a repeat of this analysis with daily time points and were able to confirm the trajectories that were observed in the shorter time course (SI Appendix, Fig. S3). Together, these data indicate that IFN-γ produced during infection by wild-type *S. aureus* is contributing to the ability of the bacteria to avoid clearance by the immune system in the liver during the early stages of bloodstream infection.

**Proinflammatory Signaling Is Delayed and Less Intense When IFN-γ Is Blocked during Sepsis.** IFN-γ is a pleiotropic cytokine of the immune system, and its depletion during infection could impact numerous downstream signaling pathways during the response to *S. aureus* bloodstream infection. In isotype antibody–treated mice, the highest level of IFN-γ was observed in the liver between 12 and 24 hpi, in excess of 500 pg/mL (Fig. 5B). Additionally, we confirmed that αIFN-γ antibodies were able to reduce IFN-γ concentration during infection up to 36 hpi (Fig. 5B). We also analyzed serum samples for aspartate aminotransferase (AST) levels as a proxy for liver damage. These data indicate that there was limited change in liver damage irrespective of IFN-γ levels; however, there did appear to be a faster drop in AST levels in the IFN-γ–depleted groups in the later time points (Fig. 5C), congruent with the reduced bacterial burden (Fig. 5A).

To gain a broader understanding of the cytokine and chemokine dynamics, liver homogenates were analyzed by multiplex
cytokine array over the course of the experiment. This demonstrated that in earlier time points (2 to 12 hpi), many signaling molecules associated with inflammation were up-regulated during infections in which IFN-γ was produced at high levels (Fig. 5D). Strikingly, between 24 and 36 hpi, many cytokines and chemokines became reduced relative to the group treated with αIFN-γ antibodies, which directly correlated with the expansion of S. aureus (Fig. 5A) and was subsequently reversed again by 96 hpi (Fig. 5D). This suggests that an inflammatory environment favorable to bacterial proliferation is sustained for a longer period during infections in which IFN-γ production is relatively high. Altogether, we infer that pathogenic production of IFN-γ results in the rapid production of a proinflammatory environment in the liver that contributes to S. aureus survival during bloodstream infection.

**Macrophage Activity in the Liver Is Subverted by SAg-Elicited IFN-γ Production.** Immune cells, such as macrophages and neutrophils, are critical for clearance of S. aureus during infection (29, 30). The cytokine and chemokine analysis indicated that wild-type S. aureus infection in HLA-DR4 mice drives an IFN-γ–dependent proinflammatory signaling cascade in the livers of animals (Fig. 5D). To determine if this response had any impact on the phagocytic cell populations in the liver, we first phenotyped immune cells isolated from this organ using flow cytometry at 24 and 96 hpi from infection with S. aureus COL Δseb. In both experiments (B and C), in vivo bacterial burden was assessed after 96 h in liver and kidneys and an assessment of gross pathological liver lesions was also performed. Each dot represents an individual mouse, and the bar indicates the geometric mean of CFUs/organ and the median for lesions/organ. Significant differences were determined using the Mann–Whitney U test or Kruskal–Wallis test with the uncorrected Dunn’s test for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

phagocytes, most likely inflammatory macrophages. To determine if these cells are the target of pathogenic IFN-γ production, we depleted macrophages in mice using clodronate containing liposomes (Clodrosome) and then performed S. aureus COL bloodstream infections with or without IFN-γ–blocking antibodies (Fig. 6B). Macrophage depletion had a negative impact on animal welfare, so end points were brought forward from 96 hpi to 72 hpi and bacterial burden was determined in both the kidneys and the liver. In the liver, we again observed, at 24 hpi in animals treated with the control liposomes, that IFN-γ depletion resulted in a higher bacterial burden (Fig. 6C). However, when we compared macrophage-depleted animals, the phenotype observed between the IFN-γ depletion and isotype antibody groups was abolished, indicating that macrophages are likely required for the IFN-γ–dependent phenotype. We also observed an increase in bacterial burden when macrophages were depleted, compared to animals treated with control liposomes and isotype antibody (Fig. 6C), indicating that these cells were important to restrict bacterial growth in the liver at this time point. In the kidneys, there was evidence that the depletion of macrophages likely resulted in greater bacterial “seeding” to this organ, although there was no difference due to IFN-γ depletion (Fig. 6C). The data from the 72-hpi time point confirmed the observation that macrophages were necessary for the IFN-γ phenotype, as again, we were able to observe a clear IFN-γ phenotype in both the kidneys and liver, yet this phenotype was mitigated by macrophage depletion (Fig. 6C). Together, these data demonstrate that the promotion of S. aureus burden by IFN-γ during bloodstream infection is mediated by macrophages.

**High Levels of IFN-γ Allow for Increased Intracellular Replication of S. aureus inside Human Macrophages.** To determine if pathogenic IFN-γ had any impact in the human system, white blood cells from healthy human donors were analyzed for their responses to SAgs and IFN-γ. First, we wanted to confirm that SAgs can elicit IFN-γ from T cells through the engagement of the TCR. To do this, we compared IFN-γ production elicited by recombinant SEB protein compared to the site-directed mutant SEB-N23A. This mutant features a mutation within the TCR binding pocket resulting in a much lower ability to engage the TCR of...
its target cells (31). As expected, SEB-N23A elicited significantly lower IFN-γ from human peripheral blood mononuclear cells (PBMCs) compared with wild-type SEB (Fig. 7A). This confirmed that to elicit IFN-γ from human PBMCs, SEB must engage and activate the T cell through binding the TCR.

Next, we wanted to establish that our experimental strains (i.e., COL and MW2) could elicit IFN-γ production from human PBMCs. We stimulated human PBMCs with a titration of wild-type bacterial supernatants grown for 8 h in brain heart infusion (BHI) broth and included supernatants from the respective SAg-deletion and complemented strains. The data clearly indicated that both SEB and SEC, produced from COL and MW2, respectively, could drive IFN-γ production in human PBMCs (Fig. 7 B and C). The deletion of seb in COL eliminated the production of IFN-γ, while there was a significant decline in the potency of MW2 Δsec. The remaining IFN-γ production was still easily detectable at lower MW2 Δsec supernatant dilutions, suggesting that other SAgs encoded by MW2 (i.e., sea, selh, selk, sell, selq, selw, and setx) are also able to elicit the production of this cytokine.

As the murine model indicated that macrophages are likely the major target of SAg-induced IFN-γ, we infected both murine and human macrophages with S. aureus and dosed these cells with varying concentrations of recombinant IFN-γ (Fig. 7 D and G). With murine macrophages, we initially tested bone marrow–derived macrophages (BMDMs) from DR4-B6 animals but observed limited S. aureus replication in these cells (SI Appendix, Fig. S5). Therefore, we moved this analysis into RAW 264.7 cells (a murine macrophage cell line), which have been shown to be permissive to S. aureus replication (32). For both S. aureus COL and MW2, we saw an overall increase in intracellular bacterial replication when murine macrophages were dosed with high levels of IFN-γ, although this was most evident with S. aureus COL, which exhibited a ∼2-log increase as IFN-γ levels increased (Fig. 7 E and F). However, in human monocyte-derived macrophages, the IFN-γ phenotype was most evident with MW2, as this strain seemed to have an improved ability at replicating inside human macrophages and also exhibited a ∼2-log increase at the highest IFN-γ concentrations (Fig. 7 H and I). Notably, the high concentrations of IFN-γ did not impact macrophage viability, and the bacteria were not simply overgrowing dead macrophages (SI Appendix, Fig. S6). Together, these data indicate that SAg-induced, IFN-γ–mediated subversion of macrophages can occur in both the murine and human system and that this mechanism appears to impair the ability of macrophages to kill intracellular S. aureus.

Discussion

Bloodstream infections caused by S. aureus represent a significant challenge in the clinic, and SAgs have been demonstrated to be important in this disease (reviewed in refs. 2 and 11). However, it has remained unclear how SAgs promote S. aureus persistence during infection and, specifically, how these toxins manipulate the immune response (10). The weak activity of SAgs in murine models has been a serious challenge for our understanding as to how these toxins promote bacterial burden during infection. While rabbit strains are more sensitive to SAg activation, they are not simply overgrowing dead macrophages (SI Appendix, Fig. S6).

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**Fig. 5.** SAg-induced IFN-γ promotes a proinflammatory environment that allows S. aureus to impede clearance during bloodstream infection. DR4-B6 mice were treated with isotype antibody or IFN-γ and mean IFN-γ ± SEM. (A) Liver burden plotted for each time point. Data shown are mean AST (pg/mL) ± SEM at each time point were determined for isotype antibody-treated, uninfected animal killed at the same time point as their respective SAg-deletion and complemented strains. The data clearly indicated that both SEB and SEC, produced from COL and MW2, respectively, could drive IFN-γ production in human PBMCs (Fig. 7 B and C). The deletion of seb in COL eliminated the production of IFN-γ, while there was a significant decline in the potency of MW2 Δsec. The remaining IFN-γ production was still easily detectable at lower MW2 Δsec supernatant dilutions, suggesting that other SAgs encoded by MW2 (i.e., sea, selh, selk, sell, selq, selw, and setx) are also able to elicit the production of this cytokine.

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**Fig. 5.** SAg-induced IFN-γ promotes a proinflammatory environment that allows S. aureus to impede clearance during bloodstream infection. DR4-B6 mice were treated with isotype antibody or IFN-γ–depleting antibodies 18 h prior to infection with S. aureus COL. Following infection, three to four animals were killed from each group at the six time points shown, and liver and blood were harvested from each animal. (A) Bacterial burden was determined at each time point and is shown as mean CFU/liver ± SEM. (B) Liver homogenate was analyzed by multiplex cytokine array, and mean IFN-γ (pg/mL) ± SEM at each time point were determined for each time point. (C) Serum was analyzed by ELISA to determine the concentration of AST. Data shown are mean AST (pg/mL) ± SEM. (D) Multiplex cytokine/chemokine array analysis was conducted on liver homogenate recovered from each animal killed during the time course. Data shown represent the log10 fold change for each cytokine between isotype antibody– and IFN-γ–depleted groups that displayed significant differences by Student’s t test. Prior to comparison, data were normalized to an antibody-treated, uninfected animal killed at the same time point as their comparator. Blue color (i.e., positive values) indicates that more cytokine/chemokine is produced in the isotype antibody infection, and red color (i.e., negative values) indicates that more cytokine/chemokine is produced in the IFN-γ–depleted infection. Broad classification for each cytokine is indicated as well as potential binding partners for chemokines.
S. aureus growth of several mechanisms may be at play in the liver to promote the stages of the menstrual toxic shock syndrome (mTSS) epidemic caused by SAgs, as there were reports during the early by eliciting pathogenic interferon-gamma production. Superantigens promote (Fig. 5)

Liver macrophages are the target of pathogenic IFN-α production. (A) Flow cytometry–based phenotyping of immune cells isolated from livers of mice infected with S. aureus strain COL at 24 and 96 hpi. Control animals (sham) were treated with HBSS only. Cells were defined based on the staining profile listed below each graph and normalized to the percentage of live cells. Isotype antibody and aIFN-γ treatments were compared; each dot represents an individual mouse, and the bar indicates the mean. Significant differences were determined using an unpaired Welch’s t test (***P < 0.005, ****P < 0.0001). (B) Schematic outlining Clodronate liposome-based depletion of macrophages along with IFN-α depletion used prior to i.v. infection of mice with S. aureus COL. (C) Bacterial burden in liver and kidneys at 24 and 72 hpi is shown. Each dot represents an individual mouse, and the bar represents the geometric mean for CFUs/organ. Significant differences were determined using the Kruskal–Wallis test with the uncorrected Dunn’s test for multiple comparisons (***P < 0.05, ****P < 0.01, *****P < 0.0001).

Cytokine/Chemokine data are consistent with these findings (Fig. 5D), and while neutrophils were trending toward lower recruitment in the IFN-γ–depleted group at 24 hpi (Fig. 6A), it appeared to be monocytes and inflammatory macrophages, rather than neutrophils in the isotype antibody–treated group, that were recruited to the liver by 96 hpi (Fig. 6A). This variation in immune-cell recruitment could promote a niche for the bacteria to reside within in the liver. Furthermore, our data also indicated IFN-γ drove an increased production of the chemokine fractalkine (CX3CL1), which has been suggested to be an attractive target for coordinating this response (41). Together, these observations, both in this study and others, suggest that SAgs, through forcing the overproduction of IFN-γ, can modulate the liver environment to create a niche that is favorable for S. aureus survival.

The liver is an important organ during bacteremia, as circulating pathogens are frequently filtered and trapped by resident Kupffer cells (37). In addition, there have been several reports that demonstrate S. aureus has evolved strategies to prevent this from occurring, including direct resistance to phagocytic killing by macrophages or through the release of Hla that can aggregate platelets to create ischemic areas in the liver and promote further bacterial growth (37, 38). It is important for the bacteria to establish in the liver, as bacteria surviving here can eventually seed other organs such as the kidneys (37, 39).

Given the pleotropic nature of IFN-γ, it is not surprising that several mechanisms may be at play in the liver to promote the growth of S. aureus. In addition to the other potential mechanisms of action defined in other studies, we have evidence that suggests a pathway in which macrophages are a clear target of pathogenic levels of IFN-γ. It has been suggested previously that macrophages may be influenced by the immune dysregulation caused by SAgs, as there were reports during the early stages of the menstrual toxic shock syndrome (mTSS) epidemic that found that macrophages from affected patients exhibited erythrophagocytosis (40). However, here we demonstrate that macrophage activity has been shown to be affected by high concentration of IFN-γ to support intracellular replication of S. aureus.

Our findings could also appear to be somewhat contradictory to several other studies that clearly demonstrate that IFN-γ contributes to the clearance of S. aureus during bloodstream infection (41, 42). Furthermore, the activity of memory CD4+ T cells supports the clearance of S. aureus by producing this cytokine along with other signals to coordinate this response (41). These studies were conducted in conventional mouse strains that are less vulnerable to the activity of staphylococcal SAgs and are more likely to represent what would occur in an immunocompetent individual with preexisting SAg-neutralizing antibodies. Indeed, this divergence is revealed by Brown et al., where mice previously exposed to S. aureus demonstrated an IFN-γ peak almost immediately after infection and subsequently dropped rapidly, whereas in mice that were not preexposed to S. aureus, IFN-γ was barely detected (41). This is contrary to what we have observed in SAg-mediated disease, as IFN-γ peaked later (24 hpi) and stayed high for much of the infection course. Altogether, this suggests that IFN-γ has a dual role during infection. Primarily, it is protective against S. aureus, but if manipulated to high and sustained levels, IFN-γ can act as a mediator to promote pathogenesis.

The implications of pathogenic IFN-γ production in human health are significant. As discussed, S. aureus is one of the most common causes of bloodstream infection, with disease often leading to life-threatening sepsis (2). Indeed, sepsis is a very serious concern in the clinic, contributing to nearly 20% of global annual deaths (43). One of the major challenges to treating sepsis is that without early intervention, this disease can rapidly move from a microbiologically mediated condition to an immunologically driven sequel, often resulting in antibiotic treatment being ineffective (3). The pathophysiology of sepsis has also proven to be highly complex, with many factors,
including the invading pathogen, contributing to outcome. Death as an outcome of sepsis can occur both through acute inflammatory processes that lead to multiorgan failure as well as chronic immunosuppressive activity (44). In both cases, IFN-γ can contribute to these outcomes as a key promoter of the proinflammatory response or due to its absence leading to the dominance of immunosuppressive pathways (45, 46). Indeed, several studies have demonstrated that once a patient enters the immunosuppressive state of sepsis, therapy with IFN-γ may actually improve outcomes; however, the opposite may be true if administered too early (47, 48).

There is also evidence to suggest that this mechanism may be at play in the context of S. aureus vaccines and could be an important consideration for vaccine design. Karauzum et al. found that whole-cell vaccines in mice promoted disease and bacterial survival through the activity of a heavily skewed Th1 immune response (49). It appeared in this bloodstream infection model that disease was promoted by the vaccines and that this was mediated by excessive production of IFN-γ subverts macrophage activity, allowing the bacterium to persist within the liver and leading to increased morbidity. Furthermore, we were able to establish that this mechanism has implications for human health, as high levels of IFN-γ were observed in patients with severe sepsis.

Fig. 7. SEB and SEC elicit IFN-γ from human cells, and excessive concentration can promote increased extracellular replication of S. aureus in macrophages. (A) IFN-γ production by PBMCs from human blood following stimulation with a titration of SEB or the SEB-N23A proteins. (B and C) IFN-γ production by PBMCs from human blood following stimulation with a titration of supernatant from S. aureus COL (B) or MW2 (C) strains as indicated. Supernatants were taken from cultures grown for 8 h in BHI prior to use in these assays. Data shown (A–C) are mean ± SEM from eight donors. Significant differences were determined from the area under each curve using a paired Friedman test for multiple comparisons (*P < 0.05, ***P < 0.001). (D) Schematic outlining the procedure for intracellular S. aureus replication in RAW 264.7 murine macrophages after dosing with recombinant murine IFN-γ. (E and F) S. aureus recovered from RAW 264.7 cells after incubation at 24 h for strain COL (E) and 12 h for strain MW2 (F) with varying concentrations of recombinant IFN-γ. Each dot represents an individual experiment, and the bar represents the geometric mean for CFUs/well. (G) Schematic outlining the procedure for intracellular S. aureus replication in monocyte-derived human macrophages after dosing with recombinant human IFN-γ. (H and I) S. aureus recovered from human macrophages after incubation at 48 h for strain COL (H) and 24 h for strain MW2 (I) with varying concentrations of recombinant IFN-γ. Each dot represents macrophages from an individual human donor, and the bar represents the geometric mean for CFUs/well. Significant differences between 0 ng/mL of IFN-γ and other concentrations were determined using the Kruskal-Wallis test with the uncorrected Dunn's test for multiple comparisons (*P < 0.05, **P < 0.01, ****P < 0.0001).
IFN-γ can promote bacterial intracellular replication in human macrophages. Altogether, this moves forward our understanding of the immunological factors at play during *S. aureus*–mediated sepsis in the context of pathogen-driven inflammation and may help inform the appropriate design of treatments and vaccines targeting *S. aureus* disease.

**Materials and Methods**

**Human Ethics Statement.** Human venous blood was taken from healthy volunteer donors in accordance with human subject protocol 110859. The full study protocol was approved by the London Health Sciences Centre Research Ethics Board (University of Western Ontario, London, ON, Canada). Volunteers were recruited by a passive advertising campaign within the Department of Microbiology and Immunology at the University of Western Ontario, and following an outline of the risks, written informed consent was given by each volunteer before each sample was taken. Following sampling, blood was fully anonymized and no information regarding the identity of the donor, including sex and age, was retained.

**Mice.** Male and female (8- to 11-wk-old) 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), or B6 mice, were used for all in vivo infection experiments. DR4-B6 animals were bred on site at the University of Western Ontario, and B6 mice were purchased from the Jackson Laboratory (Stock No. 000664). Animals for experiments were housed in single-sex cages, which did not exceed four in number. During all breeding and experiments, mice were provided food and water ad libitum and appropriate enrichment was provided in all cages. 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 the University of Western Ontario.

**Bacterial Strains, Media, and Growth Conditions.** *S. aureus* strains listed in **SI Appendix, Table S1** were grown aerobically at 37 °C in tryptic soy broth (TSB) (Difco) or BHI broth with shaking (250 rpm) supplemented with appropriate antibiotics. For solid-phase cultures, tryptic soy agar (TSA) was used (TSA containing 1.5% wt/vol agar) supplemented with the appropriate antibiotics. Escherichia coli strains were used as cloning hosts and were grown in Luria–Bertani (LB) broth (Difco) or LB agar supplemented with appropriate antibiotics at 37 °C with shaking (250 rpm). Growth curve analysis was performed using a Biotek Synergy H4 multimode plate reader.

**Construction of MW2 Δsec Mutant.** Markerless deletion of sec in *MW2* was performed using the pKOR1 allelic replacement system (51). The sec knockout was created as described previously (51), and candidate constructs were screened by PCR using primers SEC-screen-For and SEC-screen-Rev (**SI Appendix, Table S2**). See **SI Appendix, SI Materials and Methods** for more details.

**Construction of pCM29::seb and pCM29::sec Complementation Plasmids.** SEB and SEC complementation plasmids for *S. aureus* SAg null mutants were created as previously described (17), with modifications (17). See **SI Appendix, SI Materials and Methods** for more details. After this step, ligation was transformed into *E. coli* SA308 (52) for appropriate methylation before transformation of sequence positive constructs into electrocompetent *S. aureus* using a protocol previously described (53).

**Protein Expression and Purification.** Recombinant SEB was generated as described previously (54). Briefly, SEB was expressed with a His-tag in BL21 (DE3) *E. coli* and purified by nickel column chromatography. An attenuated mutant of SEB that has impaired binding to TCR was also purified. The mutant SEB carries an N–A point mutation at position 23 and is referred to as SEB(N23A) (31, 55). The experimental use of SEB was approved by the University of Western Ontario Biosafety Committee (application no. BIO-UWO-0155).

**Murine Splenocyte Analysis.** The ability of murine cells to respond to SEB was determined using IL-2 production. Mouse spleens were removed and broken into a single-cell suspension, followed by red blood cell lysis in ammonium-chloride-potassium (ACK) buffer. The remaining cells were suspended in complete RPMI (CRPMI), containing RPMI (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Wisent Inc.), 100 μg/mL streptomycin, 100 U/mL penicillin (Gibco), 2 mM l-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 100 μM nonessential amino acids (Gibco), 25 mM Hepes (pH 7.2) (Gibco), and 2 μg/mL polymyxin B (Gibco). Cell suspensions were seeded into 96-well plates at a density of 1.1 × 10^6 cells/mL. Titration concentrations of recombinant SEB were added to cells and incubated for 18 h at 37 °C with 5% CO2. Supernatants were assayed for IL-2 by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Thermo Fisher Scientific). For flow cytometry, cells were dual stained with phycoerythrin (PE)-conjugated anti-CD25 (clone PC61.5) (ebiScience) and fluorescein isothiocyanate (FITC)-conjugated anti-Vτ8 (clone KJ16) (ebiScience). Events were acquired using a FACSCan II BD (Biosciences), and data were analyzed using FlowJo version 10.7.1 (TreeStar).

**Staphylococcal Bacteremia Model.** Single bacterial colonies were picked from a TSA plate and grown in 3 mL TSB overnight (16 to 18 h). Cells were subsequently subcultured in TSB to an OD_600 (optical density at 600 nm wavelength) of 0.1 and grown to postexponential phase (OD_600 –3 to 3.5). The bacterial pellets were washed once in Hank’s balanced salt solution (HBSS) to an OD_600 of 0.15 for strain COL and 0.1 for strain MW2, corresponding to ~5 × 10^8 CFU/mL. Mice were injected via the tail vein with 5 × 10^8 CFU of *S. aureus* in a total volume of 100 μL. Mice were weighed and monitored daily. At various time points postinfection, mice were killed (maximum of 3 and 4 for MW2 and COL, respectively), and the 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.

**Antibody Depletion Protocols.** CD4^+^ and CD8^+^ T cells were depleted in animals according to a protocol described previously (23). Briefly, mice were injected with 300 μg of T cell–depleting antibodies (anti-CD4 [clone GK1.5, BioXCell], anti-CD8α [clone YTS169.4, BioXCell], or both at 150 μg each) or isotype antibody control (clone LTF-2, BioXCell) 7, 6, and 1 day before infection with *S. aureus*. For IL-17A depletion, mice were injected with a 200-μg dose of anti–IL-17A mAb (clone 17F3; BioXCell) or a mouse IgG1 isotype antibody control (clone MOPC-21, BioXCell) 3 h before *S. aureus* infection, then with a further 100-μg dose 1 h after infection, as described previously (28). For IL-10 and IFN-γ depletions, mice were treated with a 250-μg dose of anti–IL-10 mAb (clone JES5-2A3, BioXCell), anti–IFN-γ (clone XMG1.2, BioXCell), or Rat IgG1 isotype antibody control (clone CI.18, BioXCell) administered 18 h prior to infection, as described previously (55). All antibody doses were prepared in 100 to 200 μL PBS and administered by intraperitoneal (i.p.) injection.

**Detection of Cytokines and Chemokines In Vivo.** At various time points postinfection, serum supernatants and livers were collected. Supernatants were obtained from whole livers by homogenization in HBSS supplemented with the complete protease inhibitor mixture (Roche). Samples were analyzed using Mouse Cytokine Array/Chemokine Array 44-Plex (MD44, Eve Technologies). AST levels were assessed from murine serum using a mouse AST ELISA Kit (Abcam).

**IFN-γ Treatment of Mice during *S. aureus* Bacteremia.** *S. aureus* DR4-B6 animals were infected with *S. aureus* COL aseB intravenously (i.v.) at a dose of 5 × 10^8 CFU in 100 μL of HBSS prepared as described in the staphylococcal bacteremia model section. At 2 h before and 1 h after infection, animals were treated with 20 μg recombinant murine IFN-γ (Sino Biological) in 100 μL PBS by i.p. injection (40 μg total). Control animals were treated with PBS only. Animals were monitored daily and killed at 96 hpi to harvest livers. Bacterial burden was determined as described in the staphylococcal bacteremia model section.

**Flow Cytometry Analysis of Murine Cells.** Livers were extracted from mice and pushed through a 0.7-μm cell strainer. Leukocytes were isolated from livers with a 33.75% Percoll gradient (GE Healthcare). Following isolation, red blood cells were lysed using ACK lysis buffer (Gibco) and washed with PBS containing 2% FBS. Following isolation, cells were stained and analyzed as outlined in **SI Appendix, SI Materials and Methods**.

**Macrophage Depletion in Mice.** Macrophage depletion was based on a protocol previously described (56). Briefly, 200 μL Clodronate containing liposomes and control liposomes (Clodrosome + Encapsome [Encapsula Nano Sciences]) were administered to the mice i.p. 2 d and 4 h prior to infection with bacteria. At 18 h prior to infection, IFN-γ–depleting or control antibodies were also administered to the mice.

**Detection of Human Cytokines from Stimulated Human Cells.** The ability of human cells to produce cytokines was determined from stimulated PBMCs. These cells were isolated from human blood by density-based centrifugation.
following layering of the blood onto Ficoll-Hypaque plus (GE healthcare). Clear cells were isolated from blood with lympholyte-poly (Cedarlane Laboratories). Macrophage Cultures and Infections. RAW 264.7 (a murine macrophage cell line) cells were maintained in RPMI 1640 supplemented with 10% FBS and grown in 12-well plates with coverslips for experimental analysis. Primary human macrophages were derived from blood monocytes isolated from healthy human volunteers as previously described (32, 37). Briefly, mononuclear cells were isolated from blood with lymphocyte-poly (Cedarlane Laboratories) and the manufacturer’s instructions. Monocytes adhered to glass coverslips in 12-well plates (1.5 x 10^6 cells/well) and were subsequently cultured for 7 to 9 d in RPMI (Gibco) with 10% FBS (Wisent) and 0.5 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF) (R&D Systems) to allow for differentiation of monocytes into macrophages. After 5 d of differentiation, adhered cells were washed with PBS and the medium was replaced with fresh RPMI + 10% FBS containing M-CSF. Macrophages were differentiated until day 7 and used experimentally until day 10. For murine bone-derived macrophages, DR4-B6 mice were killed and leg bones were harvested for bone-marrow extraction. Bone marrow was flushed from the bones and washed with PBS prior to resuspension in RPMI suplemented with 5% FBS. Bone marrow–derived cells were adhered to glass coverslips in 12-well plates in a similar manner to human cells and stimulated with 10 ng/mL murine M-CSF. Macrophages again were differentiated until day 7 and used experimentally until day 10.

Statistical Analysis. All statistical analyses were performed using GraphPad Prism 9, and a P-value <0.05 was considered statistically significant. For all bacterial burden CFU, analysis was performed with the nonparametric Mann–Whitney U test or Kruskal–Wallis test with an uncorrected Dunn’s test for multiple comparisons, depending on group numbers. Flow-cytometry data were analyzed using Welch’s t test to determine significant differences between the isotype antibody and IFN-γ–depleted groups.

For the multiplex cytokine analysis heat map shown in Fig. 5D, each raw data point was normalized to a sample taken from an uninfected control animal that was killed at the same time point after treatment with the same antibody. Following normalization, the data from analysis at each time point were compared for statistical significance between the isotype antibody and IFN-γ–depleted using the Kruskal–Wallis’ t test. All significant values were extracted, and the mean quantity of the cytokine/chemokine detected in the isotype antibody–treated animals was divided by the quantity detected in the IFN-γ–depleted group. These values were converted to log2 values to give fold change in positive and negative values that could be plotted on a heat map.

For the human cytokine analysis performed in Fig. 7 A–C, the area under each curve for each donor was determined. These values were then compared using a paired Friedman test or paired Friedman test for multiple comparisons, depending on the number of groups. Paired tests were used due to the large variation observed between individual human donors.

Data Availability. All study data are included in the article and/or SI Appendix.

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