The Alternative Activation Pathway and Complement Component C3 Are Critical for a Protective Immune Response against *Pseudomonas aeruginosa* in a Murine Model of Pneumonia

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Pseudomonas aeruginosa is a leading cause of hospital-acquired pneumonia, and approximately 80% of patients with cystic fibrosis are infected with this bacterium. To investigate the overall role of complement and the complement activation pathways in the host defense against *P. aeruginosa* pulmonary infection, we challenged C3-, C4-, and factor B-deficient mice with *P. aeruginosa* via intranasal inoculation. In these studies, $C3^{-/-}$ mice had a higher mortality rate than $C3^{+/+}$ mice. Factor $B^{-/-}$ mice, but not $C4^{-/-}$ mice, infected with *P. aeruginosa* had a mortality rate similar to that of $C3^{-/-}$ mice, indicating that in this model the alternative pathway of complement activation is required for the host defense against *Pseudomonas* infection. $C3^{-/-}$ mice had 6- to 7-fold more bacteria in the lungs and 48-fold more bacteria in the blood than did $C3^{+/+}$ mice at 24 h postinfection. In vitro, phagocytic cells from $C3^{+/+}$ or $C3^{-/-}$ mice exhibited a decreased ability to bind and/or ingest *P. aeruginosa* in the presence of C3-deficient serum compared to phagocytic cells in the presence of serum with sufficient C3. $C3^{-/-}$ mice displayed a significant increase in neutrophils in the lungs and had higher levels of interleukin-1β (IL-1β), IL-6, IL-10, KC, and MIP-2 in the lungs at 24 h postinfection than did $C3^{+/+}$ mice. Collectively, these results indicate that complement activation by the alternative pathway is critical for the survival of mice infected with *P. aeruginosa* and that the protection provided by complement is at least in part due to C3-mediated opsonization and phagocytosis of *P. aeruginosa*.

Pseudomonas aeruginosa is a gram-negative environmental bacterium and an opportunistic pathogen that can cause pneumonia, particularly in people whose immune systems are suppressed, such as AIDS patients and cancer patients, and is a major source of hospital-acquired pneumonia (23). In addition, this pathogen causes chronic pulmonary infections in people with cystic fibrosis, leading to significant morbidity and mortality among these patients (23). A pulmonary infection with *P. aeruginosa* is characterized by a strong recruitment of neutrophils and significant inflammation in the lung parenchyma, which results in extensive damage to the lung tissue through the action of neutrophil enzymes and oxidants (23).

The complement system, which is made up of >30 serum and cell surface proteins, plays a crucial role in the host defense against infection and in the inflammatory response (17, 25). The complement system has three pathways of activation, the classical, alternative, and lectin pathways; all three pathways result in C3 activation and merge into a common pathway that results in the formation of the membrane attack complex (MAC), or C5b-9 (17). The classical pathway is primarily activated through the binding of C1q to antibody-antigen complexes. The lectin pathway is initiated when the mannose-binding lectin binds to mannose residues on bacteria or viruses,

and the alternative pathway is initiated when spontaneously activated C3 binds to activating surfaces such as those of certain bacteria or viruses (17). In a complete deficiency of C3, almost all of the biological properties mediated by complement are absent, including opsonization and phagocytosis of bacteria (mediated by C3b, C3bi, CR1, CR3, and CR4), directed migration of inflammatory cells (mediated by C3a and C5a), and amplification of the immune response (mediated by C3dg) (17, 18, 25, 41).

Although there are numerous immune and inflammatory responses that occur in an acutely infected lung, recent in vivo experiments have indicated that complement plays a particularly critical role in providing a protective immune response against *P. aeruginosa* lung infection. For example, antibodies against CR3 (CD11b/CD18) reduce neutrophil emigration into the lungs of *P. aeruginosa*-infected mice (12, 28); C5- and C5a receptor (C5aR)-deficient mice succumb more readily to *P. aeruginosa* infection, despite the robust recruitment of neutrophils to the infected lungs (3, 16, 19); and mice pretreated with cobra venom factor have increased numbers of *P. aeruginosa* in the lungs 4 h after *P. aeruginosa* pulmonary infection (10, 13).

To more fully understand the overall role of complement and its activation pathways in the host defense against an acute primary *P. aeruginosa* pulmonary infection, we subjected mice that were completely deficient in C3, factor B, or C4 to *P. aeruginosa* infection as a model of *P. aeruginosa*-induced pneumonia. The results from these studies demonstrate that an intact alternative pathway is critical to host survival of an acute

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primary *P. aeruginosa* pulmonary infection. In addition, host clearance of *P. aeruginosa* was greatly impaired in C3$^{-/-}$ mice, which appeared to be due, at least in part, to the absence of C3-mediated opsonization and phagocytosis.

MATERIALS AND METHODS

Mice. C57BL/6 mice are commonly used for animal disease models, including those for *P. aeruginosa* infection. Accordingly, complement-deficient mice that were backcrossed onto the C57BL/6 genetic background were used in all experimental procedures. The C3-deficient (C3$^{-/-}$) mice used in these studies were described previously (4). Sera from C3$^{-/-}$ mice lack detectable C3 and complement activity (4). The C3$^{-/-}$ mice were backcrossed for 12 generations onto the C57BL/6 background, and wild-type littermates (C3$^{+/+}$) were used as controls. Factor B-deficient (FB$^{-/-}$) mice have been described elsewhere (24), and alternative pathway activity is not detectable in sera from these mice (24). The FB$^{-/-}$ mice were backcrossed for 11 generations onto the C57BL/6 background, and wild-type littermates (FB$^{+/+}$) were used as controls. The C4-deficient (C4$^{-/-}$) mice were generated as described previously (8) and were found to have no detectable C4 protein in their sera (8). The C4$^{-/-}$ mice were backcrossed for at least 10 generations onto the C57BL/6 background, and their wild-type littermates (C4$^{+/+}$) were used as controls. Both male and female mice of 6 to 9 weeks of age were used for these studies, and institutional and National Institutes of Health guidelines for animal care and welfare were followed.

i.n. infection. *P. aeruginosa* strain PA103 (22), kindly provided by Barbara Iglewski, University of Rochester Medical Center, was used for most of the studies to induce pneumonia in mice via intranasal (i.n.) inoculations. A clinical strain of *P. aeruginosa* (designated PA243) that was isolated from the sputum of an intensive care unit patient with pneumonia was kindly provided by Audrey Wanger, University of Texas Medical School—Houston, and was used for mortality studies. Bacteria were cultured in tryptic soy broth (TSB) at 37°C to mid-logarithmic phase, harvested by centrifugation, washed twice in sterile, nonpyrogenic 0.9% NaCl, and resuspended in 0.9% NaCl. Mice that had been anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (0.016 ml/g of body weight) (Sigma-Aldrich, St. Louis, Mo.) (15) were given either 1 × 10^5 PA103 cells or 3 × 10^6 PA243 cells in a volume of 20 μl i.n. Control mice received 20 μl of sterile, nonpyrogenic 0.9% NaCl i.n. The number of bacteria present in the inoculum was verified by culturing serial dilutions of the inoculum on tryptic soy agar (TSA) plates.

Mortality studies. C3$^{-/-}$ mice, FB$^{-/-}$ mice, C4$^{-/-}$ mice, and their respective wild-type littermates were infected via i.n. inoculation with either PA103, PA243, or 0.9% NaCl and were observed for mortality every 12 h for 7 days. Survival curves were graphed with the GraphPad Prism (San Diego, Calif.) software package, and statistical significance was assessed by using the log rank test. All statistical analyses for these studies were performed with GraphPad Prism software.

Bacterial clearance from the lungs and blood. C3$^{-/-}$ mice and C3$^{+/+}$ mice were infected i.n. with PA103 or 0.9% NaCl, and the lungs were collected either immediately or at 24 h postinfection. For determination of the amount of the initial inoculum that was received into the lungs of the mice, six to seven mice per group were infected and then immediately euthanized. Either immediately or 24 h after infection, lungs were collected and placed in sterile 0.9% NaCl on ice. The lungs were homogenized on ice in 2 ml of sterile 0.9% NaCl by pressing the lungs through 100-μm-pore-size cell strainers (BD Biosciences, San Diego, Calif.). Serial 10-fold dilutions of the lung homogenates were plated on TSA plates to determine the numbers of CFU. Bronchoalveolar lavage (BAL) was performed on some mice 24 h after infection in order to quantitate the number of bacteria present in BAL fluid. The lungs were subjected to lavage three times with 0.5 ml of phosphate-buffered saline (PBS), and the lavage fluid was placed on ice. Serial 10-fold dilutions of the BAL fluid were plated on TSA plates to determine the numbers of CFU. Prior to BAL or harvesting of the lungs, blood was drawn from the mice at 24 h postinfection, and 100 μl of blood from the C3$^{+/+}$ mice and serial 10-fold dilutions of blood from the C3$^{-/-}$ mice were plated on TSA plates. All plates were incubated overnight at 37°C. The results are expressed as mean CFU per total lungs, mean CFU per milliliter of BAL fluid, or mean CFU per milliliter of blood ± standard errors of the means (SEM). Statistical significance between C3$^{+/+}$ and C3$^{-/-}$ mice was assessed by using the two-tailed, unpaired Student *t* test.

Bactericidal assay. Washed *P. aeruginosa* PA103 (2 × 10^8 cells/ml) or *Escherichia coli* HB101 (9 × 10^7 cells/ml) cells were incubated with either normal serum from C3$^{+/+}$ or C3$^{-/-}$ mice or heat-inactivated serum (56°C for 30 min) from C3$^{+/+}$ or C3$^{-/-}$ mice for 1 h at 37°C with gentle shaking (125 rpm). The

sera used were pooled from six C3$^{+/+}$ or six C3$^{-/-}$ mice. After 1 h, serial 10-fold dilutions of each treatment were made and were plated on TSA plates to determine the numbers of viable bacteria. The plates were incubated overnight at 37°C. The results are expressed as mean CFU per milliliter ± SEM. Statistical significance was assessed by using the two-tailed, unpaired Student *t* test.

In vitro assay for adherence and uptake of *P. aeruginosa* by mouse blood leukocytes. Total blood leukocytes were obtained from C3$^{+/+}$ and C3$^{-/-}$ mice as follows: 1-ml syringes containing 23-gauge needles were filled with 0.23 ml of dextran citrate solution (3.5% dextran T500, 70 mM sodium citrate, 34 mM citric acid in PBS), C3$^{+/+}$ and C3$^{-/-}$ mice were bled to 1 ml via cardiac puncture, the red blood cells (RBCs) were allowed to settle to the bottom of the tube for 30 min, the layer above the RBCs was placed in a new tube and centrifuged at 200 × g for 8 min, the supernatants were discarded, the residual RBCs were lysed with ACK lysing buffer (BioWhittaker, Rockland, Maine), and the cells were washed one time with PBS. The washed leukocytes were resuspended in RPMI 1640 medium containing 2.05 mM L-glutamine (VWR, West Chester, Pa.) and 10% C3$^{+/+}$ or C3$^{-/-}$ mouse serum, and the leukocytes were counted by using a hemacytometer. *P. aeruginosa* PA01, also kindly provided by Barbara Iglewski, and *P. aeruginosa* PA01 constitutively expressing green fluorescent protein (GFP) (30), kindly provided by Anne Camper, Montana State University, were opsonized in either 50% C3$^{+/+}$ or C3$^{-/-}$ mouse serum for 30 min at 37°C. The leukocytes, at a concentration of 2 × 10^6 cells/ml, were incubated with 1 × 10^8 cells of preopsonized *P. aeruginosa*/ml for 1 h at 37°C with gentle shaking (100 rpm). The cells were centrifuged as described above at 4°C, and then the cells were resuspended in PBS and kept on ice for flow cytometry analysis. Flow cytometry using a FACSCalibur apparatus (BD Biosciences) was employed to quantify the adherence and phagocytosis of bacteria by analyzing cells that were positive for GFP. The forward scatter threshold was set so as to exclude free bacteria from the analysis. Leukocytes incubated with opsonized non-GFP-expressing *P. aeruginosa* PA01 were used as negative controls, and at least 10,000 cells in all samples were analyzed. The results are expressed as percentages of positive cells ± SEM. Statistical significance was assessed by using the two-tailed, unpaired Student *t* test.

Lung histology. Mouse lungs were perfused with 0.3 ml of formalin 24 h after infection, and the tracheas were tied off with suture material. The lungs were removed from the mice and placed in formalin overnight at 4°C. The lungs were dehydrated with increasing concentrations of ethanol, embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin. The stained sections were examined by light microscopy to assess the level of inflammation.

BAL. The types and numbers of inflammatory cells present in the lungs 24 h after infection were assessed by BAL, which was performed as described above. The total number of cells present in the lavage fluid was determined by using a hemacytometer. BAL fluid was centrifuged onto slides in a cytocentrifuge (Shandon Lipshaw, Pittsburgh, Pa.), and the slides were stained with Wright-Giemsa stain (Fisher Scientific, Pittsburgh, Pa.). Two hundred cells per slide were classified according to cell type (either polymorphonuclear [PMN] cell, macrophage, or lymphocyte) based on their characteristic morphologies under a light microscope. Absolute numbers of specific cell types were calculated from the recovered BAL volume, total cell count, and percent abundance of specific cells. Statistical significance between C3$^{+/+}$ and C3$^{-/-}$ mice was assessed by analysis of variance followed by posthoc pairwise comparisons by the Tukey method.

Cytokine and chemokine analysis. The levels of the proinflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α), the anti-inflammatory cytokine IL-10, and the chemokines JE, KC, and MIP-2 were examined in mice at 24 h postinfection. BAL fluid was collected as described above and was centrifuged to remove cells. The BAL fluid supernatants were then assayed for the presence of these cytokines and chemokines by enzyme-linked immunosorbent assays (R & D Systems Inc., Minneapolis, Minn.) performed according to the manufacturer's instructions. Statistical significance between C3$^{+/+}$ and C3$^{-/-}$ mice for each individual cytokine or chemokine was assessed by using the two-tailed, unpaired Student *t* test.

RESULTS

C3$^{-/-}$ mice exhibit increased mortality after *P. aeruginosa* infection. Mice deficient in C3 lack a functional complement system and are devoid of numerous activation fragments that mediate potent biological activities in inflammation and immunity (17, 18, 25, 41). Accordingly, to assess the overall impact of complement in an in vivo model of acute primary pneumonia, we challenged C3$^{-/-}$ and C3$^{+/+}$ mice i.n. with *P. aeruginosa*
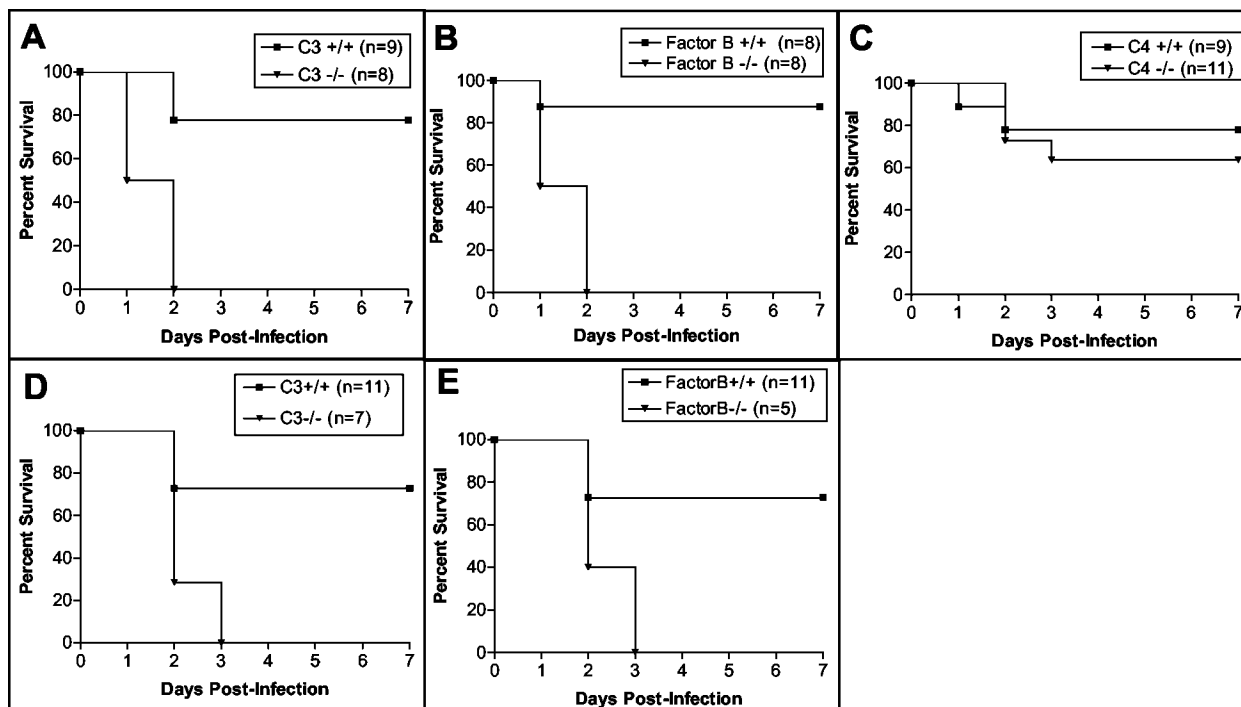
FIG. 1. Survival curves for C3$^{-/-}$, FB$^{-/-}$, and C4$^{-/-}$ mice infected via i.n. inoculation with 1×10^5 *P. aeruginosa* PA103 cells (A to C) or 3 $\times 10^6$ *P. aeruginosa* PA243 cells (D and E). Data for the C3$^{-/-}$ mice (A and D), FB$^{-/-}$ mice (B and E), and C4$^{-/-}$ mice (C) are shown with those for their respective wild-type littermates. Mice were monitored for mortality every 12 h for 7 days.

PA103 (10^5 CFU) and monitored them for mortality. Within 48 h of infection, all of the C3$^{-/-}$ mice died, compared to only 22% of the C3$^{+/+}$ mice ($P = 0.001$) (Fig. 1A). To show that the host protection provided by C3 is not unique to the PA103 strain, we also challenged C3$^{-/-}$ and C3$^{+/+}$ mice i.n. with the clinical PA243 strain (3×10^6 CFU) and monitored them for mortality. Within 72 h of infection, all of the C3$^{-/-}$ mice died, compared to only 27% of the C3$^{+/+}$ mice ($P = 0.003$) (Fig. 1D), demonstrating that complement plays a vital role in protecting the host from the lethal effects of an acute primary *P. aeruginosa* pulmonary infection.

FB$^{-/-}$ mice exhibit increased mortality after infection with *P. aeruginosa*. *P. aeruginosa* infection could potentially result in the activation of all three complement pathways, the classical, lectin, and alternative pathways. To examine the significance of the complement activation pathways in providing a protective immune response against *P. aeruginosa*, we infected FB$^{-/-}$ mice (lacking a functional alternative pathway), C4$^{-/-}$ mice (lacking functional classical and lectin pathways), and their respective wild-type littermates with *P. aeruginosa* PA103 i.n. and monitored them for mortality. Similar to the C3$^{-/-}$ mice, all of the FB$^{-/-}$ mice died within 48 h of infection, compared to only 13% of the FB$^{+/+}$ mice ($P = 0.001$) (Fig. 1B). In contrast, the C4$^{-/-}$ mice and the C4$^{+/+}$ mice displayed similar survival rates ($P > 0.05$) (Fig. 1C). To confirm the importance of the alternative pathway in this model, we also infected FB$^{+/+}$ and FB$^{-/-}$ mice with the clinical PA243 strain. Within 72 h of infection, all of the FB$^{-/-}$ mice died, compared to only 27% of the FB$^{+/+}$ mice ($P = 0.01$) (Fig. 1E). These results indicate that in this model the classical and lectin pathways are less important than the alternative pathway for a protective

immune response against primary *P. aeruginosa* pulmonary infections.

C3$^{-/-}$ mice are deficient in clearance of *P. aeruginosa* from the lungs and blood. To determine if the increased mortality seen for the C3$^{-/-}$ mice correlated with an increase in bacterial numbers in the lungs and bloodstream, we infected both C3$^{-/-}$ and C3$^{+/+}$ mice i.n. with *P. aeruginosa*. Twenty-four hours after infection, lungs were removed from one group of mice, homogenized, and plated on TSA plates, and for the other group of mice, BAL was performed and BAL fluid was plated on TSA plates. Blood was also drawn from all groups of mice at 24 h postinfection and was plated on TSA plates. Lungs from infected C3$^{-/-}$ mice contained six times more *P. aeruginosa* than lungs from infected C3$^{+/+}$ mice ($P = 0.003$) (Table 1), and BAL fluid from infected C3$^{-/-}$ mice contained seven times more *P. aeruginosa* than BAL fluid from infected C3$^{+/+}$ mice ($P = 0.02$) (Table 1). The C3$^{-/-}$ mice also had 48 times more *P. aeruginosa* present in the bloodstream 24 h after infection than did the C3$^{+/+}$ mice ($P = 0.001$) (Table 1).

Mouse serum does not affect viability of *P. aeruginosa*. In the absence of C3, complement-mediated formation of the C5b-9 MAC can no longer occur (17, 25). Hence, the increased numbers of *P. aeruginosa* in the lungs and blood of the C3$^{-/-}$ animals could potentially be due to the absence of C5b-9 lytic killing of the bacteria. To examine this possibility, we incubated *P. aeruginosa* PA103 with C3$^{+/+}$ or C3$^{-/-}$ mouse serum as well as heat-inactivated serum from these animals for 1 h at 37°C. Any remaining viable bacteria were subsequently quantitated as described in Materials and Methods. As shown in Fig. 2, *P. aeruginosa* was resistant to killing by mouse serum independent of whether C3$^{+/+}$, C3$^{-/-}$, or heat-inactivated se-

TABLE 1. Clearance of *P. aeruginosa* from the lungs and dissemination into the bloodstream after i.n. infection[a]

Mouse genotype	CFU/total lungs		CFU/ml of BAL fluid at 24 h	CFU/ml of blood at 24 h
	0 h	24 h		
C3^{+/+}	$(6.02 \pm 0.57) \times 10^4$ ($n = 6$)	$(2.61 \pm 0.96) \times 10^6$ ($n = 12$)	$(4.45 \pm 1.63) \times 10^5$ ($n = 8$)	$(6.15 \pm 2.15) \times 10^2$ ($n = 13$)
C3^{−/−}	$(7.05 \pm 0.76) \times 10^4$ ($n = 7$)	$(1.53 \pm 0.46) \times 10^{7**}$ ($n = 7$)	$(3.06 \pm 0.76) \times 10^{6*}$ ($n = 13$)	$(2.97 \pm 0.81) \times 10^{4**}$ ($n = 13$)

[a] Lungs were removed from the mice either immediately after infection (0 h) or 24 h postinfection, the lungs were homogenized in saline, serial 10-fold dilutions were made, and the dilutions were plated on TSA plates. In other experiments, the lungs were lavaged three times with 0.5 ml of PBS, serial 10-fold dilutions of the BAL fluid were made, and the dilutions were plated on TSA plates. Blood was also plated on TSA plates. Results are expressed as means $\pm$ SEM. Significant differences between C3^{+/+} and C3^{−/−} mice are indicated as follows: *, $P = 0.02$; **, $P \leq 0.003$.

rum was used. This finding indicates that mouse serum does not affect the viability of *P. aeruginosa* PA103, and hence the increased numbers of bacteria in the infected C3^{−/−} mice were not due to an impairment in C5b-9-mediated lysis.

C3^{−/−} mice are defective in adherence and uptake of *P. aeruginosa*. In the absence of C3, complement activation will not generate the C3-derived polypeptide fragments C3b and C3bi. These fragments of C3 act as opsonins and facilitate phagocytosis by adhering C3b- and C3bi-coated bacteria to macrophages and neutrophils via binding to their receptors, named CR1 and CR3/CR4, respectively (7, 31). To determine if the deficiency in clearance of *P. aeruginosa* from the lungs and blood of C3^{−/−} mice was due to a defect in adherence and phagocytosis, we conducted in vitro uptake studies using GFP-expressing *P. aeruginosa* and flow cytometry. Total blood leukocytes were harvested from C3^{+/+} and C3^{−/−} mice, and these leukocytes were incubated with *P. aeruginosa* that had been preopsonized with either C3^{+/+} or C3^{−/−} mouse serum. Leukocytes isolated from C3^{+/+} or C3^{−/−} mice incubated with *P. aeruginosa* opsonized with serum with sufficient C3 had more bacteria associated with them than leukocytes incubated with bacteria opsonized in C3-de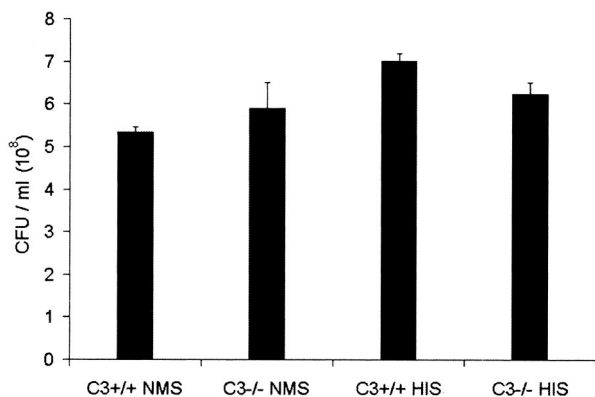ficient serum (Table 2). Our results indicate that in the absence of C3-mediated opsonization, the adherence of *P. aeruginosa* to phagocytic cells is impaired. This impairment likely results in ineffective phagocytosis, which may explain, in part, the higher numbers of *P. aeruginosa* found in the lungs and blood of C3^{−/−} mice.

C3^{−/−} mice display an increase in pulmonary inflammatory infiltrates after infection. The attenuated bacterial clearance observed for C3^{−/−} mice could be due to a reduced pulmonary cellular inflammatory response since the anaphylatoxins C3a and C5a will not be produced by complement activation in C3^{−/−} mice. To compare the level of pulmonary inflammation in C3^{−/−} mice to that in C3^{+/+} mice, we removed the lungs from both groups of mice for histology 24 h after *P. aeruginosa* infection. Lung sections that were stained with hematoxylin and eosin revealed the presence of inflammatory infiltrates in both the C3^{+/+} and C3^{−/−} mice that had been infected with *P. aeruginosa* (Fig. 3, bottom panels). A normal lung histology was evident in lung sections from control C3^{+/+} and C3^{−/−} mice infected with only saline (Fig. 3, top panels). Inflammatory cells were quantitated in BAL fluid taken from the mice at 24 h postinfection. The C3^{−/−} mice had two times more total leukocytes and PMNs than the C3^{+/+} mice ($P < 0.001$) (Table 3), demonstrating that the C3^{−/−} mice had elevated levels of inflammatory cells in the lungs after *P. aeruginosa* pulmonary infection. These results indicate that the increased numbers of *P. aeruginosa* in the C3^{−/−} mice were not due to reduced pulmonary cellular recruitment.

C3^{−/−} mice have higher levels of cytokines and chemokines after infection. To further evaluate the inflammatory response in C3^{−/−} mice and C3^{+/+} mice, we measured the levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α and the anti-inflammatory cytokine IL-10 in BAL fluid taken from mice at 24 h postinfection. The C3^{−/−} mice had 2 times more IL-1β ($P = 0.04$), 2.9 times more IL-10 ($P = 0.02$), and 2.5

FIG. 2. Serum bactericidal assay with *P. aeruginosa* and C3^{+/+} or C3^{−/−} mouse serum. *P. aeruginosa* PA103 (2×10^8 CFU/ml) was incubated with either normal mouse serum (NMS) from C3^{+/+} or C3^{−/−} mice or heat-inactivated serum (HIS) from C3^{+/+} or C3^{−/−} mice for 1 h at 37°C. After 1 h, serial 10-fold dilutions of each treatment were made and plated on TSA plates to determine the number of viable bacteria. Results are expressed as CFU per ml $\pm$ SEM. *E. coli* HB101 was used as a positive control for this experiment and was unable to grow in the presence of C3^{+/+} mouse serum but able to grow in the presence of C3^{−/−} mouse serum and heat-inactivated C3^{+/+} and C3^{−/−} mouse sera ($P < 0.03$) (data not shown). This experiment was performed three times, and the data shown are from one representative experiment.

TABLE 2. Adherence and phagocytosis of preopsonized *P. aeruginosa* by C3^{+/+} or C3^{−/−} phagocytes

Type of leukocytes	Type of serum used for GFP-PA01 incubation	% positive cells[a] (mean ± SEM)
C3^{+/+}	C3^{+/+}	55.14 ± 3.09
C3^{+/+}	C3^{−/−}	42.01 ± 0.28*
C3^{−/−}	C3^{+/+}	48.37 ± 1.31
C3^{−/−}	C3^{−/−}	37.09 ± 3.68*

[a] The percent positive cells represents the number of leukocytes that were positive for GFP by flow cytometry analysis. The negative control, which was non-GFP-PA01 incubated with C3^{+/+} serum and C3^{+/+} leukocytes, had 0.12% positive cells. *, significant difference ($P \leq 0.04$) between C3-sufficient and C3-deficient sera.
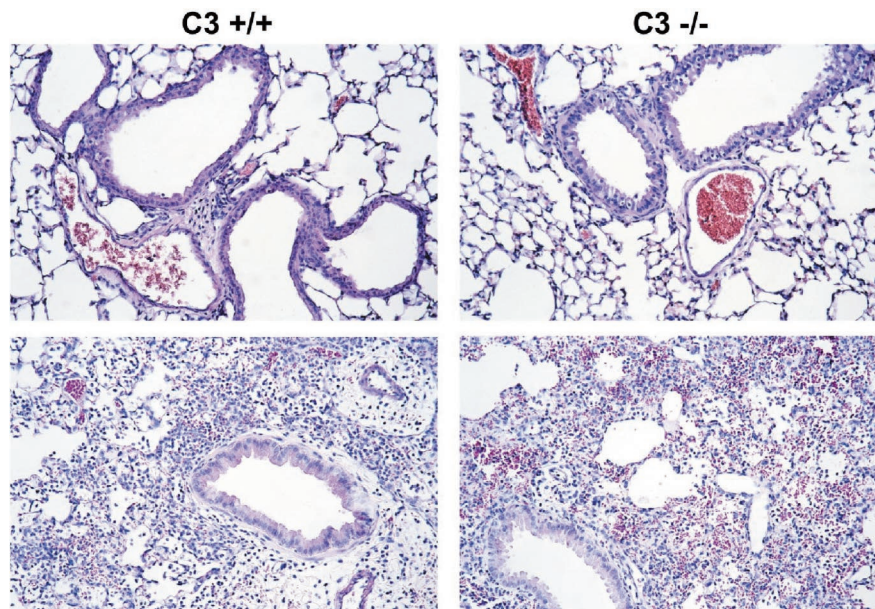
FIG. 3. Lung histology of $C3^{+/+}$ and $C3^{-/-}$ mice 24 h after i.n. infection with either *P. aeruginosa* (bottom panels) or saline (top panels). The lungs were fixed in formalin, embedded in paraffin, sectioned into 5-μm-thick sections, and stained with hematoxylin and eosin. The sections shown are representative of three separate experiments. Magnification, ×20.

times more IL-6 ($P = 0.005$) than the $C3^{+/+}$ mice (Fig. 4). The $C3^{-/-}$ mice and the $C3^{+/+}$ mice had similar levels of TNF-α ($P > 0.05$) (Fig. 4A).

To determine if the $C3^{-/-}$ mice had more production of chemokines than the $C3^{+/+}$ mice after infection, we measured the levels of KC, MIP-2, and JE in BAL fluid taken 24 h after infection with *P. aeruginosa*. The mouse chemokine JE is chemotactic for monocytes, macrophages, and lymphocytes (2, 11), and the mouse chemokines KC and MIP-2 are chemotactic for neutrophils (6, 37). The $C3^{-/-}$ mice had 3.9 times more KC ($P < 0.0001$) and 4.7 times more MIP-2 ($P = 0.01$) than the $C3^{+/+}$ mice (Fig. 5), while JE levels were equivalent between the two groups of mice ($P > 0.05$) (Fig. 5). The increased expression of KC and MIP-2 may account for the high quantity of neutrophils present in the lungs of the $C3^{-/-}$ mice.

DISCUSSION

With this study, we demonstrated for the first time with a mouse model of acute primary pneumonia the overall importance of complement activation by the alternative pathway in the immune response to primary *P. aeruginosa* pulmonary infection. Both $C3^{-/-}$ and $FB^{-/-}$ mice had much higher mortality rates than their respective wild-type littermates or $C4^{-/-}$ mice after i.n. infection with *P. aeruginosa*. The higher mortality rates of $FB^{-/-}$ mice than of $C4^{-/-}$ animals indicate that in this model of acute primary lung infection the alternative pathway of complement activation is more important than the classical or lectin pathway for protecting the host from a *P. aeruginosa* challenge. The lack of protection from the lectin pathway agrees with recent in vitro investigations which have indicated that *P. aeruginosa* does not bind human mannose-binding lectin (5, 26). Although our studies demonstrate the dominance of the alternative pathway of complement activation in providing

host protection against a primary acute *P. aeruginosa* infection, they do not address the significance of the alternative pathway compared to the classical pathway once an antibody response to this organism is generated. Recent studies have indicated that human monoclonal antibodies against *P. aeruginosa* lipopolysaccharide (LPS) (14) and both mouse monoclonal (9) and rabbit polyclonal (32) antibodies against *P. aeruginosa* PcrV protect mice (9, 14, 32) and rabbits (32) from fatal *P. aeruginosa* sepsis, suggesting that once an antibody response is mounted against *P. aeruginosa*, the classical pathway may provide significant host protection. However, whether the classical or alternative pathway will dominate upon a subsequent host exposure to *P. aeruginosa* is likely dependent on the immunocompetence of the host, which will affect the level of antibody response to this opportunistic pathogen. The model used in our studies is likely to represent what happens in an individual who is infected with *P. aeruginosa* for the first time or who has previously been exposed to *P. aeruginosa* but due to a compromised immune system is unable to mount a protective antibody response.

$C3^{-/-}$ mice harbored 6 to 7 times more *P. aeruginosa* in the lungs and 48 times more *P. aeruginosa* in the bloodstream than $C3^{+/+}$ mice at 24 h postinfection, indicating that complement significantly impacts the host clearance of *P. aeruginosa*. Although the bactericidal activity of the C5b-9 complex is important for host defense against many gram-negative bacteria (17), the results reported here demonstrate that *P. aeruginosa* PA103 is resistant to killing by mouse serum. Therefore, the increased numbers of *P. aeruginosa* in the $C3^{-/-}$ mice are not due to an impairment in MAC-mediated killing of *P. aeruginosa*. In addition, the reduced ability of the $C3^{-/-}$ mice to control the *P. aeruginosa* infection was not due to an inability of these mice to mount a neutrophil chemotactic response. In fact, a significantly elevated number of neutrophils was present

TABLE 3. Leukocyte counts and differentials in BAL fluid 24 h after i.n. infection with *P. aeruginosa*[a]

Mouse genotype	Treatment	Total leukocyte count (10^6 cells)	No. of PMNs (10^6 cells)	No. of macrophages (10^6 cells)	No. of lymphocytes (10^6 cells)
C3$^{+/+}$	Saline	0.18 ± 0.01	0	0.18 ± 0.01	0
C3$^{-/-}$	Saline	0.17 ± 0.01	0	0.17 ± 0.01	0
C3$^{+/+}$	PA103	32.47 ± 1.50	30.88 ± 1.39	1.05 ± 0.10	0.54 ± 0.10
C3$^{-/-}$	PA103	67.07 ± 6.34***	63.91 ± 5.98***	1.28 ± 0.35	1.88 ± 0.22

[a] BAL was performed on the mice 24 h after infection with either saline or PA103. Absolute numbers of specific cell types were calculated from the recovered BAL volume, total cell count, and percent abundance of specific cells. Results are expressed as means ± SEM. For the saline-treated groups, $n = 5$. For the PA103-treated groups, $n = 8$. ***, significant differences between C3$^{+/+}$ and C3$^{-/-}$ mice infected with PA103 ($P < 0.001$).

in the lungs of infected C3$^{-/-}$ animals compared to those of their wild-type littermates. Similar increases in inflammatory cells have been reported for the lungs of C5- and C5aR-deficient mice after *P. aeruginosa* pulmonary infections (3, 16, 19). Collectively, our studies with C3$^{-/-}$ mice and the studies of others with C5- and C5aR-deficient mice indicate that C5a does not play an essential role in the recruitment of neutrophils to the *P. aeruginosa*-infected lung. In the absence of an intact complement system, multiple compensatory mechanisms

exist for the recruitment of neutrophils into the lungs after *P. aeruginosa* infection. *P. aeruginosa* is itself chemotactic for neutrophils in vitro in the absence of serum (34, 39). The mouse chemokines MIP-2 and KC are CXC chemokines that are chemotactic for neutrophils (6, 37), and elevated levels of these chemokines are observed after *P. aeruginosa* pulmonary infection (21, 33, 36, 38, 44). The mouse receptor for these chemokines is CXCR2 (20, 36). Antibody neutralization of CXCR2 prior to the intratracheal inoculation of mice with *P. aeruginosa* resulted in a diminished recruitment of neutrophils into the lungs, increased mortality, and high *P. aeruginosa* numbers in the lungs and bloodstream compared to those in mice that received a control antibody (36). In our studies, the infected C3$^{-/-}$ mice had significantly higher levels of KC and MIP-2 in their BAL fluid than did the infected C3$^{+/+}$ mice. Neutrophils and mononuclear phagocytes produce MIP-2 and KC in response to LPS and IL-1 (35), so the increased levels of LPS (due to increased numbers of *P. aeruginosa*) and IL-1β in the C3$^{-/-}$ mice could account for the high levels of MIP-2 and KC in these mice. The elevated levels of these potent chemokines in the lungs of infected C3$^{-/-}$ mice may explain why C3-deficient animals exhibit a more robust recruitment of neutrophils in response to pulmonary infections than do mice with sufficient complement.

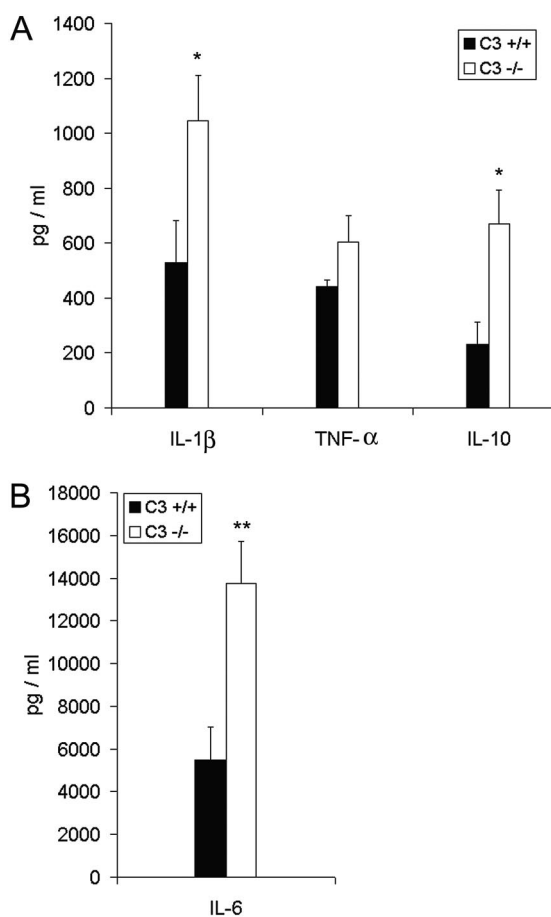Previous studies have shown that one of the defenses of the

FIG. 4. Cytokine levels in BAL fluid 24 h after infection with *P. aeruginosa*. The levels of IL-1β, TNF-α, and IL-10 (A) and the level of IL-6 (B) in BAL fluid were measured 24 h after infection with *P. aeruginosa*. BAL fluid from saline-treated control mice had no detectable levels of these cytokines. Results are expressed as means ± SEM for n values of 9 to 18. Significant differences between C3$^{+/+}$ and C3$^{-/-}$ mice are indicated as follows: *, $P ≤ 0.04$; **, $P = 0.005$.
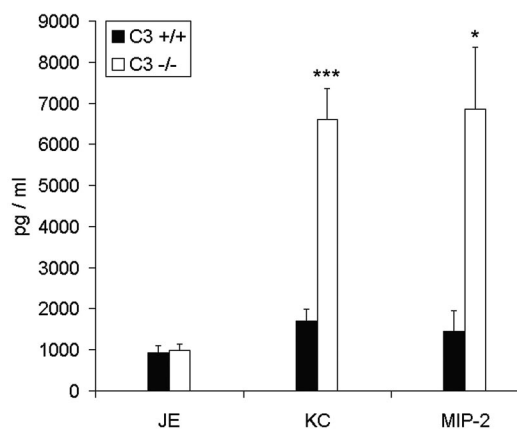

FIG. 5. Chemokine levels in BAL fluid 24 h after infection with *P. aeruginosa*. The levels of JE, KC, and MIP-2 were measured in BAL fluid 24 h after infection with *P. aeruginosa*. BAL fluid from saline-treated control mice had no detectable levels of these chemokines. Results are expressed as means ± SEM for n values of 10 to 16. Significant differences between C3$^{+/+}$ and C3$^{-/-}$ mice are indicated as follows: *, $P = 0.01$; ***, $P < 0.0001$.

host against *P. aeruginosa* infection is the phagocytic clearance of bacteria by PMNs. For example, BALB/c mice that were made to be granulocytopenic by the injection of an antigranulocyte globulin prior to infection had a significant impairment in the clearance of *P. aeruginosa* from the lungs compared to control mice (29). C57BL/6 mice injected with an antineutrophil antibody prior to infection displayed 100% mortality within 2 days of *P. aeruginosa* lung infection compared to zero mortality in 7 days for mice treated with a control antibody (36). C3b and C3bi function as opsonins by coating bacteria and then binding to CR1 and CR3/CR4, respectively, which are present on macrophages and PMNs (7, 31). Several in vitro studies have demonstrated that normal human serum increases the phagocytosis of *P. aeruginosa* by PMNs in culture (1, 27, 43), and this increase in phagocytosis was abolished when the serum was heated to inactivate complement components. Our data support the importance of complement-mediated opsonization and phagocytosis in the clearance of *P. aeruginosa*. We showed that in the absence of C3, and hence in the absence of the C3b and C3bi opsonins, the adherence of *P. aeruginosa* to phagocytic cells is impaired. This finding suggests that the exacerbated *P. aeruginosa* infections seen for $C3^{-/-}$ mice may be due at least in part to inefficient phagocytosis of the bacteria in the absence of opsonization by C3-derived polypeptide fragments. Since C5a has been shown to cause increased expression of CR1, CR3, and CR4 on phagocytic cells (16, 40, 42), a lack of efficient phagocytosis could also be involved in the elevated numbers of *P. aeruginosa* found in the lungs of C5- and C5aR-deficient mice (3, 16, 19).

In summary, the studies presented here indicate that (i) complement activation by an intact alternative pathway is essential for host survival of acute pneumonia caused by a primary *P. aeruginosa* infection, (ii) C3-mediated opsonization and phagocytosis are important mechanisms by which complement provides the host protection against *P. aeruginosa* lung infection, and (iii) a complete deficiency of C3 in mice challenged i.n. with *P. aeruginosa* results in significantly increased lung levels of the chemokines KC and MIP-2, which may cause paradoxical elevated numbers of neutrophils observed in the lungs of infected $C3^{-/-}$ mice.

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REFERENCES

1. **Bjornson, A. B., and J. G. Michael.** 1974. Factors in human serum promoting phagocytosis of *Pseudomonas aeruginosa*. I. Interaction of opsonins with the bacterium. J. Infect. Dis. **130**(Suppl.):S119–S126.
2. **Bottazzi, B., S. Walter, D. Govoni, F. Colotta, and A. Mantovani.** 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. J. Immunol. **148**:1280–1285.
3. **Cerquetti, M. C., D. O. Sordelli, J. A. Bellanti, and A. M. Hooke.** 1986. Lung defenses against *Pseudomonas aeruginosa* in C5-deficient mice with different genetic backgrounds. Infect. Immun. **52**:853–857.
4. **Circolo, A., G. Garnier, W. Fukuda, X. Wang, T. Hidvegi, A. J. Szalai, D. E. Briles, J. E. Volanakis, R. A. Wetsel, and H. R. Colten.** 1999. Genetic disruption of the murine complement C3 promoter region generates defi-
5. cient mice with extrahepatic expression of C3 mRNA. Immunopharmacology **42**:135–149.
5. **Davies, J., O. Neth, E. Alton, N. Klein, and M. Turner.** 2000. Differential binding of mannose-binding lectin to respiratory pathogens in cystic fibrosis. Lancet **355**:1885–1886.
6. **Driscoll, K. E.** 1994. Macrophage inflammatory proteins: biology and role in pulmonary inflammation. Exp. Lung Res. **20**:473–490.
7. **Ehlers, M. R.** 2000. CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes Infect. **2**:289–294.
8. **Fischer, M. B., M. Ma, S. Goerg, X. Zhou, J. Xia, O. Finco, S. Han, G. Kelsoe, R. G. Howard, T. L. Rothstein, E. Kremmer, F. S. Rosen, and M. C. Carroll.** 1996. Regulation of the B cell response to T-dependent antigens by classical pathway complement. J. Immunol. **157**:549–556.
9. **Frank, D. W., A. Vallis, J. P. Wiener-Kronish, A. Roy-Burman, E. G. Spack, B. P. Mullaney, M. Megdoud, J. D. Marks, R. Fritz, and T. Sawa.** 2002. Generation and characterization of a protective monoclonal antibody to *Pseudomonas aeruginosa* PcrV. J. Infect. Dis. **186**:64–73.
10. **Gross, G. N., S. R. Rehm, and A. K. Pierce.** 1978. The effect of complement depletion on lung clearance of bacteria. J. Clin. Investig. **62**:373–378.
11. **Gu, L., B. Rutledge, J. Fiorillo, C. Ernst, I. Grewal, R. Flavell, R. Gladue, and B. Rollins.** 1997. *In vivo* properties of monocyte chemoattractant protein-1. J. Leukoc. Biol. **62**:577–580.
12. **Gyetko, M. R., S. Sud, T. Kendall, J. A. Fuller, M. W. Newstead, and T. J. Standiford.** 2000. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary *Pseudomonas aeruginosa* infection. J. Immunol. **165**:1513–1519.
13. **Heidbrink, P. J., G. B. Toews, G. N. Gross, and A. K. Pierce.** 1982. Mechanisms of complement-mediated clearance of bacteria from the murine lung. Am. Rev. Respir. Dis. **125**:517–520.
14. **Hemachandra, S., K. Kamboj, J. Copfer, G. Pier, L. L. Green, and J. R. Schreiber.** 2001. Human monoclonal antibodies against *Pseudomonas aeruginosa* lipopolysaccharide derived from transgenic mice containing megabase human immunoglobulin loci are opsonic and protective against fatal *Pseudomonas* sepsis. Infect. Immun. **69**:2223–2229.
15. **Hogan, B., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Hopken, U. E., B. Lu, N. P. Gerard, and C. Gerard.** 1996. The C5a chemoattractant receptor mediates mucosal host defence to infection. Nature **383**:86–89.
17. **Janeway, C. A., P. Travers, M. Walport, and M. Shlomchik.** 2001. Innate immunity, immunobiology, 5th ed. Garland Publishing, New York, N.Y.
18. **Kohl, J.** 2001. Anaphylatoxins and infectious and non-infectious inflammatory diseases. Mol. Immunol. **38**:175–187.
19. **Larsen, G. L., B. C. Mitchell, T. B. Harper, and P. M. Henson.** 1982. The pulmonary response of C5 sufficient and deficient mice to *Pseudomonas aeruginosa*. Am. Rev. Respir. Dis. **126**:306–311.
20. **Lee, J., G. Cacalano, T. Camerato, K. Toy, M. W. Moore, and W. I. Wood.** 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. J. Immunol. **155**:2158–2164.
21. **LeVine, A. M., K. E. Kurak, M. D. Bruno, J. M. Stark, J. A. Whitsett, and T. R. Korfhagen.** 1998. Surfactant protein-A-deficient mice are susceptible to *Pseudomonas aeruginosa* infection. Am. J. Respir. Cell. Mol. Biol. **19**:700–708.
22. **Liu, P. V.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481–489.
23. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15**:194–222.
24. **Matsumoto, M., W. Fukuda, A. Circolo, J. Goellner, J. Strauss-Schoenberger, X. Wang, S. Fujita, T. Hidvegi, D. D. Chaplin, and H. R. Colten.** 1997. Abrogation of the alternative complement pathway by targeted deletion of murine factor B. Proc. Natl. Acad. Sci. USA **94**:8720–8725.
25. **Muller-Eberhard, H. J.** 1988. Molecular organization and function of the complement system. Annu. Rev. Biochem. **57**:321–347.
26. **Neth, O., D. L. Jack, A. W. Dodds, H. Holzel, N. J. Klein, and M. W. Turner.** 2000. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect. Immun. **68**:688–693.
27. **Peterson, P. K., Y. Kim, D. Schmeling, M. Lindemann, J. Verhoef, and P. G. Quie.** 1978. Complement-mediated phagocytosis of *Pseudomonas aeruginosa*. J. Lab. Clin. Med. **92**:883–894.
28. **Qin, L., W. M. Quinlan, N. A. Doyle, L. Graham, J. E. Sligh, F. Takei, A. L. Beaudet, and C. M. Doerschuk.** 1996. The roles of CD11/CD18 and ICAM-1 in acute *Pseudomonas aeruginosa*-induced pneumonia in mice. J. Immunol. **157**:5016–5021.
29. **Rehm, S. R., G. N. Gross, and A. K. Pierce.** 1980. Early bacterial clearance from murine lungs. Species-dependent phagocyte response. J. Clin. Investig. **66**:194–199.
30. **Rice, A. R., M. A. Hamilton, and A. K. Camper.** 2000. Apparent surface associated lag time in growth of primary biofilm cells. Microb. Ecol. **40**:8–15.
31. **Sengelov, H.** 1995. Complement receptors in neutrophils. Crit. Rev. Immunol. **15**:107–131.
32. **Shime, N., T. Sawa, J. Fujimoto, K. Faure, L. R. Allmond, T. Karaca, B. L.**

Swanson, E. G. Spack, and J. P. Wiener-Kronish. 2001. Therapeutic administration of anti-PcrV F(ab′)(2) in sepsis associated with *Pseudomonas aeruginosa*. J. Immunol. **167:**5880–5886.

33. Skerrett, S. J., T. R. Martin, E. Y. Chi, J. J. Peschon, K. M. Mohler, and C. B. Wilson. 1999. Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or *Pseudomonas aeruginosa*. Am. J. Physiol. **276:**L715–L727.

34. Sordelli, D. O., M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti. 1985. Effect of chemotactins released by *Staphylococcus aureus* and *Pseudomonas aeruginosa* on the murine respiratory tract. Infect. Immun. **49:**265–269.

35. Strieter, R. M., S. L. Kunkel, M. P. Keane, and T. J. Standiford. 1999. Chemokines in lung injury: Thomas A. Neff lecture. Chest **116:**103S–110S.

36. Tsai, W. C., R. M. Strieter, B. Mehrad, M. W. Newstead, X. Zeng, and T. J. Standiford. 2000. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine *Pseudomonas aeruginosa* pneumonia. Infect. Immun. **68:**4289–4296.

37. Van Damme, J., A. Wuyts, G. Froyen, E. Van Coillie, S. Struyf, A. Billiau, P. Proost, J. M. Wang, and G. Opdenakker. 1997. Granulocyte chemotactic protein-2 and related CXC chemokines: from gene regulation to receptor usage. J. Leukoc. Biol. **62:**563–569.

38. van Heeckeren, A. M., J. Tscheikuna, R. W. Walenga, M. W. Konstan, P. B. Davis, B. Erokwu, M. A. Haxhiu, and T. W. Ferkol. 2000. Effect of *Pseudo-monas* infection on weight loss, lung mechanics, and cytokines in mice. Am. J. Respir. Crit. Care Med. **161:**271–279.

39. Ward, P. A., I. H. Lepow, and L. J. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. Am. J. Pathol. **52:**725–736.

40. Werfel, T., M. Oppermann, M. Schulze, G. Krieger, M. Weber, and O. Gotze. 1992. Binding of fluorescein-labeled anaphylatoxin C5a to human peripheral blood, spleen, and bone marrow leukocytes. Blood **79:**152–160.

41. Wetsel, R. A., J. Kildsgaard, and D. L. Haviland. 2000. Complement anaphylatoxins (C3a, C4a, C5a) and their receptors (C3aR, C5aR/CD88) as therapeutic targets in inflammation, p. 113. *In* J. D. Lambris and V. M. Holers (ed.), Contemporary immunology: therapeutic interventions in the complement system. Humana Press, Totowa, N.J.

42. Yancey, K. B., J. O'Shea, T. Chused, E. Brown, T. Takahashi, M. M. Frank, and T. J. Lawley. 1985. Human C5a modulates monocyte Fc and C3 receptor expression. J. Immunol. **135:**465–470.

43. Young, L. S., and D. Armstrong. 1972. Human immunity to *Pseudomonas aeruginosa*. I. In-vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. J. Infect. Dis. **126:**257–276.

44. Yu, H., S. Z. Nasr, and V. Deretic. 2000. Innate lung defenses and compromised *Pseudomonas aeruginosa* clearance in the malnourished mouse model of respiratory infections in cystic fibrosis. Infect. Immun. **68:**2142–2147.