Innate Immune Responses to Systemic *Acinetobacter baumannii* Infection in Mice: Neutrophils, but Not Interleukin-17, Mediate Host Resistance[▽]

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Acinetobacter baumannii **is a nosocomial pathogen with a high prevalence of multiple-drug-resistant strains, causing pneumonia and sepsis. The current studies further develop a systemic mouse model of this infection and characterize selected innate immune responses to the organism. Five clinical isolates, with various degrees of antibiotic resistance, were assessed for virulence in two mouse strains, and between male and female mice, using intraperitoneal infection. A nearly 1,000-fold difference in virulence was found between bacterial strains, but no significant differences between sexes or mouse strains were observed. It was found that microbes disseminated rapidly from the peritoneal cavity to the lung and spleen, where they replicated. A persistent septic state was observed. The infection progressed rapidly, with mortality between 36 and 48 h. Depletion of neutrophils with antibody to Ly-6G decreased mean time to death and increased mortality. Interleukin-17 (IL-17) promotes the response of neutrophils by inducing production of the chemokine keratinocyte-derived chemoattractant (KC/CXCL1), the mouse homolog of human IL-8.** *Acinetobacter* **infection resulted in biphasic increases in both IL-17 and KC/CXCL1. Depletion of neither IL-17 nor KC/CXCL1, using specific antibodies, resulted in a difference in bacterial burdens in organs of infected mice at 10 h postinfection. Comparison of bacterial burdens between IL-17a$^{-/-}$ and wild-type mice confirmed that the absence of this cytokine did not sensitize mice to** *Acinetobacter* **infection. These studies definitely demonstrate the importance of neutrophils in resistance to systemic** *Acinetobacter* **infection. However, neither IL-17 nor KC/CXCL1 alone is required for effective host defense to systemic infection with this organism.**

Acinetobacter baumannii is a Gram-negative bacterium associated primarily with nosocomial infections. While this organism can be found in soil, there is evidence that most of the recent infections in military personnel are caused by strains that populate the hospital environment (29, 46, 52). Evidence suggests that the number of multiple-drug-resistant *A. baumannii* infections in intensive care unit (ICU) patients is on the rise, not only in North America but also in Europe and South America (35). *Acinetobacter* infections have also been a significant cause of morbidity and mortality in soldiers in intensive care units in Vietnam, Iraq, and Afghanistan. The growing drug resistance of *A. baumannii* confounds treatment of infected patients, most of whom are often the most critically ill. Deeper understanding of the pathogenesis of *A. baumannii* and the host immune response may present alternative approaches to therapy.

A. baumannii infections have been shown to manifest as bacteremia, pneumonia, urinary tract infections (UTI), and soft tissue infections. A large study examining U.S. nosocomial outbreaks from 1995 to 2002 showed that *A. baumannii* was the 10th most common etiologic agent in single-microbe bloodstream infections. *A. baumannii* bloodstream infection accounted for 1.3% of all ICU bacteremias, with an associated mortality rate between 34 and 43.4% (57). More-recent data collected by the Centers for Disease Control and Prevention reported an increase in *A. baumannii* ICU infections, with 7% of all ICU pneumonias being associated with *A. baumannii* in 2004, up from 4% in 1986, with associated increases in UTI and soft tissue infections (11). An increase in multiple-drug-resistant *A. baumannii* bloodstream infections was also recorded between 2002 and 2004 for military personnel returning from combat in Iraq and Afghanistan (5). While some studies have suggested that *A. baumannii* could be acquired from the soil, meticulous studies within the military population have genotypically linked clinical strains with those isolated from the hospital environment, including the hands of medical personnel (46). The evolving antibiotic resistance of these strains, along with the recent changes in their epidemiology, highlights the importance of a better understanding of host-pathogen interactions in regard to this organism.

Several rodent models of *A. baumannii* infection have been reported. These include pneumonia models using intranasal or intratracheal routes of infection (16, 18, 19, 36–38, 42, 43, 54), a rat soft tissue model (25, 34, 41), and a rabbit endocarditis model (39). Studies of systemic infection with *A. baumannii* have been hampered by the low virulence of bacterial strains in rodent models, leading some investigators to use a variety of

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techniques to sensitize animals to these organisms. Obana et al. (32) were the first to report infection of mice via the intraperitoneal (i.p.) route of administration, with i.p. 50% lethal dose (LD_{50}) values of $\geq 10^6$ cells for most strains tested, leading these investigators to use an artificial model in which *A. baumannii* was coated with hog gastric mucin to decrease phagocytosis of the strains and hence increase their virulence for the host. To study the use of new antibiotics against *A. baumannii* strains, Joly-Guillou et al. (17) rendered animals neutropenic by administering cyclophosphamide to increase the virulence of these strains in mice. While several investigators have approached the problem of low virulence by modifying strains or inducing immunosuppression, there are no reports in the literature comparing the virulence of strains in a systemic infection model in the absence of immunomodulation.

Despite a rising incidence of *A. baumannii* infections, the immune mechanisms that regulate infection are largely understudied. In addition to the study by Joly-Guillou et al. noted above using i.p. infection, an important role for neutrophils has been observed during both intranasal and intratracheal pneumonias (17, 36, 54). Knapp et al. (19) demonstrated that the absence of TLR4 and CD14 sensitized mice to *A. baumannii* pneumonia but noted increases in MIP-2 and MCP-1 in $TLR2^{-/-}$ mice, which correlated with a greater cell influx to the lungs. In contrast, Erridge et al. showed that UV-killed *A. baumannii* stimulated proinflammatory cytokine signaling via both TLR4 and TLR2 (9), leaving the immune dependency on TLR2 up for question. Other investigators have found that NADPH phagocyte oxidase (phox) is important for the control of bacterial burdens, while inducible nitric oxide synthase (iNOS) is dispensable for host defense (37).

In these limited studies on the immune response mounted against *A. baumannii*, there has been little investigation into the role of cytokines. Interleukin-17 (IL-17) has recently received considerable attention for its role as a proinflammatory cytokine known to induce granulopoiesis and to induce production of other proinflammatory cytokines and chemokines that enhance the accumulation of neutrophils. Within the innate arm of the immune system, IL-17 production is induced by the macrophage cytokine IL-23. A protective role for the innate induction of IL-17 has been observed in other infectious models. Neutralization of IL-17 by depleting antibodies caused an increase in the burdens of *Escherichia coli* following an i.p. injection (47) and increased fungal burdens in mouse models of *Pneumocystis carinii* (40) and *Candida albicans* (15) infections. It was also found that $IL-17R^{-/-}$ mice had greater *Klebsiella pneumoniae* burdens following intranasal infection than wild-type (WT) controls (61). These studies provided the rationale to explore the role of neutrophils and IL-17 during systemic *Acinetobacter* infection.

The present study characterized an intraperitoneal, systemic *A. baumannii* infection model in two strains of mice, C57BL/6 and C3HeB/FeJ, using several clinical isolates of *A. baumannii*. In this model, it was found that bacteria rapidly disseminated via the blood to peripheral organs, including the lung and spleen, where they replicated. Neutrophil depletion studies demonstrated an important role for these cells during systemic infection. Rapid and robust IL-17 and KC/CXCL1 responses were induced in the peritoneal cavity following infection, but

they were not found to be important in protection, as assessed using antibody neutralization and IL-17A-deficient animals.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, female or male, 6- to 8-week-old C57BL/6J and C3HeB/FeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a genetic disruption of the IL-17A gene, $IL-17a^{-/-}$ knockout (KO) mice, were obtained from Jay Kolls (LSU School of Medicine), with the permission of Yoishiro Iwakura (Institute of Medical Science, the University of Tokyo, Japan), who originally developed these animals (30). All $IL-17a^{-/-}$ and age-matched WT littermates were bred and maintained in the Central Animal Facility of Temple University. All mice were allowed to acclimate for at least 1 week before use. Rodent chow (Purina, St. Louis, MO) and fresh water were available *ad libitum*. All experiments were carried out with the approval of the Institutional Animal Care and Use Committee at Temple University. Animals were housed in a barrier facility of the Central Animal Facility.

Reagents. Rat anti-mouse Ly-6G (clone RB6-8C5) was purchased from BioXCell (West Lebanon, NH). Rat anti-mouse IL-17 (clone 50104), rat anti-mouse KC/CXCL1 (clone 124014), and recombinant mouse IL-23 were purchased from R&D Systems (Minneapolis, MN). Bacterial lipopolysaccharide (LPS) extracted from *Salmonella enterica* serovar Typhimurium was purchased from Sigma (St. Louis, MO).

***Acinetobacter baumannii*.** Clinical *A. baumannii* strains 576, 4502, 5798, 6143, and 7215 were provided by David Craft (Walter Reed Army Institute of Research, Silver Spring, MD). Organisms were stored by freezing at $-80°C$ in dimethyl sulfoxide (DMSO). To grow organisms for *in vitro* or *in vivo* experimentation, a sterile loop was touched to the frozen stock and used to streak out two blood agar (BA) plates. Plates were incubated at $37°C$ overnight. Ten isolated colonies were picked and inoculated into a 50-ml conical tube (Becton-Dickinson, Franklin Lakes, NJ), containing 10 ml of brain heart infusion (BHI) medium. The tube was incubated at $37°C$ with rotary agitation (250 rpm) for 3.5 h. The top 5 ml was drawn off and used to inoculate a 200-ml Erlenmeyer flask containing 45 ml of BHI. This flask was incubated at $37°C$ with agitation (200 rpm) on a G24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ) for 2.5 h to produce late-log-phase organisms. After 2.5 h, the flask was placed on ice to retard further growth of the bacteria. To estimate the number of organisms/ml for inoculation into mice, an appropriate dilution of the desired culture was made in 10% paraformaldehyde and counted in a Petroff-Hausser counter. The culture was diluted with 0.9% sterile, pyrogen-free saline (Hospira, Lake Forest, IL) in an endotoxin-free, sterile vial to obtain the desired concentration of organisms/ml. Viable counts on BA were used to determine the actual number of CFU per ml and to calculate the precise inoculum injected. For *in vivo* inoculation, mice were injected i.p. with the desired dose using a 26-gauge needle in a 200-μl volume.

Antibiotic resistance screening was performed on isolated pure colonies of each strain that had been plated on tryptic soy agar. Antibiotic resistance of strains was analyzed using a BD Phoenix automated microbiology system instrument (BD NMIC 127) in the Clinical Microbiology Laboratory of Temple University Hospital. BD expert rules were used in the evaluation of susceptibilities according to the Clinical Laboratory and Standards Institute (CLSI) recommendations under the supervision of Allan Truant, Director of the Clinical Microbiology, Immunology, and Virology Laboratories.

Survival studies. Susceptibility to infection was assessed by mortality. The initial LD_{50} values (Table 1) were determined by injecting 3 groups of 3 mice each, with each group receiving a different concentration of organisms. More precise values for several of the strains were obtained using 5 dosage groups of 5 mice each (Table 2). Mortality was scored for 7 days, and the LD_{50} was calculated by probit analysis. In some cases, mean survival times were compared between mice in different treatment groups by use of the method of survival distribution based on the log rank test.

Necropsy. Mice were anesthetized with 100 μl of a 50-mg/ml solution of sodium pentobarbital injected intramuscularly (i.m.). Blood samples were collected via cardiac puncture, using a 22-gauge needle, into heparinized 3-ml syringes. A 0.1-ml volume of blood or an appropriate dilution was plated on Levine eosin methylene blue (EMB) agar plates and incubated at $37°C$ overnight. The bacteria were counted and expressed as numbers of CFU/0.1 ml blood. For plasma collection, heparinized blood was centrifuged at $12,000 \times g$ for 10 min at $4°C$ and the plasma layer removed to 1.5-ml Eppendorf tubes and frozen at $-80°C$ until further use to determine levels of cytokines and chemokines. Mice were euthanized via cervical dislocation, and if desired, peritoneal exudate fluid (PEF) was collected by lavage with Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) and frozen at $-80°C$ until further use to determine levels

TABLE 1. Determination of bacterial strain virulence and antibiotic resistance in C3HeB/FeJ mice

WRAIR strain	LD_{50}[a]	Virulence level	Antibiotic susceptibility
576	1.92×10^8	Low	Susceptible to all antibiotics tested
7215	$>4.13 \times 10^6$; $<4.13 \times 10^7$	Low	Susceptible to all antibiotics tested
5798	7.5×10^6	Intermediate	Resistant to all antibiotics tested except imipenem, amikacin, and tobramycin
6143	$>5.75 \times 10^5$; $<5.75 \times 10^6$	High	Resistant to all antibiotics tested except imipenem
4502	4.3×10^6	High	Resistant to all antibiotics tested except imipenem and amikacin

[a] Bacteria were injected i.p.

of cytokines and chemokines. Organs were removed aseptically from individual animals and homogenized in 3 ml of ice-cold phosphate-buffered saline (PBS) in 14-ml round-bottom tubes (BD, Franklin Lakes, NJ), using a Tekmar Tissuemizer (Tekmar, Cincinnati, OH). Homogenates were serially diluted in sterile water, spread onto EMB agar plates, and incubated at 37°C overnight. The number of CFU was counted, and the results were expressed as numbers of CFU/0.1 g tissue or numbers of CFU/0.1 ml blood. The limit of detection in undiluted samples was 30 CFU/0.1 g organ or 1 CFU/0.1 ml blood.

Peritoneal lavage. Mice were euthanized by cervical dislocation, and the skin was pulled away to expose the peritoneum. A 1-ml volume of ice-cold Mg^{2+} Ca^{2+}-free HBSS was injected intraperitoneally. PEF was collected using a 22-gauge needle into a 1.5-ml Eppendorf tube. PEF was centrifuged at $12,000 \times g$ for 10 min at 4°C. Supernatants were collected and frozen at -80°C until further use. When infected mice were used, cells were harvested at 18 h postinfection (p.i.). Cultures containing naïve or infected cells were adjusted to the same cell number, 6×10^5 per well.

ELISA. Levels of IL-17 and KC/CXCL1 in PEF or cell culture supernatants were assessed by a sandwich enzyme-linked immunosorbent assay (ELISA). IL-17 and KC/CXCL1 antibodies and reagents were purchased from R&D Systems (Minneapolis, MN) as IL-17 or KC/CXCL1 Duoset ELISA development kits. The assay was carried out according to the manufacturer's instructions. Briefly, a 96-well Costar (Corning, NY) plate was coated with the desired concentration of capture antibody and incubated overnight at room temperature. The solution was aspirated and washed with 300 μl wash buffer three times. Reagent diluent (1% bovine serum albumin in PBS; 300 μl) was added and incubated for 1 h when the plate was washed three times. A 100-μl volume of diluted sample or standards was added to appropriate wells, and the plate was covered and allowed to incubate for 2 h at room temperature. The plate was washed three times, and 100 μl detection antibody was added to each well. The plate was covered and allowed to incubate for 2 h at room temperature, followed by three washes. A 100-μl volume of a working concentration of streptavidin-horseradish peroxidase (HRP) solution was added to each well, and the plate was covered and incubated for 20 min in the dark at room temperature. Three more washes were performed, 100 μl of color reagent solution containing a 1:1 mixture of H_2O_2 and tetramethylbenzidine was added to each well, and the plate was incubated for 20 min in the dark at room temperature. Finally, 50 μl of stop solution containing $2N\ H_2SO_4$ was added to all wells, and the absorbance was read at 450 nm using an Omega ELISA reader (BMG Labtech, Inc., Cary, NC). Levels of cytokines or chemokines in PEF were quantified using standard curves generated within each experiment of the assay. Samples were run in duplicate.

Statistical analyses. Data were analyzed by John P. Gaughan at the Biostatistics Consulting Center at Temple University using SAS version 9.1 (Cary, NC). The dependent variables, cell counts, marker levels, etc., were treated as continuous variables for all analyses. Means, standard deviations, and numbers of

observations were presented for each variable. The experimental unit was each individual animal or culture sample. The experiments used a factorial design, with each animal evaluated at individual time points. The null hypothesis was that there would be no difference between or within treatment groups over time. Prior to analysis, data were tested for normality using the Shapiro-Wilk test. If the data were significantly nonnormal, a "normalized-rank" transformation was applied to the data. The rank-transformed data were analyzed using a mixed-model analysis of variance (ANOVA) with or without repeated measures followed by multiple comparisons to detect significant differences between means (treatment groups and times). Multiple pairwise comparisons (treatment groups and times) were not adjusted for type 1 error. Two-group experiments were analyzed using t tests and the Wilcoxon rank sum test for nonnormal distributed dependent variables. Survival studies were carried out using Kaplan-Meier product limit estimation. Between-group differences were tested using the log rank test. LD_{50} estimation with 95% fiducial limits was based on probit analysis. A P value of 0.05 was used for statistical significance in all studies.

RESULTS

Characterization of systemic *A. baumannii* infection. Five clinical isolates of *A. baumannii*, obtained from the Walter Reed Army Institute of Research were tested for their relative virulence in C3HeB/FeJ female mice (Table 1). Strains were injected i.p. at three 10-fold dilutions to make a preliminary estimate of their relative virulence (Table 1). Strain 576 was found to be the least virulent ($LD_{50} = 1.9 \times 10^8$), while strain 4502 was the most virulent ($LD_{50} = 4.3 \times 10^6$). Based on these preliminary LD_{50} values, strains were classified as being of low, intermediate, or high virulence. Strains ranged over a $10^{2.5}$-fold difference in virulence, with a median LD_{50} of approximately 7×10^6, indicating that while strains of this opportunistic organism are capable of causing mortality in mice, they are only of intermediate virulence. A rough correlation between virulence and degree of antibiotic resistance was observed. The two strains that were susceptible to all antibiotics tested, strains 576 and 7215, were of the lowest virulence, while the other 3 strains exhibiting higher virulence were resistant to all but one, two, or three antibiotics. Strains 5798 and 4502 were chosen for further study, as they were of intermediate and maximal virulence, respectively, and displayed high levels of antibiotic resistance.

The LD_{50} dose of strain 5798, one of intermediate virulence, was determined more precisely in both C57BL/6 and C3HeB/FeJ male and female mice (Table 2). No statistically significant difference was found in the LD_{50} doses between males and females of either C57BL/6 or C3HeB/FeJ animals with the use of strain 5798. In addition, no statistically significant difference was found between the two mouse strains within the same gender for strain 5798. Similar studies were also carried out using the most virulent strain of *A. baumannii*, 4502. Within both strains of mice, there was no statistically significant dif-

TABLE 2. Virulence of *Acinetobacter baumannii* strains 5798 and 4502 in two mouse strains

Mouse strain	*A. baumannii* strain	LD_{50}	
		Male	Female
C3HeB/FeJ	5798	$1.3 \times 10^{7a,b}$	$4.6 \times 10^{7a,b}$
C57BL/6J	5798	$4.7 \times 10^{6a,b}$	$7.8 \times 10^{6a,b}$
C3HeB/FeJ	4502	1.0×10^6	4.3×10^{6b}
C57BL/6J	4502	6.4×10^{5a}	$2.0 \times 10^{5a,b}$

[a] Not statistically significant between males and females in each mouse strain.

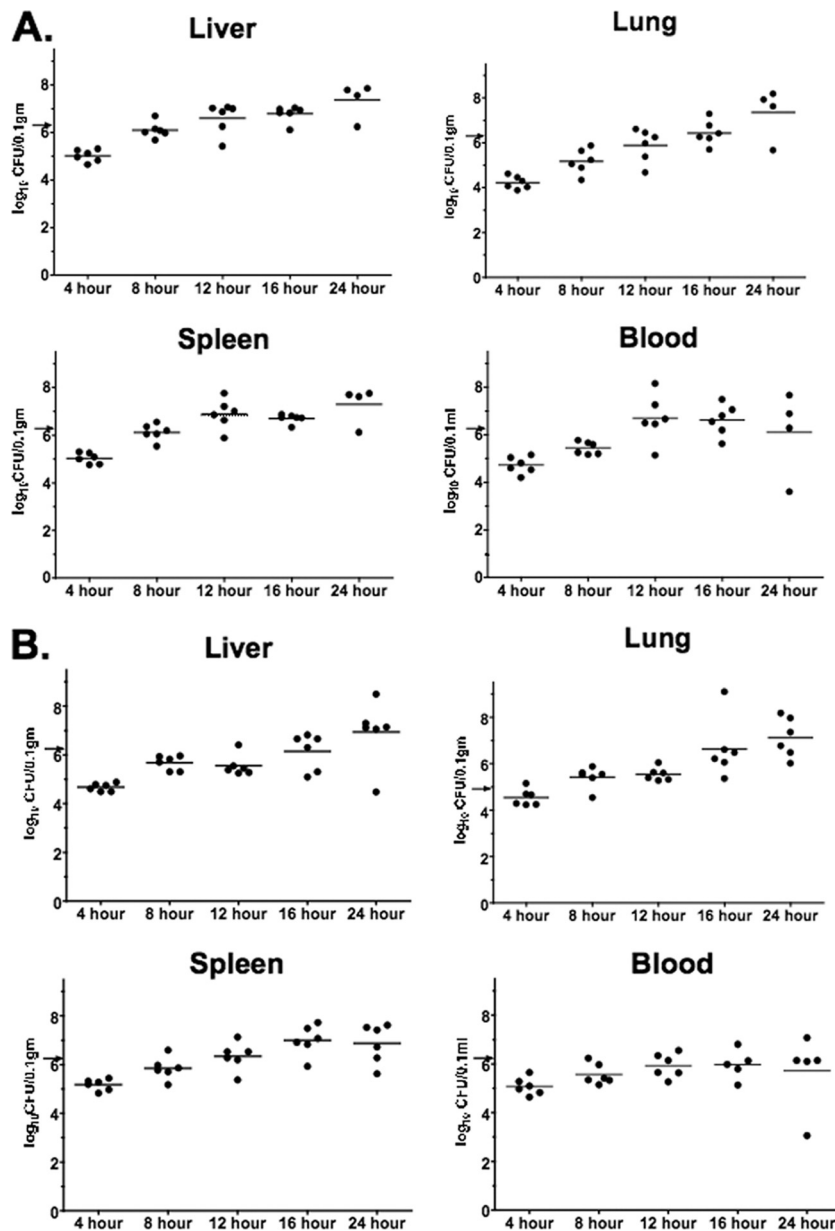[b] Not statistically significant between mouse strains by gender.

FIG. 1. Bacterial kinetics in organs of mice infected i.p. with *A. baumannii* strain 4502. (A) C57BL/6 mice challenged with a 6-LD$_{50}$ dose; (B) C3HeB/FeJ mice challenged with a 0.5-LD$_{50}$ dose. Inoculating doses are indicated by arrows on the y axis. Animals were euthanized at 4, 8, 12, 16, and 24 h postinfection, and livers, lungs, spleens, and blood samples were harvested and plated to determine the number of CFU per gram of tissue or per 0.1 ml of blood. Points represent individual animals. Lines represent median values for bacterial burdens.

ference found in the virulence of strain 4502 between male and female mice. Due to the finding that there was no difference in virulence between males and females with the use of either *A. baumannii* strain, female mice were used in all subsequent studies to avoid male dominance-related injuries that might impact immunological readouts.

Necropsy studies were carried out to follow the course of the infection after intraperitoneal inoculation. The LD$_{50}$ studies had shown that mice succumbed to i.p. infection within 48 h or they survived. Therefore, kinetics of *in vivo* growth of this strain in C3HeB/FeJ and in C57BL/6 mice were established by infecting i.p. and performing necropsies on blood samples,

liver, lungs, and spleen at various time points over the first 16 to 24 h after inoculation. Data in Fig. 1 and 2 show that in both strains of mice, both strains of *Acinetobacter* disseminated from the peritoneum by 4 h postinfection to the blood, lungs, spleen, and liver. In both strains of mice, with the use of either strain 4502 or 5798, *Acinetobacter* multiplied by 1.5 to 3.0 log$_{10}$ over the first 12 h in the organs. The data suggest that the lungs are a site of steady replication of *Acinetobacter*. Organisms were detected in the blood samples at all time points in both experiments, indicating a persisting septic state. For strain 4502 (Fig. 1), bacteria replicated more quickly in C57BL/6 mice, likely due to the higher-level inoculum. For this strain, the bacterial
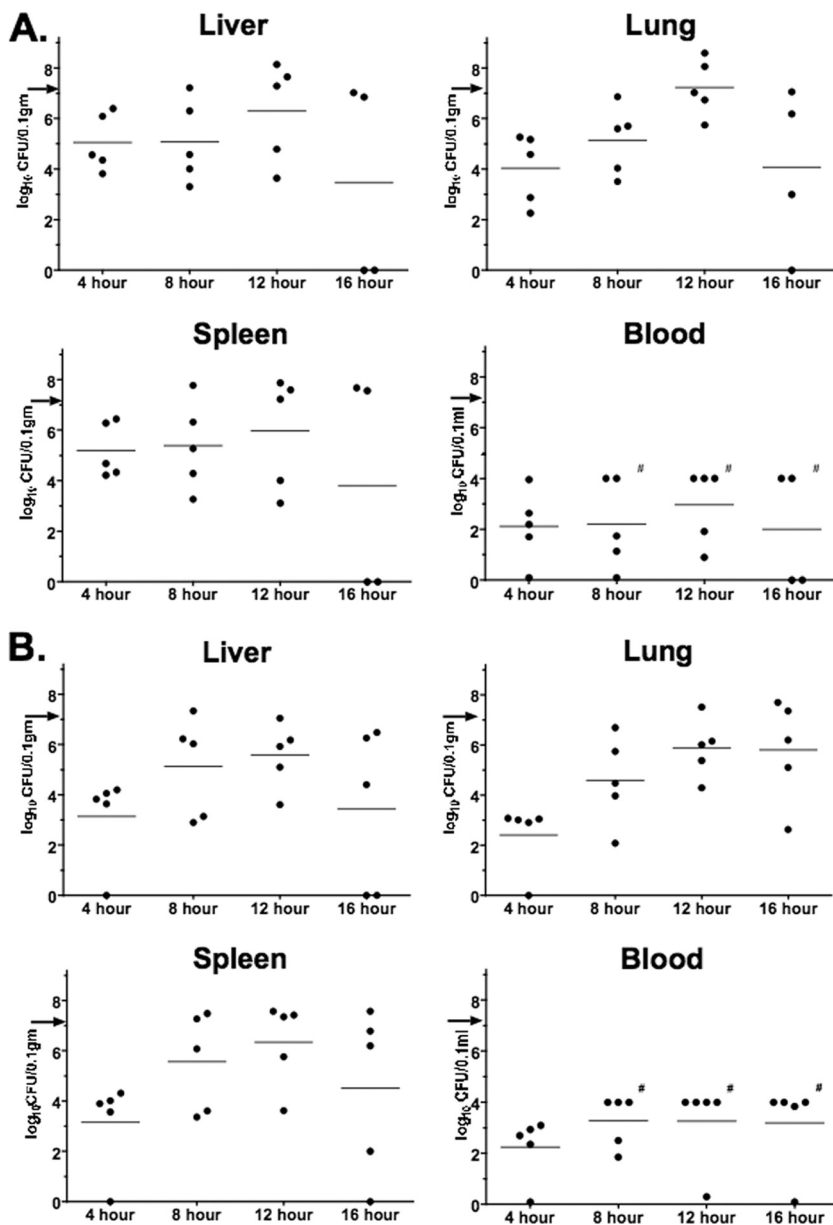
FIG. 2. Bacterial kinetics in organs of mice infected i.p. with *A. baumannii* strain 5798. (A) C57BL/6 mice challenged with a 1-LD$_{50}$ dose; (B) C3HeB/FeJ mice challenged with a 0.2-LD$_{50}$ dose. Inoculating doses are indicated by arrows on the *y* axis. Animals were euthanized at 4, 8, 12, and 16 h postinfection, and livers, lungs, spleens, and blood samples were harvested and plated to determine the number of CFU per gram of tissue or per 0.1 ml of blood. Points represent individual animals. Lines represent median values for bacterial burdens. #, counts > 10^4 CFU/ml.

burden rose over 100-fold between 4 and 12 h p.i. For *A. baumannii* strain 5798 (Fig. 2), bacterial burdens reached maximal levels matching that of the initial inoculums by 12 h p.i., followed by the beginning of clearance of bacteria over the following 4 h, as indicated by the median numbers of bacteria in organs and blood samples. Differences in the rates of multiplication and bacterial burdens at later time points are likely a function of differences in the inoculating doses between experiments.

Importance of neutrophils during *Acinetobacter* infection. Due to the rapidity of the *Acinetobacter* systemic infection, it was hypothesized that neutrophils would play an important

role in the host innate immune response to this organism when inoculated systemically. To determine the role of neutrophils, mice were rendered neutropenic by i.p. injection of anti-Ly6G antibody 24 h before, at the time of, and 24 h after infection. Depletion of neutrophils was followed by challenge with one of two doses of *A. baumannii* strain 4502. As shown in Fig. 3, depletion of neutrophils resulted in sensitization to a sublethal infection of *A. baumannii*, 4502. When challenged with a 0.05-LD$_{50}$ dose, 90% of anti-Ly6G-treated mice succumbed to infection, while all control animals survived. When neutropenic mice were challenged with a 10-fold-higher dose (0.5 LD$_{50}$), most of the animals in both groups succumbed to the infection.
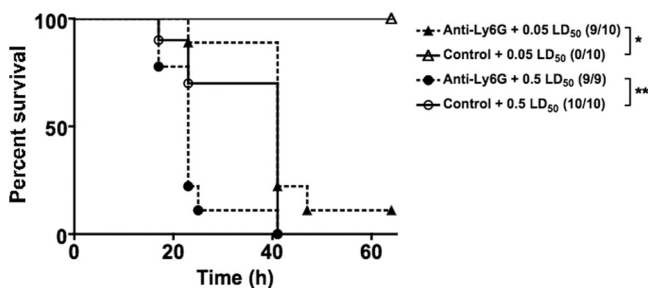
FIG. 3. Neutrophils are important for host innate immune response to systemic *A. baumannii* infection. C3HeB/FeJ mice were injected i.p. with 25 μg of either anti-Ly6G (neutrophil-specific) or control IgG2b antibody 24 h prior to, at the time of, and 24 h after i.p. injection with a given dose of *A. baumannii* strain 4502. Survival was monitored for 7 days. Numbers in parentheses represent number dead/total. *, $P < 0.0001$ for survival; **, $P < 0.05$ for survival distribution based on the log rank test.

However, the mean survival time was reduced from 35 ± 9.7 h in control animals to 23.7 ± 7 h ($P < 0.05$) in the group lacking neutrophils, a significant difference. These data support an important protective role for neutrophils in response to systemic *Acinetobacter* infection.

Production and importance of factors altering neutrophil numbers in *Acinetobacter* infection. Studies were carried out to examine the induction of factors that are chemotactic for neutrophils, following *Acinetobacter* inoculation. A major chemotactic factor for neutrophils is KC/CXCL1, which is upregulated by IL-23 or LPS-induced IL-17 production in the innate arm of the immune response (58). Peritoneal exudate cells (PECs) from *Acinetobacter*-infected or uninfected (saline-injected) mice were tested for their ability to produce IL-17 *in vitro* (Fig. 4). Cells were left untreated or stimulated with various concentrations of recombinant mouse IL-23 (rIL-23) or LPS. Stimulation of PECs from infected mice with rIL-23 resulted in markedly increased (2-fold) IL-17 production, compared to stimulation of naïve PECs. In contrast, LPS induced greater IL-17 production from naïve PECs than from PECs from infected animals, even at the highest dose of LPS tested (10 μg/ml). Further, LPS at 10 μg/ml did not induce as robust a response as IL-23 at 20 ng/ml.

The capacity of *A. baumannii* to induce IL-17 and the chemokine KC/CXCL1 *in vivo* was examined. IL-17 and KC/CXCL1 were measured in peritoneal exudate fluid (PEF) at various time points following i.p. infection. As shown in Fig. 5A, IL-17 was induced as early as 4 h postinfection. IL-17 protein concentrations in the peritoneal fluid showed a biphasic rise, with a plateau at 8 to 12 h and a second escalation in concentration between 12 and 18 h. Levels of KC/CXCL1 were similar, but not identical (Fig. 5B). For KC/CXCL1, a significant rise in protein was observed at 2 h postinfection. The kinetics of induction over the next 16 h showed a biphasic rise, similar to that seen with IL-17.

To determine the importance of these molecules for the innate immune response against *Acinetobacter*, antibody-mediated depletion studies were conducted. IL-17 was depleted by i.p. injection of 70 μg of anti-IL-17 monoclonal antibody 1 h before challenge with *A. baumannii* strain 4502, in accordance with a published protocol (21). Similarly, KC/CXCL1 was de-
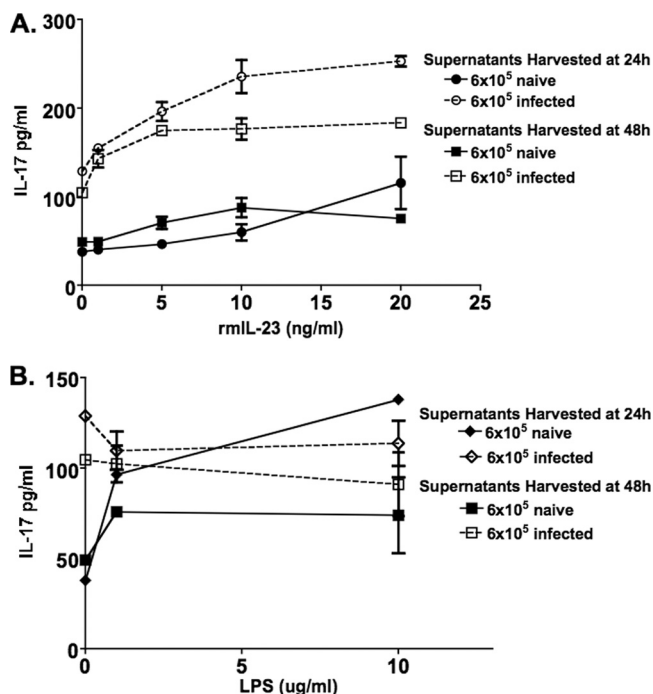

FIG. 4. *In vitro* induction of IL-17 from peritoneal exudate cells by recombinant IL-23 and LPS. PECs were collected from naive C3HeB/FeJ mice or from mice infected with a 1.2-LD$_{50}$ dose of *A. baumannii* strain 4502. Cells plated at the given concentrations were treated with recombinant IL-23 (A) or LPS (B). Culture supernatants were harvested 24 h or 48 h after stimulation and analyzed for IL-17 production by ELISA.

pleted by administration of 50 μg of anti-KC/CXCL1 (10, 21). Control groups were treated with equivalent doses of nonspecific IgG2a monoclonal antibody. At 10 h postinfection, mice were euthanized and spleens, lungs, and blood samples were necropsied to determine bacterial burdens. Depletion of either IL-17 or KC/CXCL1 individually failed to sensitize mice to *A. baumannii*, as assessed by the number of bacteria recovered from the organs or blood samples of mice infected with a 1-LD$_{50}$ dose (Fig. 6A) or a 2-LD$_{50}$ dose (Fig. 6B), in comparison to the level for control antibody-treated animals. A survival experiment was also carried out using 10 mice receiving the control antibody and 10 receiving the anti-IL-17 monoclonal antibody. The challenge dose was 0.2 LD$_{50}$ of strain 4502. All animals survived in both groups, demonstrating a lack of sensitization by the anti-IL-17 antibody.

To more definitively assess whether IL-17 is important for host defense during *A. baumannii* infection, studies were carried out with animals lacking the capacity to produce IL-17. Both WT and IL-17 knockout (IL-17a$^{-/-}$) mice were infected with *A. baumannii* strain 4502, and organs and blood samples were collected at 8 to 10 h postinfection. The lack of IL-17 caused no difference in the bacterial burdens in any organ examined or in the blood samples following a high-dose (Fig. 7A) or low-dose (Fig. 7B) challenge. The IL-17a$^{-/-}$ animals included animals of both sexes, and there was no influence of gender on the outcome. These experiments provide strong evidence that IL-17 alone is not essential for a pro-
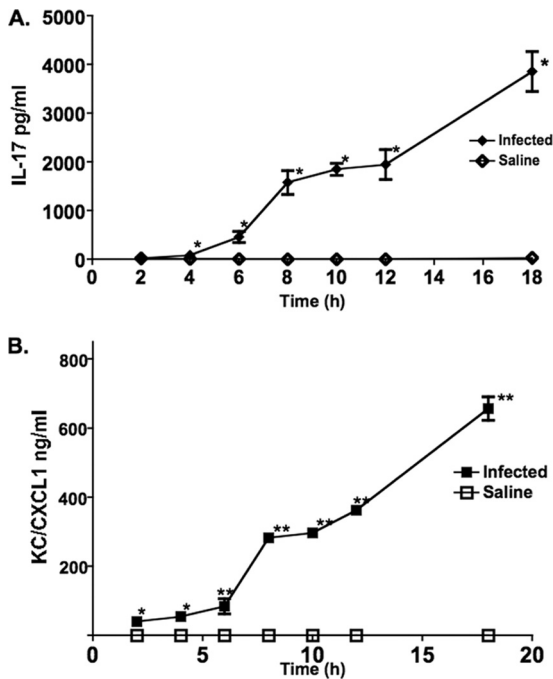
FIG. 5. Kinetics of IL-17, KC/CXCL1, and MCP-1 induction following *Acinetobacter* infection. C3HeB/FeJ mice were infected i.p. with *A. baumannii* strain 4502 in 4 experiments, with doses ranging between 1.1 and 2.3 LD_{50}s, or injected i.p. with saline. Peritoneal exudate fluid was harvested at the indicated time points and analyzed for IL-17 (pg/ml) (A) or KC/CXCL1 (ng/ml) (B) by ELISA. Points represent duplicate samples, 5 to 15 samples per time point. $*$, $P < 0.05$ for infected versus saline; $**$, $P < 0.0001$ for infected versus saline.

tective innate immune response against systemic infection with *A. baumannii*.

DISCUSSION

These studies have established an intraperitoneal, systemic infection model of *A. baumannii* in mice that rapidly results in disseminated disease, thus mimicking several of the clinical syndromes observed during the course of this infection in humans, including pneumonia and sepsis.

Five clinical strains of *A. baumannii*, obtained from the Walter Reed Army Institute of Research, were found to have a spectrum of virulence that spread over a 2.5-$\log_{10}$ difference in LD_{50} values. Classification of this organism as an opportunistic pathogen is supported by LD_{50} values in the range of 7×10^6 in two strains of mice, demonstrating an overall intermediate level of virulence. These results are consonant with results obtained by Obana (31), who tested clinical *Acinetobacter* isolates and found LD_{50} values in a similar range. Information on the clinical condition of the patients from which the five Walter Reed strains were isolated was unavailable. However, antibiograms performed on these strains showed a rough correlation between virulence and degree of antibiotic resistance, a relationship that has not previously been investigated. The tendency for *A. baumannii* strains to exhibit a high level of antibiotic resistance is well established in the literature (1, 2, 6, 7, 18, 20, 22, 27, 28, 33, 39, 49, 59). Resistance is due both to chromosomally encoded resistance genes and to ones found on
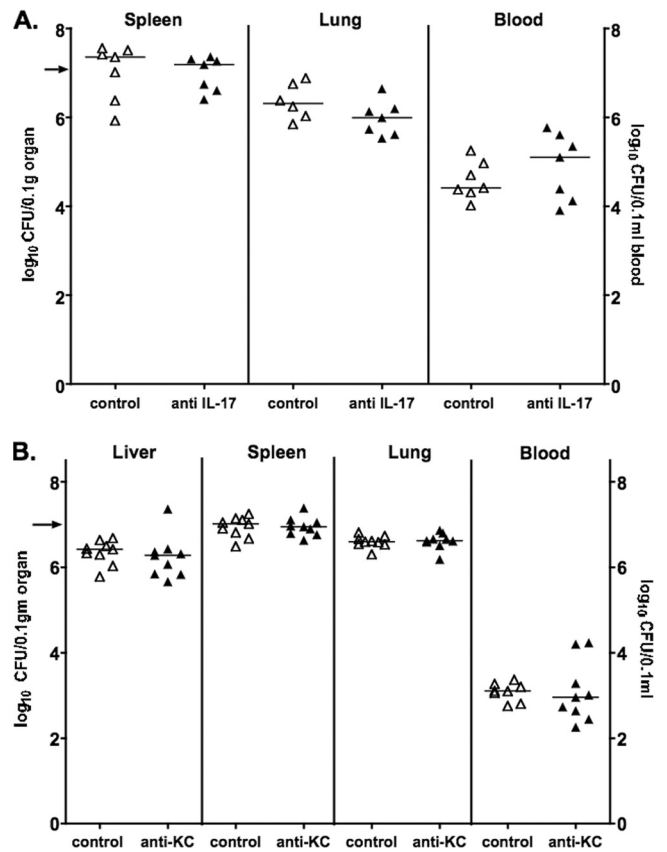

FIG. 6. Depletion of IL-17 or KC/CXCL1 by antibody neutralization does not sensitize C3HeB/FeJ mice to *Acinetobacter* infection. C3HeB/FeJ mice were injected i.p. with either anti-IL-17 MAb (A), anti-KC/CXCL1 MAb (B), or control Ig2a MAb 1 h before i.p. infection with a 1-LD_{50} dose (A) or a 2-LD_{50} dose (B) of *A. baumannii* 4502. Inoculating doses are indicated by arrows on the *y* axis. Mice were euthanized for necropsy at 10 h postinfection. Points represent individual animals. Lines represent median values for bacterial burdens. Levels of organisms in mice treated with anti-17 or anti-KC/CXCL1 are not statistically significantly different from levels in mice given control antibody in any organ at either challenge dose.

plasmids and transposons (12). In the current study, some strains remained susceptible to all antibiotics tested, while three strains were multiple antibiotic resistant, with the exception of one or more of the following antibiotics: tobramycin, amikacin, and imipenem. According to the antibiotic screens, imipenem is the one drug that was effective against all *A. baumannii* strains tested. To our knowledge, these studies are the first to directly compare the antibiotic resistance patterns with the lethality of *A. baumannii* strains in a systemic infection model, and a correlation was observed. This correlation raises concern that clinical *A. baumannii* strains with the greatest resistance to antibiotics may also have additional virulence factors.

Strains 5798 and 4502, which were found to be of the highest or intermediate virulence, were chosen to compare pathogenic potentials of *A. baumannii* in male and female mice of two different mouse strains, C3HeB/FeJ and C57BL/6J. No difference was found in the LD_{50} values for either isolate between males and females of the same strain. For both strains of
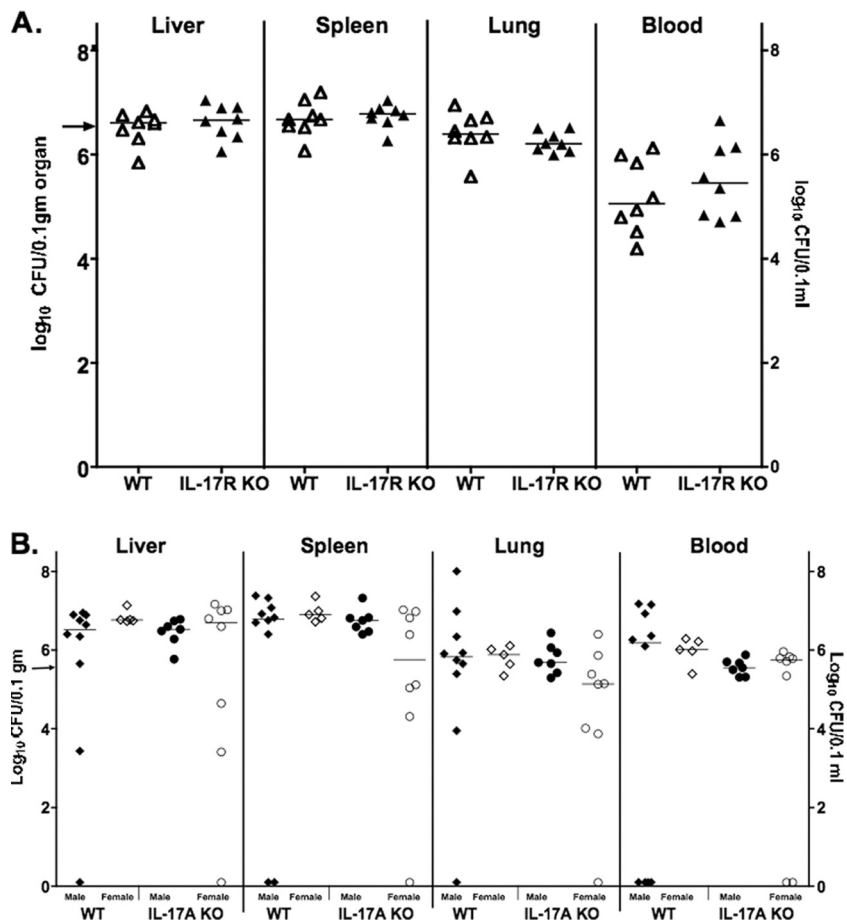
FIG. 7. IL-17A knockout mice are not sensitized to *A. baumannii*. IL-17$a^{-/-}$ and wild-type C57BL/6 mice were infected i.p. with a 17-LD$_{50}$ dose (A) or a 0.6-LD$_{50}$ dose of *A. baumannii* strain 4502. Inoculating doses are indicated by arrows on the y axis. Mice were euthanized for necropsy at 8 h (A) or 10 h (B) postinfection. Points represent individual animals. In panel B, filled symbols represent male mice, and open symbols represent female mice. Lines represent median values for bacterial burdens. Levels of organisms in any organ or blood sample of IL-17$a^{-/-}$ mice are not statistically significantly different from the level for WT mice.

bacteria in both strains of mice, there was rapid dissemination from the peritoneal cavity, the site of injection, to blood and the other organs by 4 h postinfection, showing that i.p inoculation can be considered a model of *Acinetobacter* sepsis, with resultant seeding of other organs, including the lungs. It is noteworthy that organisms actively multiplied in the lung with the use of this model.

Due to the rapidity of the infection, studies focused on the contribution of innate immune responses to host resistance to infection with this organism. Initial studies examined susceptibility to infection following selective depletion of neutrophils by treatment with an antibody to Ly6G, a neutrophil-specific marker. Depletion of neutrophils clearly sensitized mice to intraperitoneal *Acinetobacter* infection. The important contribution of neutrophils to *Acinetobacter* infection has been reported previously, with the use of other methods or routes of inoculation. In early studies to establish a mouse model of *Acinetobacter*, animals were treated with cyclophosphamide, a less selective method of depleting neutrophils (17). Interestingly, the virulence of the WRAIR strains used in the present studies, when the strains were given i.p. to normal, untreated mice, was comparable to or lower than the virulence observed

by this group, with their isolates following cyclophosphamide treatment and intratracheal inoculation. Other investigators, using an intranasal challenge to establish an *Acinetobacter* pneumonia model, found that depletion of neutrophils with the anti-Ly6G antibody resulted in sensitization to infection (54).

The effect of *Acinetobacter* infection on chemokines and chemokine-inducing pathways for neutrophils was also tested in the present experiments. Initial studies examined IL-17, a proinflammatory cytokine known to induce granulopoiesis and to induce production of other proinflammatory cytokines and chemokines that enhance the accumulation of neutrophils, particularly KC/CXCL1 (the homologue of human IL-8). The *in vitro* induction of IL-17 by rIL-23 or by LPS from mouse cells has been reported by several investigators (13, 21, 47, 60). Experiments confirmed this circuit but added an interesting result. It was observed that rIL-23-induced IL-17 production was markedly more robust when peritoneal cells were harvested from *Acinetobacter*-infected mice than from naïve mice. As peritoneal cultures from naïve and infected animals each contained 6 × 10^5 cells, either a new population of IL-23-responsive cells was recruited to the peritoneal cavity following

infection or more cells of a type that were already resident in the peritoneum of naïve animals were activated by the infection and stimulated by the IL-23. In contrast, LPS was able to induce IL-17 from peritoneal cells harvested from naïve mice, but the levels of IL-17 production never reached those elicited by rIL-23 stimulation of cells taken from *Acinetobacter*-infected animals. The lack of increased production of IL-17 observed when cells from infected animals were stimulated *in vitro* with *Salmonella* LPS may be due to the fact that the cells were already maximally stimulated *in vivo* by the *Acinetobacter* LPS, which is known to trigger TLR4 (14, 47). Another way of looking at this is that the cells from infected animals may be tolerant to additional stimulation with LPS.

It was found that *Acinetobacter* induced high levels of IL-17 *in vivo*, which increased at time points after infection, reaching a plateau between 8 and 12 h, and then continued to rise through 18 h. The early induction of IL-17 in the systemic *Acinetobacter* model is comparable to what has been observed using other infectious agents, including *E. coli* (47), *Streptococcus pneumoniae* (26), *Klebsiella pneumoniae* (13, 14, 61, 62), and *Candida albicans* (15). The present studies are the first documentation of the rapid induction of IL-17 during *A. baumannii* infection. IL-17 is known to be responsible for induction of downstream chemokines, such as KC/CXCL1, which are chemotactic for neutrophils (58). The induction of KC/CXCL1 following infection has been documented for a wide variety of pathogens, including bacterial infections such as *Pseudomonas aeruginosa* (45, 51, 56), *Chlamydia muridarum* (63), *Citrobacter rodentium* (50, 55), *Listeria monocytogenes* (53), *Streptococcus pneumoniae* (48, 54), *Leptospira interrogans* (8), and *Borrelia burgdorferi* (3). In the present studies, KC/CXCL1 induction was evaluated following systemic *A. baumannii* infection in C3HeB/FeJ mice. It was found that KC/CXCL1 appeared in PEF in a biphasic manner, similar to IL-17. The biphasic induction of these molecules may be a consequence of the inability to eliminate the bacteria in the early stages of infection, necessitating a secondary wave of cytokine/chemokine induction at time points later than 12 h postinfection.

In spite of robust induction of both IL-17 and KC/CXCL1, experiments presented in this study did not support a crucial role for these molecules in host defense to *Acinetobacter*. It was shown that depletion of either IL-17 or KC using monoclonal antibodies specific for each molecule failed to cause a significant difference in the bacterial burdens of spleens, lungs, and blood in comparison to control antibody-treated mice and did not increase mortality in a survival experiment. The protocols for choice and dose of monoclonal antibodies were based on published results which had been shown to alter resistance to other infections or to reduce neutrophil recruitment. The depletion protocol for IL-17 was found by other investigators to be sufficient to increase vaccinia virus titers (21), reduce *Schistosoma mansoni* granuloma formation (44), and reduce the severity of experimental allergic encephalitis (23). The protocol used to deplete KC levels has been reported to reduce neutrophil infiltration in the lungs and livers following induction of trauma and hemorrhage and to prevent edema formation and hepatocyte damage (10). In another model, using a concentration of anti-KC/CXCL1 antibody less than the one used in our study (50 μg versus 20 μg), IL-6 levels and lung tissue damage were reduced following induction of hemor-

rhage and sepsis (24). Another KC/CXCL1 depletion study demonstrated that KC neutralization with the monoclonal antibody used in the present study reduced detrimental neutrophil infiltration in a model of heterologous nephrotoxic nephritis (4). The possibility cannot be eliminated that the failure of the IL-17 depletion to sensitize mice to *Acinetobacter* was due to a dose that was insufficient to completely eliminate the amount of IL-17 that was induced following infection with this particular pathogen. The use of IL-17-deficient mice provided a second method to probe the function of IL-17 during *A. baumannii* infection. When these mice were injected with either a high or a low infecting dose of *A. baumannii*, no difference in bacterial burdens between IL-17$a^{-/-}$ and wild-type animals was observed. As male and female mice were used in these experiments, gender was not an important variable. This observation is in agreement with data from the IL-17 antibody neutralization studies, showing no difference in bacterial burdens when IL-17 was blocked.

The studies presented here are the first to demonstrate that IL-17 and KC/CXCL1 are both rapidly induced following *A. baumannii* infection. However, in contrast to other reports in the literature, where the absence of either one of these molecules sensitized mice to a variety of infections or pathological conditions, depletion of either molecule by itself, or inhibition of IL-17 signaling via absence of this cytokine, had no effect on *Acinetobacter* systemic infection. There is always the possibility that use of higher doses of antibody or necropsy analysis at later time points would yield different results from those obtained with the conditions tested. In addition, while the current experiments seem to rule out a role for the IL-17/CXCL1 circuit in host defense to *Acinetobacter*, it is possible that other chemokines that attract neutrophils, including MCP-1 and MIP-2, may provide a bypass pathway for recruiting neutrophils. It may be necessary to block all pathways to neutrophil recruitment to sensitize mice to *Acinetobacter*.

In summary, it has been shown that systemic infection with *Acinetobacter* results in sepsis and pneumonia, that *Acinetobacter* strains differ markedly in virulence that correlates loosely with degree of antibiotic resistance, that neutrophils are important for host defense to systemic *Acinetobacter* infection, and that neither IL-17 nor KC/CXCL1 is crucial for resistance to *Acinetobacter*. Future studies should address the importance of other pathways of neutrophil recruitment in host defense to this organism.

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