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RESEARCH ARTICLE

Protection against *Staphylococcus aureus* bacteremia–induced mortality depends on ILC2s and eosinophils

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The dysregulated, unbalanced immune response of sepsis results in a mortality exceeding 20%, yet recent findings by our group indicate that patients with allergic, type 2–mediated immune diseases are protected from developing sepsis. We evaluated CD4$^+$ Th cell polarization among patients with *Staphylococcus aureus* bacteremia and confirmed that survivors had a higher percentage of circulating Th2 cells but lower frequencies of Th17 cells and neutrophils early in the course of infection. To establish the mechanism of this protection, we used a mouse model of lethal *S. aureus* bacteremia and found that intratracheal pretreatment with the type 2–initiating cytokine IL-33 activated pulmonary type 2 innate lymphoid cells (ILC2s) and promoted eosinophilia. In addition, stimulation of type 2 immunity before lethal infection suppressed the pulmonary neutrophilic response to *S. aureus*. Mice lacking functional ILC2s did not respond to IL-33 and were not protected from lethal bacteremia, but treatment of these mice with the type 2 cytokines IL-5 and IL-13 rescued them from death. Depletion of eosinophils abrogated IL-33–mediated protection, indicating that eosinophilia is also necessary for the survival benefit. Thus, we have identified a potentially novel mechanism by which type 2 immunity can balance dysregulated septic inflammatory responses, thereby clarifying the protective benefit of type 2 immune diseases on sepsis mortality.

Introduction

Sepsis is defined as overwhelming systemic inflammation and organ dysfunction caused by a dysregulated host response to an infection (1). Sepsis affects 750,000 people per year in the United States and is a frequent cause of death in intensive care units (1, 2). *Staphylococcus aureus* (*S. aureus*) infection is an important cause of sepsis, with approximately 93,000 cases of bacteremia annually and a 20%–30% mortality rate (3). Upon infection, a proinflammatory response is rapidly elicited to eliminate this invading pathogen, often inducing the development of type 1 or type 17 inflammation (4). This combination of a rapid increase in proinflammatory cytokines with activation of neutrophils, macrophages, and polarized T cell responses can cause extensive host tissue damage while neutralizing the inciting pathogen (5). To protect against tissue damage, a compensatory, antiinflammatory, proresolving response has been postulated, which promotes restoration of homeostasis and resolution of inflammation. Thus, mortality during sepsis is thought to occur from a gross imbalance in these inflammatory responses to infection (6, 7). In support of this hypothesis, we recently discovered that an increased neutrophil/lymphocyte ratio on admission and increased type 17 responses at early time points after positive culture independently predicted increased 90-day mortality among patients admitted to the hospital with *S. aureus* bacteremia (8). These data are consistent with the hypothesis that an unbalanced, overwhelming, early proinflammatory response is detrimental during sepsis.

Patients with immunologic diseases offer a unique opportunity to understand how unbalanced immune responses to pathogens may affect sepsis outcomes. We previously analyzed the Truven MarketScan insurance claims database (with >150 million patient encounters) to determine which immune diseases were positively or negatively associated with sepsis among hospitalized patients (9). Diseases associated with inappropriate activation of the type 1 or type 17 immune responses, such as vasculitis

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and ulcerative colitis, were overrepresented among septic patients. However, to our surprise, we noted that diseases associated with activation of the type 2 allergic immune response were markedly *underrepresented* among septic patients, suggesting a possible protective effect. Zein et al. independently found a similar beneficial effect of having asthma or allergic rhinitis on the risk of development of sepsis (10). We extended these observations to a mouse model in which we pretreated mice with the innate type 2 cytokine IL-33 to induce type 2 pulmonary inflammation, followed by infection with a lethal dose of intravenous *S. aureus* (11, 12). In this mouse model, *S. aureus* causes significant tissue damage in peripheral organs, such as the lung, liver, and heart, reminiscent of that found in patients with systemic *S. aureus* sepsis (13–15). Indeed, mice pretreated with intratracheal IL-33 were protected from death induced by an *S. aureus* bloodstream infection, supporting the hypothesis that type 2 inflammation may play a critical, beneficial role during the development of sepsis (9). These results are supported by other recent publications demonstrating that systemic IL-33 can promote bacterial clearance and reduce systemic inflammation during cecal ligation puncture–induced sepsis, although the mechanism of this IL-33–induced protection remained unclear (16–18).

In the current study, we investigate whether an augmented type 2 response is protective in a prospectively collected patient cohort with *S. aureus* bacteremia, followed by use of a mouse model of lethal *S. aureus* bacteremia to identify the critical mechanisms of this protection. We found that increased eosinophil/neutrophil ratio on admission was associated with reduced 30-day mortality, as was an increase in the percentage of Th2 lymphocytes. In our model of IL-33–mediated protection against lethal *S. aureus* bacteremia in mice, we found that intratracheal IL-33 pretreatment protected against a systemic *S. aureus* infection by promoting innate lymphoid cell type 2–dependent (ILC2-dependent) eosinophilia and suppressing neutrophilia. This is the first study to our knowledge to demonstrate the importance of type 2 immunity in mediating protection against lethal systemic *S. aureus* infection. More importantly, this work suggests that manipulation of type 2 immunity may be an important therapeutic target to improve sepsis outcomes.

Results

Surviving patients of S. aureus bacteremia have higher levels of circulating Th2 cells and lower levels of Th17 cells and neutrophils. Based on our recently published work indicating a protective association between type 2 allergic diseases and sepsis, we hypothesized that survival among hospitalized patients with *S. aureus* bacteremia would be associated with increased levels of circulating Th2 lymphocytes and eosinophils (9). To test this hypothesis, we analyzed samples collected prospectively for a separate study evaluating immune responses among patients with *S. aureus* bacteremia (8). We collected blood from infected patients at several time points to determine the association of T cell immune responses with mortality and to understand the dynamics of sepsis-associated immunity (19). In the current study, we limited our analysis to 30-day mortality (rather than 90-day mortality, as previously published), reasoning that this would more closely reflect the impact of the acute bacteremia episode. Patients who were still alive 30 days after their initial episode *of S. aureus* bacteremia had an increased percentage of Th2 lymphocytes and a reduced percentage of Th17 cells and neutrophils compared with nonsurviving patients (Figure 1). Although the difference was not significant, a trend toward an increased level of blood eosinophils was found in surviving patients, and likewise, the ratio of eosinophils to neutrophils was reduced in nonsurviving patients (Figure 1C). These data demonstrate that the imbalance in proinflammatory and antiinflammatory responses associated with sepsis mortality may be reflected in an imbalance in type 2 and type 17 responses.

IL-33 treatment before S. aureus bacteremia enhances spleen eosinophilia while promoting rapid bacterial clearance in the liver and lungs. To investigate the mechanism of how the type 2 response may be mediating protection, we used our previously published mouse model of a lethal intravenous *S. aureus* infection. To induce sepsis, C57BL/6 mice were infected with 5×10^7 CFU intravenous *S. aureus* USA300, a community-associated, methicillin-resistant strain. Intratracheal IL-33 pretreatment for 3 days before infection promoted a significant reduction in *S. aureus* CFU levels in the liver and lung 6 hours after infection but did not influence bacterial levels in the spleen (Figure 2). No difference was found in bacterial burden between PBS- and IL-33–treated mice in any tissues at 18 hours after infection. To evaluate the systemic inflammatory response during intravenous *S. aureus* infection, flow cytometry was used to quantify splenic granulocyte populations at 18 hours after infection, with gating strategies in Supplemental Figure 1.

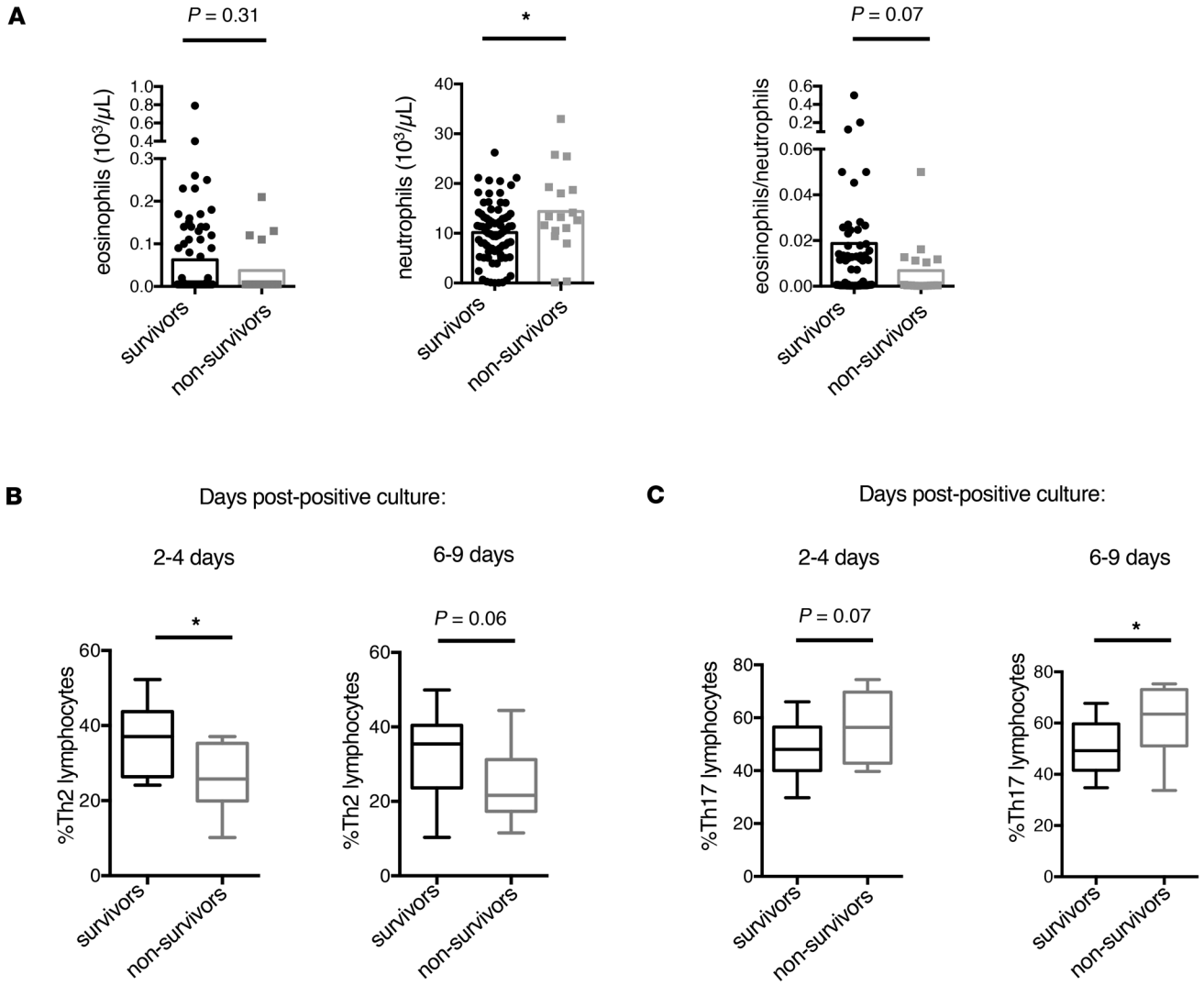

Figure 1. Patients who survive *S. aureus* bacteremia have higher levels of Th2 lymphocytes and lower levels of Th17 lymphocytes and neutrophils in the blood. (**A**) On admission (t = 0), blood was collected for complete blood cell counts with differentials, allowing eosinophil and neutrophil analysis. *P < 0.05, Mann-Whitney test. For evaluation of Th lymphocyte subsets, PBMCs were isolated from patients diagnosed with *S. aureus* bacteremia at 2 time points: 2–4 days and 6–9 days after positive culture. Flow cytometry was used to quantify Th subsets, which are expressed as a percentage of total $CD3^+CD4^+CD45RO^+$ non-Tregs, with (**B**) Th2s identified as $CCR4^+CCR6^-$ and (**C**) Th17s identified as $CCR6^+$, among survivors and nonsurvivors (matched on sequential organ failure assessment [SOFA] score and comorbidities) at 30 days after the initial *S. aureus* bloodstream culture. The box plots depict the minimum and maximum values (whiskers), the upper and lower quartiles, and the median. The length of the box represents the interquartile range. *P < 0.05, unpaired Student's t test. Sample sizes were n = 19 for surviving patients and n = 8 nonsurvivors.

Eosinophilia is a canonical sign of type 2 inflammation, while increased neutrophil levels are classically associated with acute bacterial infections and sepsis (11, 20). Intratracheal IL-33 pretreatment promoted a modest increase in spleen eosinophil levels in control mice, indicating that pulmonary administration could have an important systemic effect (Figure 3A). However, while *S. aureus* infection caused a substantial increase in splenic neutrophils, IL-33 had no impact on levels of these cells (Figure 3B). These data suggest that intratracheally administered IL-33 does not modulate the systemic levels of neutrophils to exert its protective effect.

IL-33 protects against S. aureus–mediated death by activating ILC2s to promote type 2 inflammation in the lungs. Analysis of lung histology revealed the effect of IL-33 pretreatment on airway inflammation (Figure 4A). Infected mice treated with IL-33 manifested a similar degree of edema compared with control infected mice, as measured by red blood cell accumulation in the alveolar space (Figure 4B).

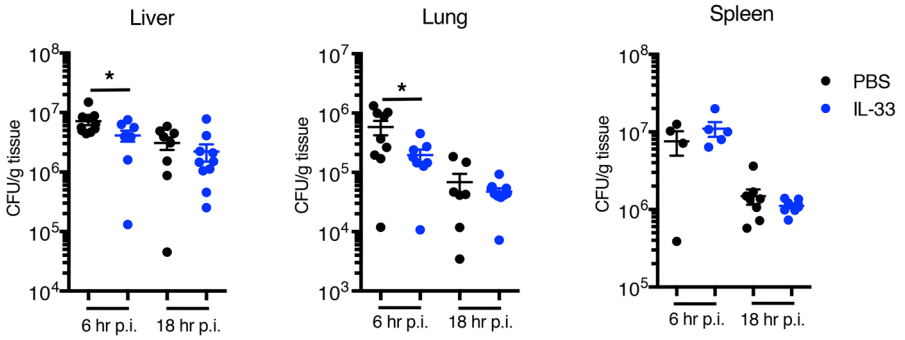

Figure 2. IL-33 pretreatment reduces bacterial burden in lung and liver during *S. aureus* bacteremia. C57BL/6 mice were treated with PBS or IL-33 and infected with 5×10^7 *S. aureus* CFU. Mice were euthanized at 6 or 18 hours after infection (p.i.). CFU were quantified in the liver, lung, and spleen using serial dilution. Colony counts were normalized to tissue weight. For the liver and lung, 2 experiments were pooled, with n = 7–10 mice per group. For the spleen, n = 4–5 mice per group for 6 hours after infection and n = 8 per group for 18 hours after infection. *$P < 0.05$, unpaired Student's t test.

However, periodic acid–Schiff (PAS) staining showed that IL-33 pretreatment induced goblet cell hyperplasia, resulting in a thick mucus layer in mice infected with *S. aureus* as well, which may act as an additional barrier of protection against invading bacteria (Figure 4C and ref. 21).

IL-33 stimulates the type 2 response in the lungs through direct activation of lung-resident ILC2s (12). We used flow cytometry to evaluate the inflammatory response in the lungs at 18 hours after infection with *S. aureus*, with our gating strategy shown in Supplemental Figure 1. In our model, IL-33 promoted lLC2 expansion whether or not the mice were infected with *S. aureus* (Figure 5A). Moreover, *S. aureus* alone did not increase ILC2 numbers in the absence of IL-33 treatment. However, neither IL-33 treatment nor *S. aureus* infection affected other innate lymphocyte populations, such as NKTs and γδ T cells (Supplemental Figure 2, A and B), nor did it affect adaptive T lymphocyte populations (Supplemental Figure 2, C and D). IL-33 pretreatment also increased the percentage of ILC2s that were actively producing the type 2 cytokines IL-5 and IL-13, whether or not the mice were infected with *S. aureus* (Figure 5B). In addition, intratracheal IL-33 induced an increase in serum type 2 cytokine levels, as measured by multiplex analysis (data not shown).

To determine whether ILC2s were required for either survival during lethal bacteremia or for IL-33–mediated protection, we measured survival of mice deficient in the transcription factor promyelocytic leukemia zinc finger protein (PLZF). PLZF$^{-/-}$ mice lack functional ILC2s because of cell-intrinsic developmental defects, resulting in reduced type 2 cytokine production and reduced downstream eosinophilia during airway inflammation (12). Although PLZF$^{-/-}$ mice have intact adaptive T cell responses (12), they also have reduced functional NKTs (22). PLZF$^{-/-}$ mice infected with *S. aureus* exhibit reduced survival compared with wild-type littermates, suggesting that either functional ILC2s or NKTs are important in protection (Figure 6).

To differentiate the roles of ILC2s and NKTs, survival analysis was performed with CD1d$^{-/-}$ mice, which are deficient in NKTs but have normal ILC2 responses. The absence of NKTs did not influence survival (Supplemental Figure 2E), suggesting that the lack of functional ILC2s rendered PLZF$^{-/-}$ mice more susceptible to infection. In support of this hypothesis, treatment with IL-33 did not rescue infected PLZF$^{-/-}$ mice from death. Moreover, no difference was found in the levels of spleen NKTs after IL-33 treatment during sepsis (data not shown). Thus, these data suggest that ILC2s are required for protection against *S. aureus* infection.

To determine whether ILC2 production of IL-5 and IL-13 was critical for protection against sepsis, we pretreated PLZF$^{-/-}$ mice with these cytokines before *S. aureus* infection (Figure 6). IL-5 and IL-13 treatment rescued PLZF$^{-/-}$ mice from death, indicating that the defect in PLZF$^{-/-}$ILC2s could be overcome by the restoration of these type 2 cytokines. Thus, we conclude that IL-33–induced production of IL-5 and IL-13 from ILC2s is an important mechanism by which type 2 immune responses can protect against lethal bacteremia.

Enhanced eosinophilia and suppression of S. aureus–induced neutrophilia are necessary for protection during bacteremia. To determine the downstream effects of IL-33–mediated ILC2 activation on the immune response to *S. aureus* bacteremia, we quantified the levels of lung granulocyte populations. Eosinophils

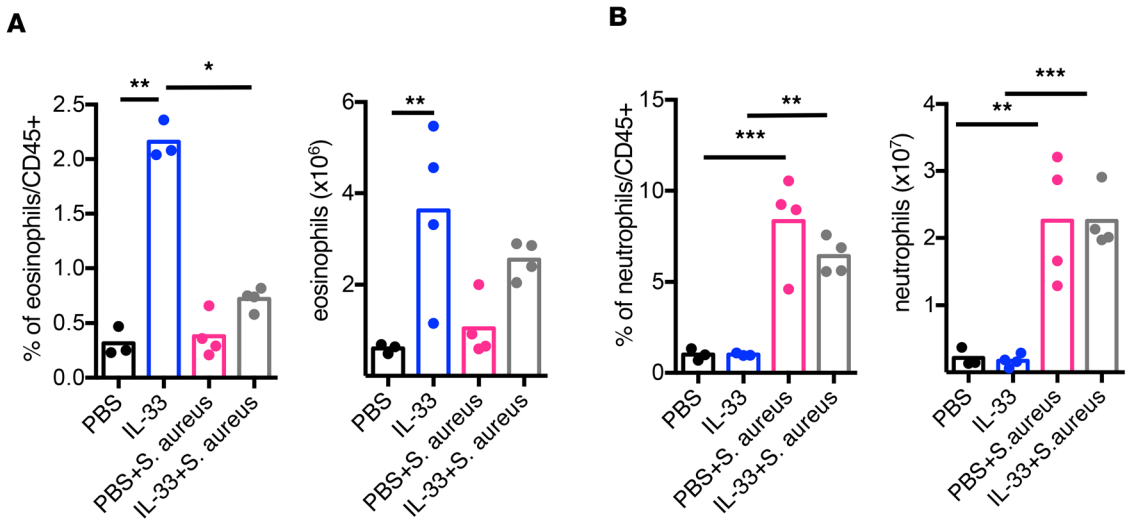

Figure 3. Intratracheal IL-33 pretreatment induces splenic eosinophilia but does not affect splenic neutrophilia during *S. aureus* bacteremia. C57BL/6 mice were treated with PBS, IL-33, PBS and *S. aureus*, or IL-33 and *S. aureus* and euthanized 18 hours after infection. Shown are representative graphs depicting the frequency and total cell number of spleen (**A**) eosinophils ($CD45^+CD11c^{lo}SiglecF^+SSC^{hi}$) and (**B**) neutrophils ($CD45^+CD11b^{hi}Ly6G^{hi}F4/80^-$). All percentages were normalized to live $CD45^+$ cells. Nonsignificant comparisons are not shown. Shown are representative graphs of 2 experiments, with a sample size of 3–4 mice per group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, 1-way ANOVA with Sidak's multiple-comparisons test.

were increased in wild-type $PLZF^{+/+}$ mice treated with IL-33, regardless of whether they were infected with *S. aureus* (Figure 7, A–C). IL-33 pretreatment led to a marked reduction in the percentage and number of neutrophils in infected wild-type mice compared with uninfected mice. These results imply that IL-33 may be protective by reducing *S. aureus*–induced lung neutrophilia. Interestingly, examination of the ratio of eosinophils to neutrophils in the lung suggested that a balanced number of granulocytes was associated with survival (Figure 7C). Similar results were found in C57BL/6 mice (Supplemental Figure 3).

Similar to previously published data (12), $PLZF^{-/-}$ mice had fewer eosinophils compared with wild-type mice upon pretreatment with IL-33, demonstrating their diminished capacity to initiate type 2 pulmonary inflammation (Figure 7A). Notably, in the absence of functionally intact ILC2s and pulmonary eosinophilia, $PLZF^{-/-}$ mice demonstrated augmented *S. aureus*–induced neutrophilia despite IL-33 pretreatment (Figure 7, B and C). These data suggest that a reduction in the ratio of eosinophils to neutrophils may result in increased mortality.

Given that ILC2-dependent eosinophilia is associated with a decline in *S. aureus*–induced neutrophilia, we posited that either *S. aureus*–induced mortality because of a surplus of neutrophils or, alternatively, the lack of eosinophils renders the animal more prone to unchecked neutrophilic inflammation. To decipher these possibilities, we first depleted neutrophils to determine whether inhibition of neutrophilia promoted survival. Systemic neutrophil depletion with the monoclonal antibody Ly6G (clone 1A8) resulted in almost immediate death upon infection (Supplemental Figure 4), indicating that a neutrophilic response is necessary, presumably to contain the infection. To determine the importance of eosinophils in IL-33–mediated protection, we adapted an inducible depletion model using a conditional eosinophil-deficient strain of mice (iPHIL), as developed by Jacobsen et al. (23). The eosinophils from iPHIL mice express the human diphtheria toxin (DT) receptor under control of the eosinophil peroxidase promoter. Inducible depletion of eosinophils was achieved by administering DT to iPHIL mice 1 day before *S. aureus* infection and then every other day throughout the experiment. Remarkably, depletion of eosinophils during infection eliminated IL-33–mediated protection and enhanced bacteremia-induced mortality (Figure 8). These data indicate that eosinophils are required for IL-33–mediated protection against bacteremia-induced death.

Thus far we have demonstrated that *S. aureus* induces neutrophilia, yet neutrophils are required for survival. However, IL-33–dependent, ILC2-mediated eosinophilia inhibits neutrophilia and protects against lethal infection. To determine whether IL-33 pretreatment could rescue neutrophil-depleted

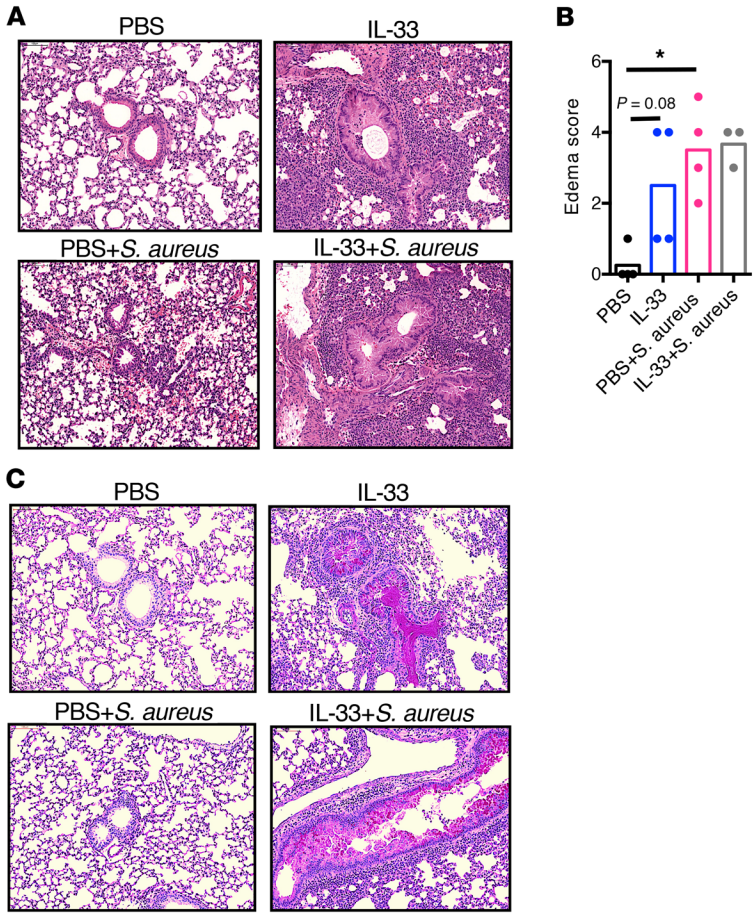

Figure 4. Intratracheal IL-33 pretreatment does not affect *S. aureus*–induced pulmonary edema. C57BL/6 mice were treated with PBS, IL-33, PBS and *S. aureus*, or IL-33 and *S. aureus* and euthanized 18 hours after infection. Shown are representative images of the left lung at ×10 magnification stained for (**A**) H&E, with (**B**) blinded scoring and quantification for edema, or (**C**) PAS staining to show goblet cell hyperplasia and mucous production. Sample size was 4 mice per group. *$P < 0.05$, unpaired Student's *t* test.

mice from *S. aureus*–induced mortality, C57BL/6 mice were treated with IL-33, followed by neutrophil depletion with anti–mouse Ly6G. When mice were depleted of neutrophils, IL-33 treatment modestly increased survival compared with isotype controls (Supplemental Figure 4). These data indicate that eosinophils may be partially able to compensate for the lack of neutrophils in this experiment.

Discussion

Our study demonstrates that increased type 2 immune responses during *S. aureus* bacteremia in both patient samples and mouse models are associated with improved outcomes. Mechanistically, in our mouse model we have shown that a preexisting type 2 response can protect against systemic *S. aureus* infection via ILC2-dependent cytokine secretion and subsequent eosinophilia. We propose a model to explain our findings wherein activation of the type 2 response counterbalances the pathogen-induced proinflammatory response and the resulting tissue/organ damage characteristic of sepsis (Figure 9). This model incorporates theories of immune balance proposed by Eberl and Gause et al. with the theory of sepsis as an immune response "see-saw" proposed by Hotchkiss (4, 6, 24). Additionally, this may explain the apparent protective benefit of allergic diseases on survival from sepsis that we and others have observed (9, 10). During bacterial infections, neutrophils are necessary for controlling infections because they are recruited to the site of inflammation in response to activation of type 17 immune responses (20). However, inflammation must also be regulated; overproduction of proinflammatory cytokines during sepsis can redirect the protective nature of neutrophils to become detrimental and induce tissue damage. Analysis of our bacteremia patient samples suggests that

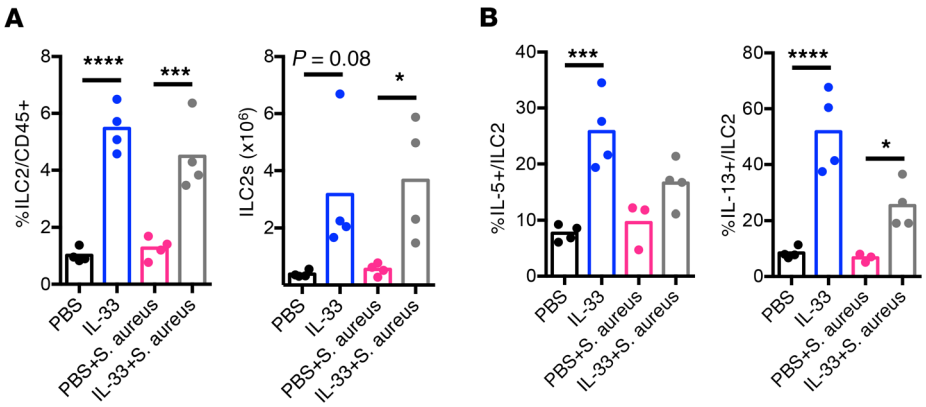

Figure 5. Intratracheal IL-33 pretreatment mediates ILC2 expansion and cytokine production during *S. aureus* bacteremia. C57BL/6 mice were treated with PBS, IL-33, PBS and *S. aureus*, or IL-33 and *S. aureus* and euthanized 18 hours after infection. ILC2s were defined as lineage$^-$CD45$^+$CD90$^+$CD127$^+$ST2$^+$. (**A**) Graphs depicting the frequency and total cell number of ILC2s. Percentages of ILC2s were normalized to live CD45$^+$ cells. (**B**) Intracellular staining was used to quantify percentage and total cell number of ILC2s expressing IL-5 and IL-13. Nonsignificant comparisons are not shown. Shown are representative graphs of 2 experiments, with a sample size of 4 mice per group. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$, 1-way ANOVA with Sidak's multiple-comparisons test.

both an elevated neutrophil response and increased type 17 immune activation are associated with mortality. Indeed, the driver of inflammation in our bacteremia patients appears to be intrinsic to the host response itself, rather than the persistence of bacteria, given that there were no differences in the number of days that blood cultures were positive in either the survivors or nonsurvivors (8). Similar to what we observed in patient samples, *S. aureus*–mediated sepsis in mice causes increased neutrophilia and edema, both of which are hallmarks of lung inflammation. Importantly, some neutrophilia is clearly necessary: Removal of neutrophils before infection accelerated mouse mortality, and patients with neutropenia are at high risk for morbidity and mortality because of *S. aureus* bacteremia (25).

Mice pretreated with IL-33 before *S. aureus* infection have an eosinophil/neutrophil ratio in the lungs that closely resembles that of uninfected mice, in contrast with a markedly reduced eosinophil/neutrophil ratio upon infection with *S. aureus* alone. These data suggest that the type 2 eosinophilic response may balance the necessary but destructive *S. aureus*–associated neutrophilic response. In support of this notion, we observed an association between an increased eosinophil/neutrophil ratio in patients and reduced mortality. Furthermore, in the absence of ILC2 cytokine secretion and airway eosinophilia, IL-33 treatment failed to suppress *S. aureus*–induced neutrophilia in PLZF$^{-/-}$ mice. Of note, despite this being a systemic infection, survival during *S. aureus* bacteremia in mice was associated with suppression of pulmonary, but not systemic, neutrophilia. Consistent with prior work indicating that the lungs are

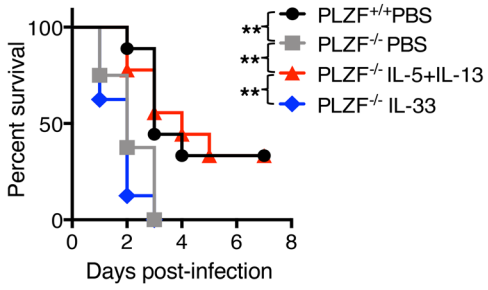

Figure 6. IL-5 and IL-13 cytokine production by ILC2s is required for survival during *S. aureus* bacteremia. Shown are Kaplan-Meier survival curves for PLZF$^{+/+}$ and PLZF$^{-/-}$ mice treated with intratracheal PBS, intratracheal IL-33, or intraperitoneal IL-5 and IL-13 for 3 days. All groups were intravenously infected with *S. aureus* on day 4 and monitored for survival for 7 days after infection. For each group, $n = 8–9$ mice. PLZF$^{+/+}$ PBS versus PLZF$^{-/-}$ PBS, **$P < 0.01$; PLZF$^{-/-}$ PBS versus PLZF$^{-/-}$ IL-5 and IL-13, **$P < 0.01$; PLZF$^{-/-}$ IL-5 and IL-13 versus PLZF$^{-/-}$ IL-33, **$P < 0.01$, log-rank (Mantel-Cox).

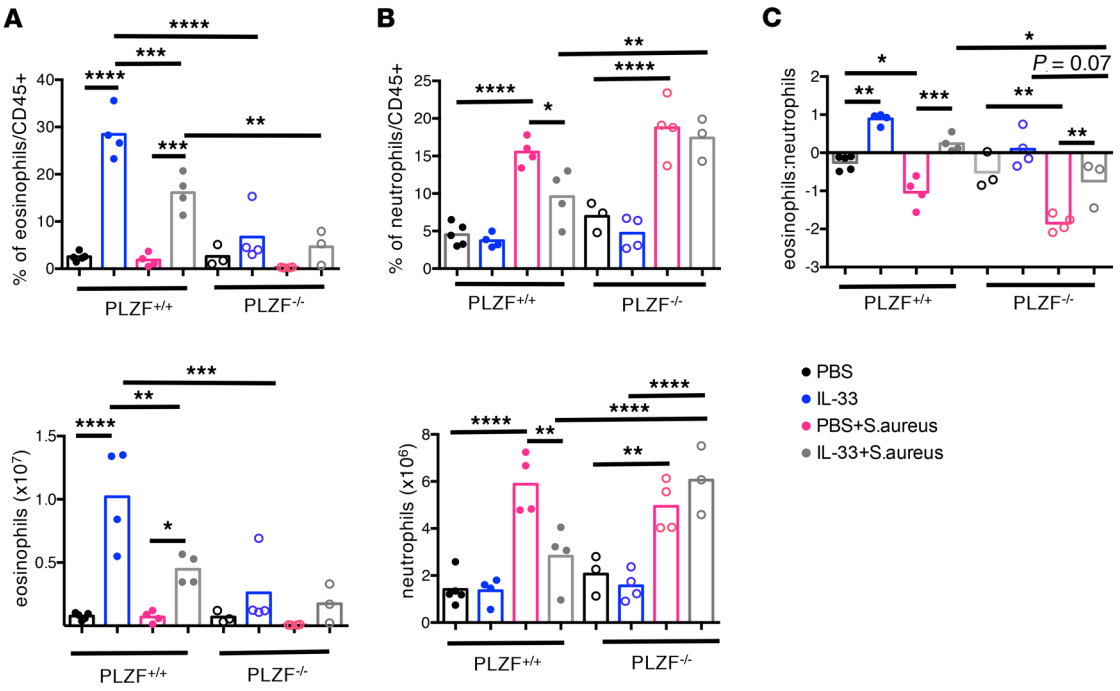

Figure 7. Mice lacking functional ILC2s have impaired IL-33–mediated eosinophilia and enhanced neutrophilia during *S. aureus* bacteremia. PLZF$^{+/+}$ or PLZF$^{-/-}$ mice were treated with PBS or IL-33, followed by an intravenous *S. aureus* infection. Control groups received PBS or IL-33 treatment alone. All groups were euthanized at 18 hours after infection. Shown are graphs depicting the frequency and total cell number of lung (**A**) eosinophils or (**B**) neutrophils. All percentages were normalized to live CD45^{+} cells. (**C**) Ratio of lung eosinophils to neutrophils, expressed using logarithmic transformation. Statistical significance was determined using a 1-way ANOVA with Sidak's multiple-comparisons test. Nonsignificant comparisons are not shown. Shown are representative graphs of 2 experiments, with a sample size of 3–5 mice per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, 1-way ANOVA with Sidak's multiple-comparisons test.

a site for neutrophil recruitment in bacteremia mouse models of sepsis (26), our data suggest that mortality may result from the inflammatory response specifically in the lungs. Interestingly, IL-33 promoted a modest reduction in bacteria detected in the liver and lungs but not the spleen during early infection. However, the mechanism underlying this increase in bacterial clearance and the suppression of neutrophilia in the lungs remains unknown.

In our mouse model, IL-33 pretreatment primarily activated the innate type 2 response because treatment with IL-33 was short term and low dose. Moreover, intratracheal IL-33 targeted lung-resident ILC2s, which highly express the IL-33 receptor ST2 at baseline. This model is distinct from prior studies of IL-33 and sepsis, in which IL-33 is administered systemically either at the time of infection or after sepsis has been induced (16, 18). Although we also observed a decrease in bacterial burden with IL-33 pretreatment 24 hours before infection, the mechanism likely does not involve direct action on recruited neutrophils, given the extremely short half-life of both IL-33 and neutrophils (27, 28). In addition, we did not observe a notable impact of IL-33 pretreatment on the adaptive immune system during bacteremia, though this may not be surprising given that naive mice have relatively low numbers of ST2-expressing Th2 cells (29). However, our previous work demonstrated that activation of the adaptive type 2 immune response using a house dust mite sensitization/challenge model also protects against lethal bacteremia, indicating that there may be multiple ways to engage the beneficial type 2 immune response during infection (9).

Traditionally, an overactive type 2 response has been associated with diseases such as asthma, atopic dermatitis, and food allergies (11). However, recent studies have found that the type 2 response has antiinflammatory and wound-healing benefits as well (24). A growing body of evidence indicates that release of amphiregulin during innate type 2 responses is critical for suppressing inflammation and inducing tissue repair (30). IL-13 and IL-5 have both been demonstrated to protect against acute lung injury, and several recent studies have noted that type 2 cytokines may be protective during sepsis (21, 31–35). Consistent with these studies, we observed that treatment with

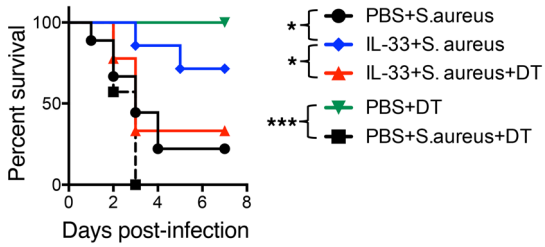

Figure 8. Eosinophils are required for survival during *S.aureus* bacteremia. iPHIL mice were treated with PBS and *S. aureus*, IL-33 and *S. aureus*, IL-33 and *S. aureus* plus DT, PBS plus DT, or PBS and *S. aureus* plus DT. Mice were monitored for survival 7 days after infection. Shown are Kaplan-Meier survival curves for each treatment group. Sample sizes were 5–10 mice for each treatment group. $*P < 0.05$; $***P < 0.001$, log-rank (Mantel-Cox).

IL-5 and IL-13 rescued PLZF$^{-/-}$ mice from sepsis, indicating that 1 potential mechanism of IL-33–mediated protection is through ILC2 cytokine production. It remains unclear, however, whether the elevated Th2 cells observed in surviving human patients reflect a specific response to *S. aureus* bacteremia or whether they reflect either genetic or environmental differences that nonetheless appear to confer a survival advantage.

Although we have demonstrated both a tendency toward higher eosinophil/neutrophil ratios among bacteremia survivors and that ILC2-dependent increases in the eosinophil/neutrophil ratio are protective in mice, we do not know how eosinophils are acting to protect against lethality. Studies have shown that eosinophils have antibacterial properties, possibly through the formation of extracellular traps (26, 36). Eosinophils also express pattern recognition receptors, including several Toll-like receptors, and it has been suggested that they may phagocytose bacteria (37). Thus, eosinophils may be key in reducing the *S. aureus* bacterial load, which could, in turn, lead to a reduction in neutrophilia. Alternatively, patients with asthma often have exclusively neutrophilic-predominant or eosinophilic-predominant subtypes of asthma, indicating that these inflammatory responses may be mutually inhibitory (38). Last, there are likely multiple adaptive and innate pathways that result in increased eosinophilia; in our human samples, we identified increased adaptive Th2 cells as associated with survival, but in our mouse model, we found that innate ILC2s were critical for protection.

Our studies are among the first to suggest a mechanism for how the type 2 response may be protective against bacteremia, and in turn, sepsis. This study has several important clinical implications: First, biologic therapies that target type 2 inflammation for the treatment of asthma and allergies may inadvertently render patients more susceptible to infection and sepsis. Conversely, activation of type 2 immunity during acute infection may represent a novel therapeutic strategy to combat the overwhelming proinflammatory neutrophilic response associated with lethal sepsis. Interindividual differences in the capacity to mount a type 2 immune response may underlie the wide heterogeneity in hospital course and outcomes observed for patients with otherwise common infections, such as *S. aureus* bacteremia. Finally, this study further supports the importance of considering additional immunologic comorbidities (such as asthma and allergy) in developing risk prediction and illness severity models. Future work will ascertain whether the presence of type 2 immune activation and allergic disease prevents the initial development of sepsis-associated organ dysfunction or whether it accelerates resolution of sepsis-associated organ dysfunction. In summary, our findings reveal an unappreciated role for the contribution of type 2 immunity to the inflammatory response during sepsis, thereby representing an exciting new avenue for study of this deadly disease.

Methods

Patient sample collection. For complete details of sample collection and study design, please refer to ref. 8. Samples were collected from patients admitted to the University of Chicago between July 1, 2013, and October 24, 2014. All adult inpatients with at least 1 positive blood culture for *S. aureus* within the previous 4 days were approached for participation. Blood samples were drawn into EDTA tubes at 2 distinct time points (2–4 and 6–9 days after positive culture). It was necessary to include day ranges within time points because there was variability in the time it took blood cultures to turn positive. PBMCs were isolated via differential centrifugation over Histopaque-1077 (Sigma-Aldrich) and samples were cryopreserved until analysis.

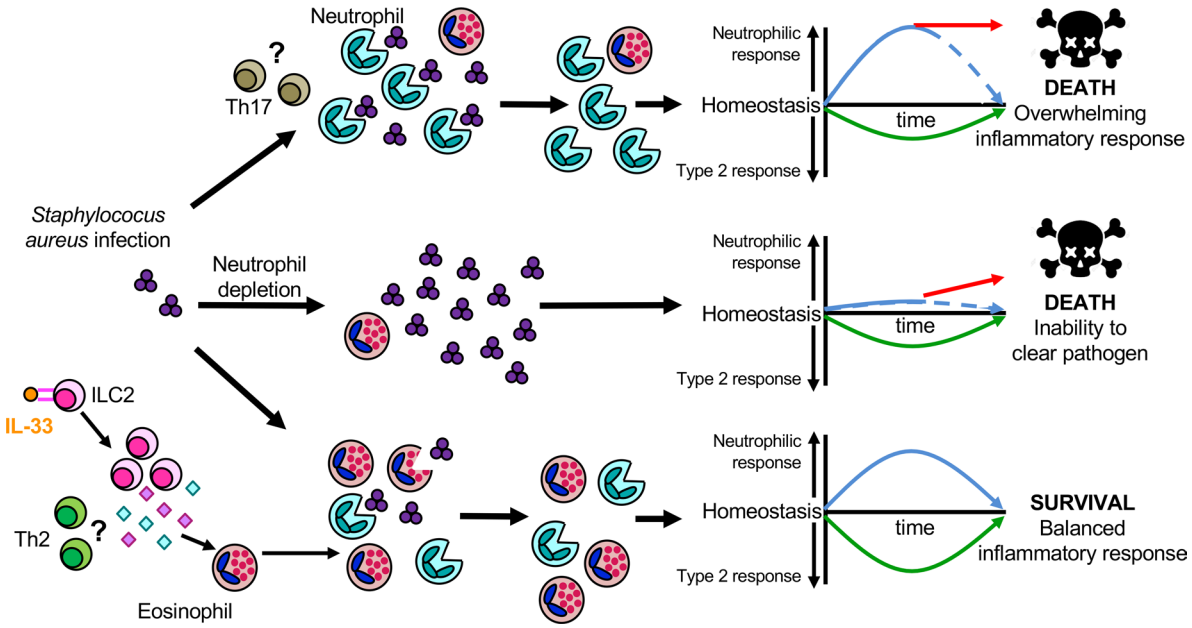

Figure 9. The type 2 immune response protects against systemic *S. aureus*–induced sepsis by balancing overwhelming neutrophilia. Although neutrophils are required for survival during *S. aureus* bacteremia for bacterial clearance, excessive neutrophilia is associated with mortality, perhaps through neutrophil degranulation, neutrophil extracellular trap (NET) formation, and tissue damage. Intratracheal pretreatment with IL-33 induces pulmonary ILC2 proliferation and IL-5 and IL-13 cytokine production, resulting in eosinophilia. Eosinophilia inhibits *S. aureus*–induced neutrophilia, and removal of eosinophils results in overwhelming neutrophilia and death. Thus, we propose that a balanced inflammatory response is crucial to allow the host to clear the infecting pathogen and attenuate tissue damage (modified from ref. 7).

By 30 days after the first positive culture, 8 of the first 65 patients had died. We therefore identified 19 survivors with otherwise similar comorbidities and illness severity (as measured by SOFA score) on whom to perform flow cytometry analysis of PBMC helper T cell subsets to understand immune factors associated with mortality. For this analysis, thawed PBMCs were washed twice in PBS and incubated with a viability dye for 15 minutes (Zombie Aqua, Biolegend). Cells were incubated for 10 minutes with pooled human IgG to block nonspecific antibody binding (FcX, Biolegend), and cell surface staining was performed using fluorescently conjugated antibodies: CD3-FITC (clone HIT3A), CD4-APC (clone OKT4), CCR6-BrilliantViolet605 (clone G034E3), CD45RO-BrilliantViolet711 (clone UCHL1), CD25-APC/Cy7 (clone BC96), CD127-PE/Cy7 (clone A019D5) (all from Biolegend), and CCR4-PE (clone L291H4, BD Biosciences). Flow cytometry data were acquired on an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.). Details regarding the flow cytometry data can be found in reference 8, but briefly, conventional $CD3^+CD4^+$ T cells were first separated from Tregs based on the expression of CD25 and CD127. Conventional T cells were further characterized as $CD45RO^+$, a marker of effector memory T cells, which were then divided into Th subsets using chemokine receptors, with Th2 cells being identified as $CCR4^+CCR6^-$ and Th17 cells as $CCR6^+$ (19). The amounts of Th2 and Th17 lymphocytes were expressed as a percentage of total conventional $CD3^+CD4^+CD45RO^+$ cells.

Because the objective of the current study was to determine whether a Th2 response was protective against mortality associated with an acute episode of bacteremia, we compared the percentage of Th2 lymphocytes in patients who were still alive at 30 days after positive culture with those who had died. We reasoned that patients who died within the first 30 days after *S. aureus* bloodstream infection did so, in part, because of complications of their bacteremia and not secondary infections or comorbidities.

Blood eosinophils and neutrophils were determined from the complete blood count with differential that all patients had drawn on admission.

Mouse studies. C57BL/6J mice were purchased from The Jackson Laboratory and maintained in house. $PLZF^{-/-}$ mice and $CD1d^{-/-}$ mice on a C57BL/6J background were a gift from Albert Bendelac (University of Chicago). iPHIL mice on a C57BL/6J background were a gift from Nancy and James Lee, Mayo Clinic, Scottsdale, Arizona, USA. Female mice, 6–8 weeks old, were used for all experiments. All animals were housed in specific pathogen-free conditions.

Murine model for S. aureus systemic infection. Overnight *S. aureus* cultures were grown in 3 ml of tryptic soy broth until reaching stationary phase (13). A 1:100 dilution of the overnight culture was grown in tryptic soy broth with shaking until reaching an OD between 0.45 and 0.6. Bacteria were washed and resuspended in PBS. For IL-33 pretreatment, mice were intratracheally administered 100 ng of murine recombinant IL-33 (Biolegend) or PBS for 3 days. On day 4, mice were infected with 5×10^7 USA300 CFU/100 μl by retro-orbital injection as previously described (9). For survival analysis, mice were monitored for 7 days after infection, with weight and survival recorded throughout the duration. Lung inflammation was quantified using flow cytometry or histologic analysis. For flow cytometry analysis, mice were euthanized at approximately 18 hours after infection. To quantify CFU levels in the liver, lung, or spleen, mice were perfused with 6 ml of PBS at the indicated times. Tissues were removed and homogenized in 1 ml of PBS. For all tissues, serial dilutions were plated on tryptic soy agar plates and counted. Colony counts were normalized to tissue weight.

Survival analysis using IL-5 and IL-13 administration. PLZF$^{-/-}$ mice were treated with intraperitoneal IL-5 and IL-13 (Biolegend, 1 μg) for 3 days. The following day mice were intravenously infected with 5×10^7 USA300 *S. aureus* and monitored for survival for 7 days after infection.

Flow cytometry analysis. Lungs were processed as previously described (32). Briefly, perfused lungs were minced and digested with 150 U/ml Collagenase D (Gibco) for 1 hour in DMEM supplemented with 5% FCS. Spleens were processed into a single-cell suspension using mechanical dissociation. Red blood cells (RBCs) were lysed using ACK lysis buffer, resuspended in a single-cell suspension, and counted. All antibodies were purchased from Biolegend unless otherwise stated. For all cell populations, 0.5×10^6 to 1×10^6 cells were used for staining, with anti-CD16/32 (clone 2.4G2) used for blocking. Cells were washed with PBS containing 0.1% sodium azide and 0.2% bovine serum albumin.

The following antibodies were used for cell surface and intracellular staining of ILC2s: CD45.2-APC-Cy7 (clone 30-F11), ST2-PE or BrilliantViolet 421 (clone DIH9), Sca-1 PerCP-Cy5.5 (clone D7), CD90.2-785 (clone 30-H12), CD127-PE-Cy7 (clone A7R34) (all Biolegend); IL-5-APC (clone TRFK5), and IL-13-PE (clone eBio13A) (both from eBioscience/Thermo Fisher Scientific). For quantification of ILC2s, the following antibodies were used as lineage markers (all in biotin): CD3 (clone 17A2), CD4 (clone GK1.5), CD8 (clone 53-6.7), NK1.1 (clone PK136), CD49b (clone DX5), CD11b (clone M1/70), CD11c (clone N418), Gr1 (clone RBC-8C5), TCRβ (clone H57-597), and CD19 (clone 6D5) (all Biolegend). For quantification of granulocytes, the following antibodies were used for cell surface staining: CD45.2-APC-Cy7 (clone 30-F11), Ly6G-BrilliantViolet 711 (clone 1A8), CD11b-BrilliantViolet 510 (clone M1/70), CD11c-BrilliantViolet 605 (clone N418), F4/80 FITC (clone BM8) (all Biolegend); and Siglec F-PE (clone E50-2440, BD Biosciences). To identify T cell subsets, the following antibodies were used: CD45.2-APC-Cy7 (clone 30-F11), CD3 APC (clone 145-2C11), CD11c FITC (clone N418), CD11b FITC (clone M1/70), CD19 FITC (clone 6D5), TCRβ PE-Cy7 (clone H57-597), CD8 PerCP-Cy5.5, (clone 53-6.7) (all Biolegend); and TCRγδ-Biotin (clone eBioGL3, eBioscience/Thermo Fisher Scientific). Invariant NKTs (iNKTs) were identified using PE-conjugated CD1d^{PBS57} tetramers (NIH Tetramer Facility at Emory University, Atlanta, Georgia, USA). Streptavidin was used in BrilliantViolet 421 (catalog 405226), BrilliantViolet 510 (catalog 405233), or FITC (catalog 405202; all from Biolegend).

For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) for 30 minutes, followed by the addition of Brefeldin A (1 μg/ml) for an additional 4–5 hours before staining. PMA, ionomycin, and Brefeldin A were purchased from Sigma-Aldrich. For intracellular staining, cells were fixed with 1% paraformaldehyde and permeabilized with BD Biosciences permeabilization buffer. All data were collected using a BD Biosciences LSRFortessa and analyzed using FlowJo software (Tree Star, Inc.). All instruments are maintained by the University of Chicago Flow Cytometry and Antibody Technology Core Facility. Isotype controls, no PMA/ionomycin added, or no anti-cytokine antibody added, were used as negative controls for all cytokine staining.

Histologic analysis. Lungs were perfused and the left lobe was fixed in formalin and used for staining with hematoxylin and eosin (H&E) or PAS. Edema and inflammation were quantified by blindly scoring H&E staining, while mucus accumulation was assessed by evaluating PAS staining. Images were taken and analyzed with Pannoramic Viewer (3DHISTECH). Lung edema was scored as previously described for 1 lung lobe per mouse: 0, no RBCs present; 1, small patch of airway with RBCs infiltrating; 2, one-eighth of the lung lobe contains RBCs; 3, one-fourth of the lung lobe contains RBCs; 4, half of the lobe contains RBCs; and 5, the entire lobe shows RBCs in airways. For all histology scoring, the scorer was blinded to the mouse treatment, and slides were presented in a random order. The same lung lobe was scored for each mouse (32).

Eosinophil depletion. Eosinophils were depleted using DT (Sigma-Aldrich) administration in homozygous iPHIL mice, according to previously published methods (23). iPHIL mice were intratracheally treated with PBS or IL-33 (100 ng) on days –3, –2, and –1. To ensure thorough depletion of eosinophils, mice were administered DT intraperitoneally (15 ng/g body weight) on days –1, 1, 3, and 5. On day 0, mice were infected with 5×10^7 *S. aureus* USA300 CFU/100 μl via retro-orbital injection. Survival analysis was performed until day 7. Flow cytometry was used to confirm that eosinophils were efficiently depleted in the lungs and spleen (data not shown).

Neutrophil depletion. To systemically deplete neutrophils, mice were intraperitoneally injected with the monoclonal Ly6G antibody (0.5 mg/mouse) (clone 1A8 Bio X Cell) 1 day before infection. Control mice received isotype antibody (0.5 mg/mouse) (catalog BP0090, Bio X cell). The following day, mice were infected with 5×10^7 USA300 CFU/100 μl via retro-orbital injection. Survival analysis was performed until 7 days after infection. Flow cytometry was used to confirm that neutrophils were efficiently depleted in the lungs and spleen (data not shown).

Statistics. All statistical analyses were performed with GraphPad Prism software, and P values less than 0.05 were considered significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). For comparisons of 2 groups, an unpaired Student's 2-tailed t test (parametric) or Mann-Whitney test (nonparametric) was performed. To determine whether data were parametric or nonparametric, variances were compared using an F test. For comparisons of 3 or more groups, a 1-way ANOVA with Sidak's multiple-comparisons post hoc test was conducted. Survival analysis was performed using a log-rank (Mantel-Cox) test. Error bars represent ±SEM.

Study approval. For collection of blood samples from patients with *S. aureus* bacteremia, written informed consent was obtained from the patients or their surrogates before their inclusion in the study. The University of Chicago Institutional Review Board approved this study. For all mouse studies, animal procedures and housing were approved by the University of Chicago Animal Resources Center and conformed to the principles set forth by the Animal Welfare Act and the NIH guidelines for the care and use of animals in biomedical research.

Author contributions

PAK, AIS, and PAV conceived and designed the study. PAK, TJL, TSD, TGK, DFC, CLH, and JAG conducted experiments and acquired data. PAK, TJL, CLH, JAG, AIS, and PAV analyzed and interpreted the data. PAK, AIS, and PAV drafted the content of the manuscript. PAK, TJL, TSD, TGK, DFC, JAG, AIS, and PAV approved the final manuscript.

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