Induction of Acute Pleural Inflammation by Staphylococcus aureus. I. CD4+ T Cells Play a Critical Role in Experimental Empyema

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Bacterial empyema is a frequent complication of pneumonia in patients with acquired immunodeficiency syndrome (AIDS). A model of Staphylococcus aureus empyema was developed that closely resembles bacterial empyema in patients infected with human immunodeficiency virus (HIV). Results show a compartmentalized chemokin expression in bacterial empyema. The chemokine levels were higher in the pleural compartment than in the peripheral circulation. Polymorphonuclear leukocyte counts, murine GRO-α (KC), and macrophage inflammatory protein-2 levels were significantly (P < .001) lower in CD4+ knockout (CD4 KO) mice pleural fluid than in CD4+ wild-type (CD4 WT) mice. The CD4 KO mice had poorer bacterial clearance than CD4 WT mice. During S. aureus infection, interleukin-10 levels increased in the CD4 KO mice, whereas interferon-γ levels were increased in CD4 WT mice. CD4+ T cell depletion results in a decreased pleural chemokine response, decreased neutrophil influx into pleural space, and impaired bacterial clearance in empyema.

The presence of bacterial organisms in the pleural space is defined as empyema, a well-described complication of pneumonia [1]. Bacterial pneumonia is more frequent in patients infected with human immunodeficiency virus (HIV) than in normal hosts. In patients with AIDS, bacterial pneumonia is one of the most common causes of pleural effusions [2]. Bacterial infections occur frequently and have a propensity to recur in patients with AIDS [3, 4]. An increased incidence of Staphylococcus aureus bacteremia has been described in patients with AIDS and pneumonia [5]. Patients with AIDS who develop empyema do not have an aggressive neutrophil influx into the pleural space as do non-AIDS patients. The possible mechanisms for this effect were thought to be defective neutrophil chemotactic ability [6].

The C-X-C chemokines are chemotactic for neutrophils and play an important role in neutrophil infiltration in inflammatory diseases. Effective host defense against bacterial invasion is characterized by the recruitment and activation of polymorphonuclear leukocytes (PMNL), which are dependent on the coordinated local expression of chemokines. In bacterial empyema, recruited neutrophils represent important phagocytic cells involved in pleural antibacterial host defense. We [7], and others [8], have demonstrated that parapneumonic effusions contain a novel chemokine from the C-X-C family, namely interleukin-8 (IL-8), that has specific chemotactic properties for neutrophils. An exact murine homologue for IL-8 does not exist [9]. In mice, the primary C-X-C chemokines are macrophage inflammatory protein-2 (MIP-2) and murine GRO-α (KC) [10, 11]. KC and MIP-2 are specifically chemotactic for neutrophils and are important for a neutrophil-predominant inflammatory response. MIP-2 and KC cause neutrophil activation, including the induction of the respiratory burst [11, 12]. The expression of MIP-2 and KC is increased during acute inflammation of the lung [13, 14].

Helper T cells are crucial for defense of the lung against specific pathogens. T helper–1 (Th1) and T helper–2 (Th2) cells are subsets of CD4+ T lymphocytes defined phenotypically by their ability to preferentially generate a distinct set of cytokines. During intracellular infections by bacteria and viruses, Th1-like cells preferentially develop, whereas during helminthic infection and responses induced by environmental allergens, Th2-like cells predominate [15]. IL-10, a novel lymphokine secreted by Th2 cells, inhibits a broad spectrum of inflammatory responses [16, 17]. In contrast, IFN-γ, a lymphokine secreted by Th1 cells, counters the IL-10–mediated inhibition [18]. Several reports indicate that CD4+ T cells regulate antigen-induced leukocyte recruitment to the lungs and airway [19–21]. However, it is not clear whether the CD4+ cells regulate acute inflammatory processes in bacterial empyema. In the present study, we present a model of pleural empyema. We demonstrate that S. aureus induces KC and MIP-2 expression in the pleural space and that CD4+ T cells regulate the chemokine expression and play a significant role in antimicrobial host defense.

Materials and Methods

Animals, antibodies, and reagents. Female CD4+ knockout (CD4 KO) mice with C57BL/6 background (5–6 weeks of age),
and wild-type (WT) C57BL/6 (CD4 WT) mice (Jackson Laboratories, Bar Harbor, ME) were used in this study. Penicillin, streptomycin for cell culture, and nonspecific rat IgG were purchased from Sigma (St. Louis). Goat anti-mouse KC and goat anti-mouse MIP-2 antibodies were obtained from R & D Systems (Minneapolis). Rabbit anti-goat IgG-ABC reagent kit (Vector Laboratories, Burlingame, CA), monoclonal antibodies against \textit{S. aureus} (Chemicon International, Temecula, CA), rabbit polyclonal antiserum to \textit{S. aureus} (American Qualex, San Clemente, CA), F12-K medium (Gibco Laboratories, Grand Island, NY), and fetal bovine serum (Harlan Sprague Dawley, Indianapolis) were also used in this study.

**Bacterial culture and development of empyema in mice.** Mouse virulent strain of \textit{S. aureus} (ATCC-14154) was purchased from American Type Culture Collection, (Rockville, MD). \textit{S. aureus} was cultured as reported elsewhere [22]. Briefly, a loopful of \textit{S. aureus} was inoculated into 5 mL of nutrient broth and incubated overnight at 37°C. The number of colony-forming units (cfu) of cultured bacteria in the original stock was determined by plating known volumes of serial dilutions over nutrient agar plates.

The CD4 WT and CD4 KO mice used in our experiments were found to be culture negative for \textit{S. aureus}, and neither strain of mice had any preexposure of \textit{S. aureus} as tested by ELISA for circulating antibodies to \textit{S. aureus} antigens. A total of $5 \times 10^6$ cfu of viable \textit{S. aureus} were inoculated into the pleural space of each mouse in 0.2 mL of sterile saline. CD4 KO and CD4 WT mice were infected with \textit{S. aureus} at the same time. For each time period studied, a total of 6 CD4 KO mice and 7 CD4 WT mice were injected. Equal numbers of control mice were injected with intrapleural saline at each time period studied. To minimize variability in handling the animals, we simultaneously killed three batches of both CD4 KO (saline- and \textit{S. aureus}-injected) and CD4 WT (saline- and \textit{S. aureus}-injected) mice for each time period and analyzed the data. Mice (CD4 KO and CD4 WT) were killed at 6, 12, 24, 48, and 72 h after inoculation and blood, pleural fluid (PF), spleen, and the liver were collected. The experiments were designed for 72 h, since the acute inflammatory response in CD4 WT mice subsided and they successfully cleared bacteria from pleural space and blood by this time point. The PF, spleen, and liver were cultured on nutrient agar plates for \textit{S. aureus}. PF and serum were saved for the estimation of chemokines/cytokines.

**Cytokine ELISA.** KC, MIP-2, IL-10, and IFN-γ levels in the mice PF and serum were estimated by sandwich ELISA (Quanti-
Figure 3. *Staphylococcus aureus*-mediated polymorphonuclear leukocyte (PMNL) recruitment into the pleural space of CD4 wild-type (WT) and CD4 knockout (KO) mice. Values at each time period represent the combined results (mean ± SE) of 3 separate experiments of CD4 WT (saline-injected, n = 7; *S. aureus*-injected, n = 7) and CD4 KO (saline-injected, n = 6; *S. aureus*-injected, n = 6) mice. Asterisk (*) denotes P < .001; superscript dollar sign ($) denotes *S. aureus*-injected CD4 KO vs. CD4 WT mice.

Figure 4. No. of colony-forming units of *Staphylococcus aureus* recovered from CD4 wild-type (WT) and CD4 knockout (KO) mice over time. Values at each time period represent the combined results (mean ± SE) of 3 separate experiments of CD4 WT (saline-injected, n = 7; *S. aureus*-injected, n = 7) and CD4 KO (saline-injected, n = 6; *S. aureus*-injected, n = 6) mice. Asterisk (*) denotes P < .001; CD4 KO vs. CD4 WT mice.

Kinase (R&D Systems, Minneapolis) according to the manufacturer’s instructions. Briefly, 50 μL aliquot of PF or serum were added into 96-well microtiter plates that had previously been coated with anti-cytokine/chemokine polyclonal antibody and incubated at room temperature for 2 h. The wells were blocked with 2% bovine serum albumin in PBS. The chemokines were detected with peroxidase-conjugated specific antibodies (KC, MIP-2, IL-10, and IFN-γ) by incubating at 37°C for 2 h. The microtiter plates were rinsed with PBS–Tween-20 (0.05% Tween-20) and developed with substrate o-phenylenediamine + H2O2. The color intensity was measured at 450 nm with an ELISA reader. The antibody against mouse KC has a sensitivity to detect <2 pg/mL and did not cross-react with IFN-γ, IL-10, MIP-2, MIP-1α, MIP-1β, C10, or JE/MCP-1. The antibody against MIP-2 used in this study did not cross-react with mouse IFN-γ, IL-10, KC, MIP-1α, MIP-1β, C-10, or JE/MCP-1 and has a sensitivity to detect <1.5 pg/mL. The antibodies against IFN-γ did not cross-react with IL-4, IL-10, KC, MIP-2. The antibodies against IL-10 did not cross-react either with IFN-γ, IL-4, KC, or MIP-2 and has a sensitivity to detect <2 pg/mL.

Assessment of bacterial clearance and pleural fluid PMNL counts. The mice were killed at various time intervals, and the PF, liver, and spleen were removed aseptically. The liver and spleen were squeezed through a sterile steel mesh and suspended in 2 mL of PBS. Aliquots of 100 μL (PF, spleen, or liver suspension) were plated on nutrient agar plates and incubated at 37°C. After a 24 h incubation, the total number of *S. aureus* colonies were enumerated and expressed as total number of cfu recovered. The PF was centrifuged briefly, the cell pellet washed 3 times in PBS. The total cells counted using a hemocytometer. To evaluate PMNL in pleural fluid, cytospin smears were prepared on glass slides and stained by using a Geimsa stain. The number of PMNL present in the pleural fluid was evaluated by calculating the number of PMNL noticed in a total of 1000 cells counted in randomly selected fields.

Statistical analysis. The significance of difference of bacterial clearance between CD4 WT and CD KO mice group means was tested by Student’s t test. The significance of difference between the groups (mean) was tested by Kruskal-Wallis 1-way analysis of variance on ranks. A P < .05 was considered significant.

Results

**KC and MIP-2 levels were lower in CD4 KO mice than in CD4 WT mice empyema.** The KC and MIP-2 levels were barely detectable in saline-injected CD4 WT and CD4 KO mice
at all the time points studied. *S. aureus* induced KC and MIP-2 expression in both CD4 WT and CD4 KO mice in a time-dependent manner. In *S. aureus*-injected CD4 WT mice, both KC and MIP-2 levels were several times higher, compared with those seen in CD4 KO mice (figure 1A, B and figure 2A, B). The maximal pleural chemokine levels were noticed upon 12 h after inoculation of *S. aureus*. In both (CD4 WT and CD4 KO) groups, KC and MIP-2 levels were significantly (*P* < .001) higher in the pleural fluids, compared with their serum levels.

**Neutrophil influx into the pleural space was higher in CD4 WT mice empyema.** The PF neutrophil counts were significantly (*P* < .001) higher in CD4 WT mice empyema (figure 3) than in CD4 KO mice. The neutrophil influx into the pleural space peaked 12 h after intrapleural instillation of *S. aureus*. During this period, KC and MIP-2 levels in PF also reached their peak concentrations. In CD4 KO mice the PF neutrophil influx was significantly (*P* < .001) lower than that seen in the WT mice until 48 h after *S. aureus* inoculation, whereas at 72 h after *S. aureus* inoculation, a significantly (*P* < .05 vs. CD4 WT) higher number of neutrophils persisted in the pleural spaces of CD4 KO mice.

**Bacterial clearance was higher in CD4 WT mice empyema than CD4 KO mice empyema.** CD4 WT mice were able to clear >50% of *S. aureus* as early as 12 h, and at 72 h after inoculation, no bacteria were noticed in the pleural spaces (figure 4). In this group, the spleen and liver were culture negative by 12 h after inoculation. During the 6- and 12-h exposure periods, the number of bacterial colonies noticed in the PF culture were significantly (*P* < .001) higher in CD4 KO mice than in CD4 WT mice. In the CD4 KO mice, *S. aureus* were noticed in the spleen and liver at 48 h after inoculation. In addition, although there were neutrophils in the pleural spaces of the CD4 KO mice, bacteria were found in the pleural spaces even at 72 h after inoculation.

**CD4 KO mice have higher IL-10 levels in serum and PF during bacterial empyema.** The IL-10 levels in *S. aureus*-injected CD4 WT mice were close to those seen in saline-injected mice.
(figure 5A, B). In S. aureus-injected CD4 KO mice, the IL-10 levels were significantly (P < .001) higher than in S. aureus-injected CD4 WT mice and saline-injected CD4 KO. In the S. aureus-injected CD4 KO mice, the IL-10 levels in the PF were several times (P < .001) higher than their serum levels.

CD4 WT mice have higher IFN-γ levels in serum and PF during bacterial empyema. In S. aureus-mediated empyema, the IFN-γ levels were significantly (P < .001) higher in CD4 WT mice than in the CD4 KO mice (figure 6A, B). In CD4 WT mice sera, the IFN-γ levels were 2- to 3-fold higher (P < .001) than that seen in the CD4 KO mouse sera. In CD4 WT mice PF, the IFN-γ levels were 5- to 6-fold higher than those seen in CD4 KO mouse PF. However, in S. aureus-injected CD4 KO mice, the serum IFN-γ levels were relatively higher (P < .001) than those of saline-injected CD4 KO mice.

Discussion

Animal models using the pleural space for a model of bacterial infection are excellent for studying the inflammatory cascade of cytokines, as well as for evaluating the response of local cells to the infecting organism in the pleural space [23]. The pleural space lends itself to the study of interaction between cells to the infecting organism in the pleural space [23]. The presence of pleuropulmonary bacterial infections in patients with AIDS. Between 40% and 60% of patients with bacterial pneumonia develop pleural effusions [24]. Effective host defense against bacterial invasion is characterized by the vigorous recruitment and activation of inflammatory cells, which are dependent upon the coordinated expression of both pro- and anti-inflammatory cytokines. Proinflammatory mediators, such as MIP-2 and KC, mediate acute inflammatory responses in bacterial empyema. MIP-2 and KC mediate PMNL chemotaxis and augment the ability of PMNL to phagocytose and kill bacteria. KC and MIP-2 activate neutrophils and induce the respiratory burst [11, 12]. Passive immunization with MIP-2 and KC antibodies inhibited neutrophil accumulation in vivo [25, 26], and inhibition of MIP-2 bioactivity in vivo decreased the lung PMNL influx in bacterial pneumonia [27]. In addition, recent data indicate that mice expressing KC in a tissue-specific manner have increased neutrophil accumulation in those tissues [28]. In CD4 WT mice, we noticed an increase in the PF MIP-2 and KC levels and also increased influx of PMNL into the pleural space after S. aureus instillation. Bacterial killing was much higher in this group. In CD4 KO mice, the pleural MIP-2 and KC levels were low, and they did not clear the bacteria even 72 h after inoculation. This indicates that CD4+ T cell deletion results in altered pleural chemokine responses. The poor bacterial clearance in the CD4 KO mice may be partly due to impaired neutrophil bactericidal capacity, as seen in HIV infection where numbers of CD4+ T cells are substantially reduced [29]. However, despite low KC and MIP-2 levels in CD4 KO mice, the presence of PMNL in their PF indicates that other chemokines may be responsible in part for PMNL influx.

During inflammatory processes in the lung, the infected macrophage is a rich source of chemokines that induce infiltration of leukocytes to the site of infection. However, in pleural infection, pleural mesothelial cells, the first cells to respond to the presence of bacteria, are considered to be a rich source of chemokines [30]. Various other cell types, including fibroblasts and epithelial cells, have been reported to secrete MIP-2 [31]. Several microbes induce chemokine secretion in a variety of cell types. S. aureus induces IL-8 and MIP-1 secretion in neutrophils [32] and GRO secretion in alveolar macrophages [33]. In mice, intraperitoneal challenge of mycobacterial antigen results in an acute and extensive accumulation of neutrophils [34]. Infection of bone marrow-derived macrophages with Listeria monocytogenes induces MIP-2 and KC expression [35]. In the present study, the pleural mesothelial cells, the elicited PMNL, and the mononuclear phagocytes may be the source of MIP-2 and KC in response to S. aureus in the pleural space.

The decreased chemokine response in CD4 KO mice indicates that endogenously released helper cytokines may regulate the pleural chemokine response in empyema. IL-10 is an anti-inflammatory cytokine that suppresses the ability of several cell types to release other proinflammatory factors. The major sources of IL-10 include B cells and monocytes [17, 36]. B cells from patients with AIDS secrete a significantly larger quantity of IL-10 than B cells from non-AIDS patients [36]. Neutral-
zation of IL-10 results in enhanced MIP-2 production and bacterial clearance in acute bacterial pneumonia [37]. IL-10 can cause macrophage deactivation [16]. Peripheral blood neutrophils, when activated by lipopolysaccharide, do not produce IL-8 in the presence of IL-10 [38]. IL-10 inhibits tumor necrosis factor-α– and IFN-γ–induced IL-8 in smooth-muscle cells [39] and in macrophages inhibits Listeria monocytogenes–triggered MIP-2 and KC expression [35]. In vivo, IL-10 suppresses the herpes simplex virus type 1–induced MIP-2 expression [40]. IL-10 also inhibits phagocytic and bactericidal capacity of neutrrophils [41]. In our studies, the increased IL-10 levels noticed in CD4 KO mice empyema fluid may be responsible for the suppression of MIP-2 and KC responses and for decreased bacterial clearance. In a similar study, Standiford et al. noticed an increased expression of IL-10 in the lung in response to the intratracheal instillation of Klebsiella pneumonia [27].

In CD4 WT mice empyema, the IFN-γ levels were high and IL-10 levels were low. IFN-γ enhances IL-8 production in smooth-muscle cells [39] and KC and MIP-2 production in macrophages [35]. IFN-γ potentiates the bactericidal capacity of PMNL [42]. The lack of IFN-γ in CD4 KO mice may be partially responsible for reduced bacterial clearance in this group. Patients with AIDS who were treated with IFN-γ have a reduced incidence of bacterial infection [43]. The increased IFN-γ levels in the CD4 WT mice may be responsible in part for increased pleural KC and MIP-2 levels and for successful elimination of bacteria. The major source of IFN-γ is CD4+ T cells, CD8+ T cells, and NK cells [44, 45]. Although the CD8+ cells were present in CD4 KO mice, their relative contribution of IFN-γ may be not enough to counter the IL-10–mediated immunosuppression as the IL-10 levels were several times higher in this group.

In conclusion, our studies indicate that, in S. aureus empyema, MIP-2 and KC are produced in the pleural space that mediates PMNL recruitment and activation. Expression of these chemokines may be regulated in part by CD4+ T cells through endogenous release of helper cytokines.

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References

25. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL. Elevated...


