CD4$^+$ T Cells Promote the Pathogenesis of *Staphylococcus aureus* Pneumonia

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We postulated that the activation of proinflammatory signaling by methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA300 is a major factor in the pathogenesis of severe pneumonia and a target for immunomodulation. Local activation of T cells in the lung was a conserved feature of multiple strains of *S. aureus*, in addition to USA300. The pattern of V$\beta$ chain activation was consistent with known superantigens, but deletion of SelX or SEK and SEQ was not sufficient to prevent T-cell activation, indicating the participation of multiple genes. Using Rag2$^{-/-}$, Cd4$^{-/-}$, and Cd28$^{-/-}$ mice, we observed significantly improved clearance of MRSA from the airways and decreased lung pathology, compared with findings for wild-type controls. The improved outcome correlated with decreased production of proinflammatory cytokines (tumor necrosis factor, KC, interleukin 6, and interleukin 1$\beta$). Our data suggest that T-cell–mediated hypercytokinemia induced by infection with MRSA strain USA300 contributes to pathogenesis and may be a therapeutic target for improving outcomes of this common infection in a clinical setting.

Keywords. MRSA; *Staphylococcus aureus*; T cell; lung; pneumonia.

*Staphylococcus aureus*, especially the epidemic community-acquired methicillin-resistant *S. aureus* (MRSA) strain USA300, is the major cause of healthcare-associated pneumonia, as well as primary infection and complications of influenza in previously healthy hosts [1–3]. Many cell types participate in the innate immune response to inhaled pathogens in the respiratory tract, usually by activating NF-κB and Jak-STAT signaling in response to recognition of extracellular and endocytosed microbial ligands [4]. *S. aureus* pneumonia is characterized by an especially intense proinflammatory response, dominated by neutrophils and their damaging products. The mechanism by which this response is activated is not fully understood, and the components of immunoregulation are not fully known.

Much of the pathology in staphylococcal lung infection is attributed to an excessive influx of neutrophils and their toxic products [5–7]. It is apparent, though, that in many cases excessive numbers of neutrophils do not correlate with damage and that other host components are involved [7–11].

It is also well recognized that staphylococcal superantigens, especially toxic shock syndrome toxin (TSST-1) and staphylococcal enterotoxin B (SEB), trigger a systemic inflammatory response, contributing to tissue damage as a result of dysregulated T-cell activation and excessive cytokine production [12, 13]. Superantigens function by cross-linking T-cell receptors with major histocompatibility complex (MHC) class II molecules present on antigen-presenting cells, leading to rapid T-cell activation and proinflammatory signaling, which is further exacerbated by expansion of specific T-cell V$\beta$ chain types unique to each superantigen [14, 15]. SEB and TSST-1 have shown promise as vaccine candidates against cognate strains [16, 17].

*S. aureus* expresses several different superantigens with encoded repertoires unique to each strain [18]. *S. aureus* USA300 FPR3757, a sequenced strain, encodes 3 characterized superantigens, SEK, SEQ, and SelX, and has several putative superantigen genes [19, 20]. While each superantigen has exhibited the expected properties in vitro, the contribution of each to the severe pathology of MRSA pneumonia has not been
established. Participation of host T-cell recruitment and signaling in the pathophysiology of acute staphylococcal pneumonia could provide a target to ameliorate the substantial and often irreversible lung injury associated with this infection. Despite the availability of several antibiotics that are highly active against MRSA strains, morbidity and mortality remain high [1]. In other diseases of excessive T-cell signaling, particularly rheumatologic and autoimmune diseases, drugs blocking T-cell activation pathways have been highly effective in preventing pathology [21]. T-cell responses in S. aureus pneumonia, similarly, could be a target of immunomodulatory therapy for these acute and severe infections.

In the experiments described herein, we demonstrate that pathological consequences of T-cell signaling by S. aureus contribute significantly to lung pathology. Mice lacking specific T-cell populations clear S. aureus USA300 infection from the airways much more efficiently than wild-type controls. The participation of S. aureus superantigens in this process is demonstrated, and we identify that T-cell signaling contributes to pathogenesis, representing a potential therapeutic target. These studies begin to explore the potential of host immunomodulation to prevent lung damage in the context of acute S. aureus pneumonia.

MATERIALS AND METHODS

Mice Studies
C57Bl/6J, Rag2−/−, Cd4−/−, andCd28−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Bacteria were grown and mice infected as described previously [8]. CD4+ T cells from lung tissue specimens were isolated by positive selection, using the Dynabeads Flowcomp CD4 kit (Invitrogen). Cytokine levels and analysis of cell populations were quantitated as described before [8].

Construction of a USA300 sekq Mutant
A double knockout of sek and seq was conducted using the pIMAY system [22]. Adjacent regions of sek and seq were cloned into pIMAY, underwent restriction modification using the Escherichia coli SA08B system (Lucigen), and inserted into USA300. Mutants were then generated and selected for as described previously [22].

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Vβ Expression
PBMCs were isolated from whole-blood specimens, using Ficoll-Hypaque density gradient centrifugation (Sigma). Lymphocytes were incubated with filter-sterilized culture supernatant from S. aureus for 11 days. RNA was isolated, and Vβ expression levels were determined using a quantitative real-time polymerase chain reaction method [23].

Histopathological Analyses
Mouse lungs were fixed overnight in 4% paraformaldehyde before being embedded in paraffin. Slides of paraffin sections were stained with hematoxylin-eosin and imaged on an Olympus CX41 microscope, and images were captured using a Canon Powershot 55IS camera.

Statistical Analyses
The statistical significance of data that followed a normal distribution was determined using a 2-tailed Student t test; for data that did not follow a normal distribution, a nonparametric Mann-Whitney test was used. Animal experiments with multiple comparisons were conducted using a Mann-Whitney test with Bonferroni correction. Dichotomous survival was assessed using the Fisher exact test. Statistic analyses were performed with Prism software (GraphPad, La Jolla, CA). Additional information is available in the Supplementary Materials.

RESULTS

S. aureus Strains Activate CD4+ T Cells
To establish whether S. aureus MRSA strain USA300 (the strain responsible for the current MRSA epidemic in the United States) activates T cells, we examined its ability to induce an influx of CD4+ cells into the airway and lung tissue (Figure 1A and 1B). A small but significant influx of CD4+ cells into the airway was observed (Figure 1A), with <1% of cells expressing CD4. This percentage was less than that observed among cells from lung tissue specimens, indicating that T-cell responses at this early stage of infection were likely associated with resident cells in the lung. There was no significant change in CD4+ T-cell numbers in the lung 24 hours after S. aureus inoculation (Figure 1B).

We next screened the CD4+ T cells from the lungs of uninfected and USA300-infected wild-type mice for markers of activation (Figure 1C). There were significant increases in expression of CD25, CD69, and especially CD134, which, on average, increased from 1.3% to 9.8% of CD4+ T cells (P < .001; Figure 1C). Significant increases in activation were also observed in CD4+ T cells isolated from the mediastinal lymph nodes (Figure 1D). The activation was not immediate, with no significant changes in CD69 expression at 4 hours (2.38% of CD4+ T cells), but by 24 hours, CD69 was significantly upregulated (12.56% of CD4+ T cells; P < .01, compared with expression at 4 hours).

To determine whether the activation of T cells was specific to USA300 or a universal characteristic of S. aureus, we examined in vivo the activation of T cells by several different S. aureus strains (Figure 1E). These included community-acquired and hospital-acquired strains, methicillin-resistant and methicillin-susceptible strains, and laboratory and atop dermatitis strains [24–27]. We observed that each strain was able to induce significant activation on CD4 for each of the markers tested. These
data indicate that the activation of T cells in acute pneumonia is a general property of *S. aureus*.

**Activation of CD4+ T Cells Reflects a Response to Staphylococcal Superantigen**

Given the small numbers of T cells and relatively short time to activation, we postulated that this reflected T-cell activation by superantigens known to be expressed in USA300 MRSA [19, 28].

*S. aureus* USA300 expresses several superantigen-like proteins, as well as the superantigens SEK and SEQ [19, 29, 30]. The distribution of the different Vβ types on these activated (CD134+) CD4+ cells in the murine lung was determined, and Vβ 8.1/8.2 and 8.3 were significantly upregulated in response to *S. aureus* infection (range, 0.13%–3% [P < .05] and 0.63%–4.4% [P < .05] of activated CD4+ cells, respectively; Figure 2A); all have been reported to respond to *S. aureus* enterotoxin B, a superantigen [31].

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**Figure 1.** *Staphylococcus aureus* strains rapidly activate T cells. A and B, CD4+ T cells from bronchoalveolar lavage fluid (BALF; A) and lung tissue specimens (B) from uninfected mice or mice infected with 10^7 colony-forming units of *S. aureus* USA300 for 24 hours. ***P < .001, compared with uninfected mice. Each point represents a mouse. Lines display medians. C and D, Percentages of CD4+ T cells in the lung (n = 9; C) and mediastinal lymph node (D), with activation markers as labeled (n = 7, 5, and 10 for the uninfected [UN] group, mice euthanized at 4 hours, and mice euthanized at 24 hours). E, Percentages of CD4+ T cells with positive activation after infection with several different *S. aureus* strains (UN, n = 6; 36–477, n = 9; Newman, n = 8; USA100, n = 8; USA400, n = 8; and USA500, n = 8; wild-type mice were infected with 10^7 colony-forming units for 24 hours). **P < .01 and ***P < .001, compared with uninfected mice. Abbreviation: PBS, phosphate-buffered saline.
also examined the Vβ repertoire on human PBMCs exposed to S. aureus supernatants (Figure 2B). Significant increases in expression of Vβ5, Vβ6, and Vβ18 were observed (Vβ5, 9% vs 55% [P < .001]; Vβ6, 1.4% vs 16% [P < .01]; and Vβ18, 1.9% vs 6.4% [P < .01]) in PBMCs incubated with S. aureus supernatant, compared with controls (Figure 2B). Further experiments using USA300 strains with mutations in sek and seq indicated that Vβ5 expansion was due to SEK, whereas SEQ was responsible for increased Vβ6 and Vβ18 expansion (Figure 2B), consistent with work done using purified SEK and SEQ [23, 32].

Because of the ability of SEK and SEQ to influence Vβ activation, we sought to determine their role in acute pneumonia due to S. aureus USA300. The genes encoding SEK and SEQ are adjacent on the chromosome, and because of their separate roles in Vβ activation we constructed a double mutant [19, 22]. In our model of acute pneumonia, mice infected with sekq did not display differences in bacterial clearance (Figure 2C), cellular recruitment (Supplementary Figure 1A and 1B), or alveolar leakage (Supplementary Figure 1C). We observed a 38% decrease in CD69 expression on CD4+ cells (P < .05; Figure 2D), as well as a 26% reduction in KC (P < .05) in the bronchoalveolar lavage fluid (BALF; Figure 2E), while expression of other cytokines remained unchanged (Supplementary Figure 1D). USA300 S. aureus also expresses a core genome superantigen, SelX, present in non-USA300 isolates [20]. We tested the effects of the wild-type and selX null mutant in our model of pneumonia but found no differences in outcome (data not shown). Thus, we attribute the activation of these subsets of CD4+ T
cells to one or more of the multiple USA300 gene products expected to have superantigen-like behavior as identified by genomic analysis and expected T-cell signaling by antigen-presenting cells [19, 28, 33, 34].

**Rag2^−/−** Mice Have Improved Staphylococcal Clearance

We next examined the host contribution of T cells to pathogenesis in acute staphylococcal pneumonia. *Rag2^−/−* null mice that do not produce B or T cells were infected with USA300. *Rag2^−/−* mice had a significantly reduced bacterial burden in both the airway (BALF, 6.3-fold reduction; *P < .001) and lung tissue (3.5-fold reduction; *P < .05; Figure 3A and 3B), compared with infected wild-type mice. Consistent with the improved clearance, at a higher inoculum *Rag2^−/−* mice were protected against mortality (Figure 3C). Although there was significantly enhanced staphylococcal clearance, the USA300-infected *Rag2^−/−* mice displayed markedly reduced inflammation (Figure 3E). As an indicator of reduced lung damage, total protein levels in BALF were reduced by 32% in *Rag2^−/−* mice, compared with infected wild-type mice (*P < .05; Figure 3D). Histopathological examination of lung tissue from infected wild-type mice demonstrated loss of alveolar architecture, hemorrhage, and consolidation of lung parenchyma (Figure 3E), in contrast to the sections from the *Rag2^−/−* mice, which displayed considerably reduced levels of consolidation and hemorrhage (Figure 3E).

The phenotype observed with the *Rag2^−/−* mice was not due to a compensatory change in the types of cells recruited into the airways. Classification of these immune cells in BALF by flow cytometry showed no significant differences in numbers of neutrophils, macrophages, or natural killer (NK) cells in wild-type and *Rag2^−/−* mice (Figure 4A). Although macrophages can express CD4, we did not observe differences in macrophage numbers [35]. We did observe a significant albeit slight increase in the number of dendritic cells in the BALF of infected *Rag2^−/−* mice, but numbers of dendritic cells in this model have not been found to correlate with improved host outcome [36]. No
significant differences in immune cell populations in lung tissues were observed (Figure 4B).

Quantification of the proinflammatory cytokines tumor necrosis factor (TNF), KC, and interleukin 6 (IL-6) in the airway fluid revealed significant reductions (by 50% [P < .001], 85% [P < .01], and 52% [P < .01], respectively) in S. aureus–infected Rag2−/− mice (Figure 3F). The T-helper type 17 (Th17) cytokine interleukin 17 (IL-17) was completely abrogated (P < .001), while an increase in the Th1 cytokine IFN-γ was observed (Figure 3F), although no role for IFN-γ in staphylococcal pneumonia has been reported [37]. Levels of cytokines involved in chemotaxis and cytotoxicity (CXCL10 and granzyme B) were similar in wild-type and Rag2−/− mice (Figure 4C). The significant reduction in IL-17 and TNF levels further suggested that this cytokine effect was T-cell associated.

**CD4+ T Cells Contribute to S. aureus–Induced Pathology**

To distinguish which T-cell population was involved in exacerbating the pathology associated with acute S. aureus pneumonia, we studied wild-type and Cd4−/− mice. Cd4−/− mice also exhibited improved outcomes in our model of acute pneumonia. There was significantly improved clearance of S. aureus USA300 from both BALF (5-fold; P < .05) and lung tissue (4-fold; P < .05) in Cd4−/− mice, compared with wild-type mice (Figure 5A and 5B). The infected lungs of Cd4−/− mice also had reduced inflammation. The total protein level in BALF was significantly less than in the wild-type mice, as was consolidation of the lung parenchyma, demonstrated by histopathological analysis (Figure 5C and 5D). We excluded the possibility that the improved outcome in Cd4−/− mice was due to a compensatory increase in another cell population in the airway or lung tissue, analogous to results obtained with the Rag2−/− mice (data not shown).

To determine whether the improved outcome of the Cd4−/− mice was due to differences in cytokine abundance, we examined levels of several cytokines in the BALF. The proinflammatory cytokines TNF, KC, and IL-6 were all significantly reduced in S. aureus–infected Cd4−/− mice (by 69% [P < .01], 86% [P < .01], and 61% [P < .05], respectively), compared with wild-type controls (Figure 5E), similar to what was observed with

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**Figure 4.** Wild-type (WT) and Rag2−/− mice display equivalent cell recruitment to the airway and lung tissue after infection with *Staphylococcus aureus* USA300. A and B, Cells from bronchoalveolar lavage fluid (BALF; A) and lung tissue specimens (B) were stained and analyzed by flow cytometry. Each point represents a mouse. Data are from 3 independent experiments. Lines display medians. *P < .05, compared with WT mice. C, Proinflammatory cytokine production by Rag2−/− mice. Mice were infected with *S. aureus* for 24 hours before BALF samples were collected and cytokine levels measured using an enzyme-linked immunosorbent assay. Each point represents a mouse. Data are from 3 independent experiments. Lines display medians. Abbreviations: NK, natural killer; PBS, phosphate-buffered saline.
Rag2\(^{-/-}\) mice (Figure 3G). Levels of IL-17 were significantly reduced (by 47%; \(P < .05\)) but not as completely as in Rag2\(^{-/-}\) mice, likely because of production from other cell types, such as \(\gamma\delta\) T-cells and NK T cells [38]. In this model, \(\gamma\delta\) T-cells do not appear to play a role since \(Cd4^{-/-}\) mice (which have \(\gamma\delta\) T-cells) have a phenotype analogous to that of Rag2\(^{-/-}\) mice.

**T Cells Contribute to Cytokine Production**

Cytokine arrays were performed on BALF from wild-type, Rag2\(^{-/-}\), and \(Cd4^{-/-}\) mice that had been infected with *S. aureus*. In Rag2\(^{-/-}\)-infected mice, there was a general diminution of cytokine expression, compared with the wild-type controls (Figure 5G). A slightly smaller yet largely overlapping subset of cytokines was also reduced in \(Cd4^{-/-}\) mice, compared with wild-type mice (Figure 5F).

We further identified the cytokines contributed by CD4\(^{+}\) T cells. We positively isolated CD4\(^{+}\) T cells from uninfected and infected mice and noted significant induction of several cytokines (Figure 5F and 5G). A subset of those produced (Figure 5F) were seen as reduced in the T-cell knockout mice: interleukin 1\(\beta\) (IL-1\(\beta\)), macrophage inflammatory protein 1\(\alpha\) (MIP-1\(\alpha\)), MIP-2, KC, interleukin 2 (IL-2), interleukin 1\(\alpha\), interleukin 16, granulocyte macrophage colony-stimulating factor, and TNF. These data demonstrate that CD4\(^{+}\) T cells are...
significant contributors to the acute inflammatory response to 
*S. aureus* respiratory infection.

**T-Cell Signaling Through CD28 Contributes to MRSA Pneumonia**

In addition to interaction with MHC class II molecules, T cells require a second so-called hit to be stimulated; this occurs via the receptor CD28. Staphylococcal superantigens are known to interact with CD28 [39–41], and CD28 null mice are protected against toxic shock syndrome [42]. Antigen-presenting cells interact with CD28 via CD80/CD86, which is balanced via the negative regulatory pathway by means of the CTLA-4 receptor [43]. We used the *Cd28−/−* mouse to examine the effect of preventing T-cell costimulation [39–41]. *Cd28−/−*–infected mice had 58% fewer bacteria (*P* < .01; Figure 6A) in their lungs and decreases in all CD4 activation markers (Figure 6B). We observed significant reductions in levels of proinflammatory cytokines (Figure 6C). TNF levels were reduced by 51% (*P* < .01), IL-6 levels were reduced by 40%, IL-1β levels were reduced by 53% (*P* < .05), and KC levels were reduced by 60% (*P* = .0541) in *Cd28−/−* mice, compared with USA300-infected wild-type mice (Figure 6C). These reductions in bacterial and cytokine production were evident in lung sections from wild-type and *Cd28−/−*–mice (Figure 6D). Lungs from *Cd28−/−*–infected mice showed significantly less consolidation and improved alveolar architecture. We observed some modest changes in cell populations (Supplementary Figure 2B). Thus, activation of the costimulatory T-cell receptor is a major contributing factor to acute inflammation in response to MRSA infection.

**DISCUSSION**

We analyzed a mouse model of acute *S. aureus* pneumonia in multiple knockouts and demonstrated that activation of resident pulmonary CD4+ T cells has a major role in the pathogenesis of acute USA300 pneumonia contributing to a cytokine storm and severe pulmonary pathology [44]. In these mouse...
models, as is also often the case in staphylococcal pneumonia in humans, the overexuberant proinflammatory response to these organisms that is activated early in pathogenesis is often lethal; animals that survive the first 24 hours generally clear the infection. While there are well-recognized limitations to murine models of *S. aureus* pneumonia, the ability to manipulate specific populations of immune effectors and monitor outcome provides the opportunity to define potential targets of deleterious host responses to infection.

Much of the pathology in staphylococcal lung infection is attributed to the influx of neutrophils and their associated toxic products [5–7]. We observed comparative levels of neutrophils and other leukocytes in both wild-type and knockout infected mice. Our data suggest that T-cell signaling is responsible for much of this early lung damage in the setting of *S. aureus* USA300 infection, an exceptionally virulent pathogen [1, 45]. Although some studies have shown that products of CD4+ T cell cells, such as IL-17 [37], are important in acute pneumonia or other models of infection (eg, central nervous system infection) [46], our study shows that the collective influence of CD4+ T cells contributes early in infection. We also observed that IL-17 was still produced in *Cd4−/−* mice, likely because of γδ T cells, which have been shown to play a role in *S. aureus* pneumonia [47]. The activation of T cells was a conserved attribute across several different strains of *S. aureus*. The pathologies that we observed—increased pulmonary edema and loss of normal lung architecture—are consistent with local effects of the increased cytokines associated with these activated T cells and a cytokine storm [44]. The cytokines involved, IL-2, TNF, KC, IL-1β, MIP-1α, and MIP-2, which were present in lower levels in knockout mice, were produced by activated CD4+ T cells and correlated with poor outcome, as supported by other studies on type I interferon–regulated T-cell cytokines [8, 36, 48]. The toxicity of these cytokines interferes with the efficient clearance of pathogens from the airways.

The systemic expression of *S. aureus* superantigens is a well-established mechanism of severe host damage and is best illustrated by the morbidity and mortality associated with TSST-1 expression and cytokine storms [49]. Although USA300 lacks the best-characterized superantigens, TSST-1 and SEB, its expression of multiple open reading frames with superantigen properties contributes to its virulence as a pulmonary pathogen. The T-cell activation observed in the lung within a short time frame of 24 hours is likely the result of superantigens. USA300 has >16 open reading frames predicted to encode superantigens [19], and mutations in the superantigen SelX and even an SEK/SEQ double-knockout were not attenuated in their virulence, although some decreased cytokine production was observed. It seems likely that, because of the redundancies in superantigen production, any single mutation may not be sufficient to alter the overall virulence phenotype. We did note the ability of USA300 to stimulate Vβ chain expression consistent with the domains recognized by SEK and SEQ on human PBMCs, which confirms the potential to USA300 to signal through this mechanism in humans.

Superantigen-mediated inflammatory signaling mediated by the abundance of host T cells with Vβ chains recognized by USA300 superantigens could help explain the variability in patient outcomes to USA300 pneumonia. We predict that patients with greater numbers of T cells bearing the appropriate SEK/SEQ or other USA300 superantigen–specific Vβ chains would be at greater risk for severe pathology due to T-cell activation and cytokine production. Modulation of the host’s ability to respond to superantigen stimulation by deleting the costimulatory receptor CD28 recapitulated the improved outcome seen in the *Cd4−/−* and *Rag2−/−* null mice, consistent with the potential involvement of superantigens and a role for T-cell signaling in the pathogenesis of *S. aureus* pneumonia.

The recognition that T-cell activation and associated cytokine production contribute substantially to lung pathology in the setting of acute MRSA pneumonia provides a potential target for immunomodulatory therapy. Many conditions and diseases involving excessive T-cell activity are managed by modulating the signaling pathways that control T-cell activation, including organ transplantation, rheumatologic disease, and autoimmune disease. Peptides to inhibit CD28 have had efficacy in models of toxic shock [50]. CTLA-4, the coinhibitory receptor that responds to CD80/CD86 on antigen-presenting cells, regulates the activation status of CD28 [21]. The relative affinity of the staphylococcal superantigens for CD28 appears to be the predominant factor in their activation.

These observations suggest that, in the setting of acute MRSA pulmonary infection, limiting the initial participation of activated T cells should improve outcome in the context of acute infection. The failure of conventional vaccination strategies based on highly expressed and antigenic staphylococcal virulence factors suggests that innate immunity, as opposed to adaptive immunity, may predominate in maintaining protection against this ubiquitous commensal pathogen. Thus, treatment that targets potentially deleterious host responses to MRSA in the lower airway that induce a local cytokine storm may be a useful adjuvant to antimicrobial therapy.

### Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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References


