Participation of CD11c⁺ Leukocytes in Methicillin-Resistant *Staphylococcus aureus* Clearance from the Lung[▽]

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Staphylococcus aureus causes especially severe pulmonary infection, associated with high morbidity and mortality. In addition to the effects of specific virulence factors, it appears that the intensity of the host proinflammatory response, particularly in the initial stages of infection, contributes substantially to pulmonary damage. We tested the hypothesis that the CD11c⁺ leukocytes are important in the host response to pulmonary infection with methicillin-resistant *S. aureus* (MRSA) USA300. Clodronate-induced depletion of the alveolar macrophage population resulted in increased numbers of dendritic cells (DCs) and CD4⁺ cells in bronchoalveolar lavage (BAL) fluid and was associated with significantly increased mortality by 18 h following *S. aureus* inoculation but had no effect on bacterial load or polymorphonuclear leukocyte (PMN) numbers in the lung. These clodronate-treated mice also had increased expression of interleukin-17A/F (IL-17A/F) and CXCL10 but not of gamma interferon (IFN- γ) or tumor necrosis factor (TNF). Depletion of the dendritic cell population in mice expressing a CD11c-enhanced green fluorescent protein (EGFP)-diphtheria toxin receptor (DTR) transgene was associated with an increased bacterial load in the lung but not increased mortality. Both DCs and airway epithelial cells produced CXCL9, -10, and -11 in response to *S. aureus*. Pretreatment of mice with an anti-CXCR3 antibody prior to inoculation with MRSA substantially reduced CD4⁺ cells and decreased pulmonary inflammation at 18 h postinfection compared to pretreatment with an IgG control. The results of these experiments suggest that CD11c⁺ cells, the induction of CXCR3 ligand expression, and subsequent CD4⁺ cell recruitment have an important role in the pathogenesis of severe MRSA pulmonary infection.

Staphylococcus aureus bacteria, particularly the epidemic methicillin-resistant USA300 strains, have recently been associated with severe invasive pneumonias in previously well individuals, as well as being a complication of influenza (15). These pneumonias are characterized by a predominant polymorphonuclear leukocyte (PMN) response with significant areas of necrosis and hemorrhage (6). Numerous studies have addressed which of the several virulence factors expressed by the USA300 strains are most important in the pathogenesis of pulmonary infection. In the C57BL/6J mouse model of acute pneumonia, the contributions of α -hemolysin (Hla) (3) and protein A (SpA) (8) have been well documented. The importance of Panton-Valentine leukocidin (PVL) has also been confirmed in a rabbit model of infection; rabbits, like humans, have highly PVL-susceptible leukocytes (6). Besides the direct toxicity of staphylococcal toxins, some of the pathological consequences of staphylococcal virulence factors are due to the intensity of the host immune response. Both *Tnfr1*^{-/-} (8) and *Ifnar*^{-/-} (17) mice are highly resistant to *S. aureus* pneumonia and even to the USA300 strains expressing these virulence factors, suggesting that the intensity of the host immune response to infection is critical in the pathogenesis of severe pneumonia.

Exactly which components of the innate immune response contribute to lethality in these models of acute *S. aureus* pneumonia has not been clearly delineated. PMNs have long been

thought to be critical for efficient staphylococcal clearance (19, 22), and the increased incidence of severe staphylococcal infection in humans with disorders of PMN function or activation, such as chronic granulomatous disease (10, 12), confirm this observation. PMNs themselves can also contribute to the pathology associated with staphylococcal infection. A substantial component of the lung pathology associated with *S. aureus* USA300 pneumonia has been attributed to the PVL-associated release of cytotoxic components from PMN granules (5). In models of soft tissue infection, the importance of T cells and local CXC chemokine production in PMN recruitment in response to *S. aureus* has been demonstrated (18). These studies suggest that CXC chemokines, while increasing PMN accumulation at the site of infection, may decrease the ability of the recruited PMNs to kill ingested *S. aureus*. Studies of acute pulmonary inflammation have indicated a role for the CXCR3 chemokines, and CXCL10/IP-10 specifically, in directing the chemotaxis and activation of T cells and associated pathology in the lung (11, 16, 25).

Defects in human Th17 signaling are also associated with immunodeficiency syndromes characterized by increased susceptibility to infection due to staphylococci and other pyogenic bacteria (4). Cytokines more typically associated with T cell signaling cascades are important in the clearance of extracellular bacteria from the airways. Mice with mutations in the Th17 cascade (interleukin-17A [IL-17A] or -F and IL-22) appear to have impaired staphylococcal clearance from the lung, despite increased cytokine production and increased inflammation (1). In contrast, the type I interferon (IFN) cascade contributes directly to pulmonary pathology in response to *S. aureus*. *Ifnar*^{-/-} mice had significantly increased survival following infection with an *S. aureus* USA300 inoculum that

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caused 80% mortality in wild-type mice (17), although the differences in mortality were not associated with increased (or decreased) numbers of PMNs. Thus, the participation of additional leukocyte populations that are recruited and activated in the initial stages of staphylococcal pneumonia may be critical in determining the outcome of the infection.

In the experiments described in this report, we manipulated leukocyte populations in the murine lung that we postulated would be important in clearing *S. aureus* and characterized the subsequent response to USA300 infection. While the accumulation of CD11c⁺ cells, both dendritic cells (DCs) and macrophages, in the lung contributed to bacterial clearance, increased numbers of DCs, CXCR3 chemokine production, and CD4⁺ T-cell recruitment were associated with fatal outcomes, suggesting that the early production of CXCR3 ligands may exacerbate the inflammation typical of severe *S. aureus* pneumonia.

MATERIALS AND METHODS

Pneumonia model. C57BL/6J mice and mice expressing a CD11c-enhanced green fluorescent protein (EGFP)-diphtheria toxin receptor (DTR) transgene [B6.FVB-Tg (Itgax-DTR/EGFP)57Lan/J] were obtained from the Jackson Laboratories, genotyped, and bred or held in-house for 1 week prior to experiments. Seven- to 10-week-old male and female mice were anesthetized with ketamine (10 µg/ml) and xylazine (0.5 µg/ml) and intranasally inoculated with 10⁷ to 10⁸ CFU of logarithmically growing *S. aureus* strain USA300(LAC) in PBS. After 16 to 24 h, mice were euthanized by CO₂ asphyxiation. Bronchoalveolar lavage (BAL) fluid (3 1-ml amounts of Hanks' balanced salt solution [HBSS] instilled intratracheally), lungs, and spleens were collected, and the bacterial burden was determined by plating serial dilutions of BAL fluid or organ homogenate on Luria-Bertani agar. CXCR3 was blocked by intraperitoneal application of 80 µg Armenian hamster anti-mouse CXCR3 antibody (BioLegend) 16 h before infection. Normal hamster IgG was used as a negative control. A lactate dehydrogenase (LDH) assay was performed on BAL fluid according to the manufacturer's instructions (Roche). Total protein in BAL fluid was determined by using Bradford reagent. All animal experiments were approved by the institutional animal care and use committee of Columbia University.

Lung macrophage and dendritic cell populations were depleted by intranasal application of 50 µl clodronate liposomes or 100 ng diphtheria toxin (DT), respectively (9, 21). Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany, to Nico van Rooijen and was packaged in his laboratory at Vrije Universiteit, VUMC Amsterdam, Netherlands. Phosphate-buffered saline (PBS)-liposomes or PBS alone were used as negative controls. Mice were infected 16 to 24 h after application of the depletion agent, and mice were sacrificed 16 to 24 h after infection. Cell depletion was verified by flow cytometry.

Flow cytometry. Samples of lung single-cell suspensions or BAL fluid were washed in PBS, subjected to red blood cell (RBC) lysis, and blocked with Fc Block (BD Biosciences) as previously described (17). The cells were stained with fluorochrome-labeled antibodies to Ly6G (clone RB6-8C5), CD11c (clone N418), major histocompatibility complex class II (MHCII) (clone M5/114.15.2), CD4 (clone GK1.5), and/or CD8 (clone H35-17.2) from eBiosciences. After washing with PBS and fixation in 4% paraformaldehyde, the samples were read on a FACSCalibur or LSRII (BD) running FACSDiva software. The FlowJo software package was used for analysis and quantification of appropriate cell populations after gating for forward scatter and side scatter characteristics. Absolute cell numbers were determined by using Dragon green fluorescent microspheres (Bangs Laboratories) as an internal calibrator.

Cytokine analysis. IL-17A/F, CXCL10, tumor necrosis factor (TNF), and gamma interferon (IFN-γ) levels were detected in individual BAL fluid samples by enzyme-linked immunosorbent assay (ELISA) using reagents from eBiosciences.

Real-time PCR. RNA was isolated by using a Qiagen RNeasy mini kit and subjected to DNase treatment according to the manufacturer's instructions. cDNA was made from 1 µg of RNA by using an iScript synthesis kit (Bio-Rad). For quantitative real-time PCR, amplification was performed with Power SYBR green master mix in a Step One Plus thermal cycler (real-time PCR system; Applied Biosystems).

Histopathology. Mouse lungs were fixed overnight in 4% paraformaldehyde before being embedded in paraffin. For histology, lung sections were stained with hematoxylin and eosin (H&E) and imaged on an Olympus Cx41 microscope with attached Canon Powershot S5IS camera.

Cell culture. Dendritic cells were generated from bone marrow isolated from femurs and tibias. DCs were cultured for 7 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, streptomycin, penicillin, and 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech). Murine nasal epithelial cells in primary culture were grown as described previously (17).

Statistical analyses. Sample data with normal distribution were analyzed using Student's *t* test. Sample data that did not follow normal distributions were analyzed with the nonparametric Mann-Whitney test. Data from mortality studies were subjected to Fisher's exact test.

RESULTS

Clodronate-treated mice are highly susceptible to *S. aureus* pneumonia. Alveolar macrophages are often considered the major surveillance cell type in the lung, available to respond immediately to inhaled pathogens. We used clodronate treatment to deplete these actively phagocytic cells, and then characterized the responses to acute USA300 infection of the clodronate-treated mice compared with those of mice treated with PBS containing liposomes. The clodronate-treated mice had a significant reduction in the macrophage (MHCII⁺ and CD11c⁺) population of lymphocytes and a concomitant increase in the proportion of DCs (MHCII⁺ CD11c⁺), as has been demonstrated previously by others (13) (Fig. 1A). Following a sublethal *S. aureus* infection (10⁷ CFU), there were no significant differences in bacterial counts in the lung, BAL fluid, or spleen of the clodronate-treated mice and the PBS-treated controls (Fig. 1B). As expected, there were significantly fewer alveolar macrophages in the lungs (Fig. 1C), similar numbers of PMNs (Fig. 1D), and significantly increased numbers of DCs (Fig. 1E). The clodronate-treated mice also had increased numbers of CD4⁺ T cells (*P* = 0.0513) but not of CD8⁺ cells (Fig. 1F and G). When challenged with a 10-fold-higher dose of USA300 (10⁸ CFU), the clodronate-treated mice had significantly increased mortality, close to 90% compared with the 10% mortality of the untreated controls (Fig. 2; *P* < 0.0001).

BAL fluid analysis from *S. aureus*-infected, clodronate-treated mice demonstrated significantly increased levels of CXCL10 (*P* = 0.0078) and IL-17A/F (*P* = 0.0384) but no significant differences in IFN-γ or TNF levels (Fig. 3) compared to the results for untreated infected mice. Controls treated with clodronate liposomes but not infected had between 1/10 and 1/3 the level of cytokine induction in the infected mice (data not shown). The upregulation of DC-T cell signaling, with significantly increased numbers of DCs (CD11c⁺/MHCII⁺), CD4⁺ T cells, and amounts of the T cell chemokine CXCL10, suggested that DC-CXCL10 production may contribute to the excessive mortality in the clodronate-treated mice.

Depletion of CD11c⁺ cells does not increase mortality from *S. aureus* pneumonia. The contribution of DCs (CD11c⁺ cells, including inflammatory monocytes) to the response to USA300 pulmonary infection was studied by comparing the responses of mice expressing the CD11c-EGFP-DTR transgene following treatment with diphtheria toxin (DT) or with PBS (21). Depletion of the CD11c⁺ population was evaluated by flow

FIG. 1. Effects of clodronate on the immune response to *S. aureus* USA300 pulmonary infection. (A) Depletion of the CD11c⁺ population of lymphocytes resulted in a 6-fold increase in the CD11c⁺MHCII⁺ cells (DCs) in BAL fluid, as illustrated by flow cytometry analysis, in response to the intranasal administration of clodronate liposomes. Axes display raw intensity values. (B) Levels of *S. aureus* (CFU) recovered from lung lysate, BAL fluid (BALF), and spleen of clodronate-treated (Clod) and PBS-treated mice. (C to G) Levels of alveolar macrophages (C), polymorphonuclear monocytes (PMNs) (D), DCs (E), CD4⁺ cells (F), and CD8⁺ cells (G) in BAL fluid following intranasal inoculation of 10^7 CFU *S. aureus* USA300. Mice were given clodronate and, 16 to 24 h later, infected for 16 to 24 h with *S. aureus*. Each dot represents the results for an individual mouse. Horizontal bars show the median values for the groups. Data from two independent experiments are shown.

cytometry confirming the effect on the MHCII⁺/CD11c⁺ dendritic cell population (Fig. 4A). At 18 h postinfection (10^7 CFU), there was no DC response in the DT-treated animals and no differences in the numbers of macrophage (CD11c⁺/MHCII⁺) cells, but there were significantly fewer PMNs (Ly6G⁺/CD11c⁺) (Fig. 4B). There were significantly higher bacterial counts in the lungs ($P = 0.0023$) and BAL fluid ($P = 0.0082$) but not in the spleens of the DT-treated mice (Fig. 4C). The CD11c⁺-depleted mice had significantly decreased CXCL10 ($P = 0.0015$) but no differences from the levels of IL-17A/F or IFN- γ and TNF cytokines in the controls (Fig. 3). Mortality was assessed in mice treated with a higher inoculum (10^8 CFU) and did not differ in the mice lacking the CD11c⁺ population of lymphocytes (Fig. 2).

Induction of CXCR3 ligands in response to *S. aureus*. The association of increased amounts of the CXCR3 chemokines, particularly CXCL10, and poor outcome in response to staphylococcal pneumonia was noted in the experimental groups. CXCR3 is widely expressed in the lung, available on lymphocytes, NK cells, DCs, and epithelial cells. Exposure of either DCs or airway epithelial cells to *S. aureus* significantly stimulated the expression of CXCR3 chemokines (Fig. 5). In the clodronate-treated group, we noted an increase in the CD4⁺ population in the lung by 18 h postinfection (Fig. 1). FACS analysis of BAL fluid from these mice indicated that less than 10% of the CD4⁺ cells at 18 h postinfection were also CXCR3⁺ (data not shown), suggesting that additional lymphocyte populations are responding to these chemokines. Thus, *S.*

FIG. 2. Mortality associated with USA300 infection in mice with manipulated populations of leukocytes. Percent mortality at 18 h following an intranasal inoculation of 10^8 CFU *S. aureus* USA300 in untreated control C57BL/6J (Un) mice, CD11c-EGFP-DTR mice treated with diphtheria toxin (DT), and mice treated with clodronate liposomes (Clod). Data are combined from at least two independent experiments.

aureus induces DC and epithelial production of the CXCR3 chemokines that in turn activate several cell types with the corresponding CXCR3 ligand receptors.

CXCR3 ligands contribute to staphylococcus-induced pathology in the lung. The CXCR3 chemokines have been associated with inflammation in models of immune-mediated pneumonia (11, 16). Our data suggested that production of the CXCR3 ligands in response to *S. aureus* infection contributes to the excess pathology associated with staphylococcal pneumonia. To test this hypothesis, we monitored the effects of pretreating mice with anti-CXCR3 antibody or a control IgG prior to infection with 10^7 CFU of *S. aureus* (Fig. 6). Although there was no difference in PMN numbers in the BAL fluid (Fig. 6A) or bacterial counts in the lung (Fig. 6C), there was a significant decrease in the numbers of CD4⁺ cells in the BAL fluid ($P = 0.0379$) (Fig. 6B). There were also increases in total protein and LDH in the CXCR3-treated mice (Fig. 6D and E). Routine H&E staining of the lungs indicated substantial protection associated with the anti-CXCR3 antibody but not the IgG control (Fig. 6F); there was preservation of the airspaces, less edema, and less cellular infiltrate in the anti-CXCR3-pretreated lungs. These findings are consistent with the observations that leukocyte populations that contribute to CXCR3 chemokine expression can be associated with excessive inflammation in response to *S. aureus* infection in the lung.

DISCUSSION

S. aureus causes an especially severe pneumonia, marked by an intense proinflammatory response that interferes with respiration and causes excessive mortality (15). While PMNs are required to effect bacterial clearance (19), accumulating data suggest that the regulation of PMN recruitment and their activation status may be critical in determining the outcome of the *S. aureus* infection (17, 18). In our studies, significantly increased mortality was observed in clodronate-treated mice; these mice had an increased DC population, increased CXCR3 ligand expression, and increased CD4⁺ population, as well as decreased macrophages. When CXCR3 was blocked, decreased CD4⁺ cell recruitment and lung pathology were ob-

FIG. 3. Cytokine and chemokine analysis of BAL fluid harvested from clodronate-treated (Clod) and DT-treated mice. Amounts of CXCL10, IFN-γ, IL-17A/F, and TNF were determined by ELISA of BAL fluid samples from USA300-infected mice. Each dot represents the results for one mouse, and the horizontal lines depict the median value for each group. Un, untreated controls.

served. Since many cells in the lung (DCs, T cells, and epithelial cells) express CXCR3 (16), it is likely that several distinct cell types respond to the CXCR3 ligands and contribute to the pathological inflammation elicited by USA300 infection.

The regulation of inflammation in the response to bacterial infection in the lung is especially crucial, as there is a narrow range of PMN recruitment that is sufficient to clear a bacterial inoculum but not so great as to cause fatal pneumonia. Since there are many redundancies in the innate immune response to bacterial pathogens in the lung, we did not expect to see a single leukocyte population as critical in staphylococcal clearance. For example, depleting macrophages with clodronate resulted in increased numbers of DCs, as has been reported by others (13). Thus, it is difficult to link a single population of immune cells with pathological responses to infection. We found that mortality from *S. aureus* pneumonia correlated with increased numbers of DCs, increased production of CXCR3 chemokines, and CD4⁺ T cell recruitment into the lungs but not with the numbers of PMNs recruited into the airways or lungs. Blocking CXCR3 in clodronate-depleted animals did not reduce lethality (data not shown), indicating the complexity of the signaling involved. The association of excess DC signaling and pathology is apparently not limited to the response to *S. aureus*, as an expanded DC population caused excess pathology in a similar murine model of pneumococcal pneumonia (24).

The role of CXC chemokines in the pulmonary pathology associated with *S. aureus* infection has been previously described (2, 7). These investigators demonstrated that *S. aureus* alpha-toxin, which is expressed by the USA300 strain used in our studies, induces the expression of the CXC chemokines and the associated pathology (2). Exactly how alpha-toxin, which binds to ADAM-10, activates these chemokines is not

FIG. 4. Effect of CD11c⁺ depletion on the response to USA300 infection. (A) Representative flow cytometry scatter plots from CD11c- and MHC class II-stained BAL fluid samples from mice expressing a CD11c-EGFP-DTR transgene treated with DT or PBS. Axes display raw intensity values. (B) The numbers of alveolar macrophages, polymorphonuclear leukocytes, and dendritic cells in the BAL fluid of mice were determined by flow cytometry. (C) Bacterial counts were determined in lung, BAL fluid (BALF), and spleen of DT- or PBS-pretreated transgenic mice. Each dot represents the results for one mouse, and the horizontal bars shown the median value for each group. Representative data from three independent experiments are shown.

clear (23). *In vitro* studies have demonstrated induction of CXC chemokine expression by airway epithelial cells in response to *S. aureus* and their function to upregulate CXCR3 expression and the subsequent induction of T cell chemotaxis (7). Our model system similarly finds that increased amounts of the CXCR3 chemokines are especially linked to pulmonary pathology. As several different cell types in the lung respond to

the CXCR3 ligands, including the abundant epithelial cells, it is difficult to link excessive inflammation with a single cell type.

The apparently deleterious effects of excessive CXCR3 chemokine production in the lung is in contrast to what occurs systemically, as studied in models of sepsis. The administration of Fms-like tyrosine kinase 3 ligand (Flt3L) in a thermal injury model of sepsis increased DC numbers and chemokine expression, resulting in improved bacterial clearance, both locally in the burn wound and in the blood (20). In the setting of polymicrobial sepsis from cecal ligation and puncture, type I IFN signaling and its positive regulation of CXCL10 expression were critical in stimulating PMN phagocytosis and enhancing survival (14). The rate of mortality in IFNAR null mice from polymicrobial sepsis could be reversed by CXCL10 treatment. However, as the IFNAR null mouse is substantially protected from *S. aureus* pneumonia (17) and has diminished T cell accumulation and CXCL10 expression (A. Prince, unpublished data), it appears that the consequences of CXCR3 accumulation may be less beneficial in the lungs as the primary site of infection.

The contribution of other cytokine signaling cascades, particularly IL-22 and IL-17A/F, while undoubtedly contributing to host-pathogen interactions in the setting of staphylococcal pneumonia, did not consistently correlate with either staphylococcal clearance or lethality. Since the model of infection

FIG. 5. *S. aureus* USA300 induces CXCR3 ligand expression. Real-time PCR was performed, demonstrating the induction of CXCR3 ligands in response to USA300 in bone marrow-derived dendritic cells (A) and murine primary nasal epithelial cells (B). Data shown are representative of two independent experiments ($n = 3$). Graphs show the mean values with standard deviations. *, $P < 0.05$ compared to results for unstimulated controls.

FIG. 6. CXCR3 blockade decreases pulmonary inflammation. C57BL/6J mice were pretreated with either anti-CXCR3 or an irrelevant IgG prior to intranasal infection with 10^7 CFU of USA300. (A and B) BAL fluid was analyzed by FACS to determine PMN (A) and CD4⁺ cell (B) numbers. (C) Bacterial counts enumerated from lung homogenates. Lines indicate median values. Each dot represents the results for an individual mouse. (D and E) Total protein (D) and LDH (E) were assayed in BAL fluid. OD490nm, optical density at 490 nm. (F) Representative hematoxylin-and-eosin-stained lung sections harvested at 18 postinfection are shown. 40×, 100×, magnification.

used in our studies was limited to the very early responses to these organisms, it is possible that the involvement of the Th17 cytokines manifests in later stages of infection. Future studies using *Cxcr3*^{-/-} and *Cd4*^{-/-} mice, as well as examining immune cell trafficking at later stages of infection, will help to identify additional components of this cascade and, perhaps, appropriate targets to ameliorate the excessive immune signaling that contributes to the pathology of *S. aureus* pneumonia.

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