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Neutrophils Play an Important Role in Host Resistance to Respiratory Infection with *Acinetobacter baumannii* in Mice[▽]

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***Acinetobacter baumannii* has emerged as a major cause of both community-associated and nosocomial pneumonia, but little is known about the cellular and molecular mechanisms of host defense against respiratory infection with this bacterial pathogen. In this study, we examined the role of neutrophils in host resistance to pulmonary *A. baumannii* infection in a mouse model of intranasal (i.n.) infection. We found that neutrophils were rapidly recruited to the lungs following i.n. inoculation of the pathogen and declined to baseline level upon clearance of the infection. Depletion of neutrophils using monoclonal antibody RB6-8C5 prior to infection resulted in an acute lethal infection that was associated with enhanced bacterial burdens in the lung ($P < 0.05$) and extrapulmonary dissemination to the spleen. The increased susceptibility to *A. baumannii* in neutropenic mice was associated with a delay in the mRNA expression and production of early proinflammatory cytokines such as tumor necrosis factor alpha, interleukin-6, keratinocyte chemoattractant protein, monocyte chemoattractant protein 1, and macrophage inflammatory protein 2 (MIP-2) in the lungs and development of severe bronchopneumonia and lymphoid tissue destruction in the spleen. Moreover, i.n. administration of the neutrophil-inducing chemokine MIP-2 to normal mice induced a pulmonary influx of neutrophils and significantly enhanced the clearance of *A. baumannii* from the lungs ($P < 0.01$). These results imply that neutrophils play a critical role in host resistance to respiratory *A. baumannii* infection.**

Acinetobacter baumannii is a ubiquitous gram-negative bacterium that can survive for prolonged periods in the environment. During the last decade, this organism has emerged as a major cause of both community-associated and nosocomial infections worldwide (11, 14). The overall 30-day mortality of *Acinetobacter* infection can be as high as 49%, with the respiratory tract being implicated as an important portal of entry (18). Moreover, infections with *A. baumannii* have become increasingly difficult to treat because of its rapid development of resistance to multiple antibiotics (9). Despite its clinical importance, little is known about the cellular and molecular mechanisms of host defense against respiratory *A. baumannii* infection (9, 17, 26).

Innate immunity plays a crucial role in determining the outcome of respiratory infection with many bacterial pathogens, including *A. baumannii* (17, 35). In this regard, it has been recently shown that the CD14/Toll-like receptor 4 pathway is important in the control of intranasal (i.n.) *A. baumannii* infection in mice (17). However, little else is known about the nature of the innate cellular response to *A. baumannii* infection. Neutrophils play an important role in early control of acute bacterial infections by killing bacterial pathogens through powerful oxidative and nonoxidative mechanisms and through the production of inflammatory and immunoregulatory cytokines and chemokines (24). However, the contribution of neutrophils in host resistance to respiratory *A. baumannii* infection has not been directly investigated, although some indirect evidence implies that they may play an important role. For instance, clinical studies have shown that *A. baumannii* is one of the most frequently isolated gram-negative bacteria in neutropenic febrile patients in nosocomial settings (16). Experimental studies have also shown that neutrophils and neutrophil-recruiting chemokines are present at the site of *A. baumannii* infection (15, 17, 26), and neutrophil granule extract is bactericidal to other species of *Acinetobacter* (12, 22).

In the current study, as the first step to furthering our understanding of the role of innate immunity against respiratory *A. baumannii* infection, we determined the importance of neutrophils in the control of i.n. initiated infection with *A. baumannii* in mice. We found that neutrophils were rapidly recruited to the lungs after infection and that depletion of these cells exacerbated disease, resulting in an acute and lethal outcome. This increased susceptibility was associated with increased bacterial replication and extrapulmonary dissemination of the pathogen and a decrease in the early proinflammatory cytokine responses in the lung.

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MATERIALS AND METHODS

Mice. Eight- to 12-week-old specific-pathogen-free female C57BL/6 and BALB/c mice were purchased from Charles Rivers Laboratories (St. Constant, Quebec). The animals were housed under specific-pathogen-free conditions in a small animal containment level 2 facility and given free access to sterile water and certified mouse chow. The animals were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, and the experimental procedures were approved by the institutional animal care committee.

***A. baumannii* and i.n. inoculation.** Fresh inocula were prepared for each experiment from a frozen stock of *A. baumannii* (ATCC 17961; American Type Culture Collection, Manassas, VA). Stock vials of *A. baumannii* were thawed and regrown in tryptic soy broth medium for 3.5 h at 37°C with rotation (100 rpm), centrifuged at 12,000 × *g* for 15 min, resuspended in phosphate-buffered saline,

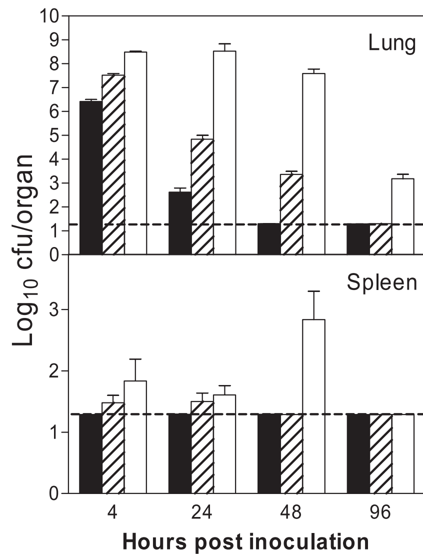


FIG. 1. Bacterial burdens in the lungs and spleens of C57BL/6 mice inoculated by the i.n. route with 10^6 (solid bars), 10^7 (shaded bars) or 10^8 (open bars) CFU of *A. baumannii*. The data are presented as mean $\log_{10}$ CFU/organ ± SD ($n = 5$) and represent one of at least two experiments with similar results. The detection limit (dashed lines) for the bacterial burdens was 1.3 $\log_{10}$ CFU/organ.

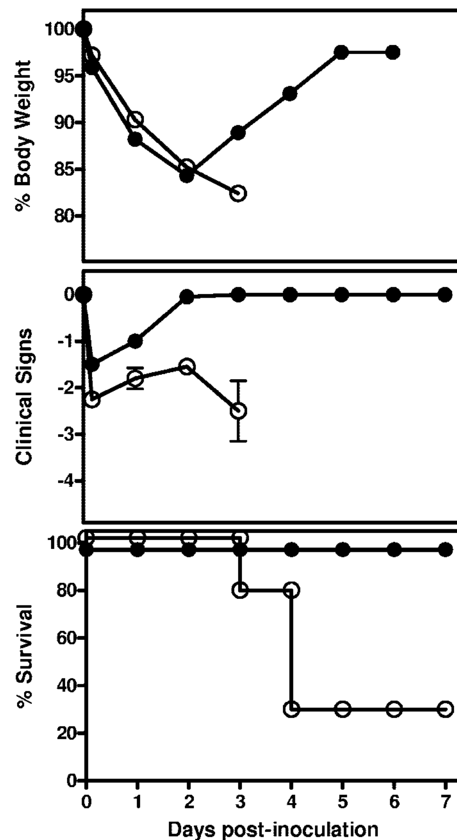


FIG. 2. Effect of RB6 treatment on the body weight, clinical scores, and survival of mice after i.n. inoculation with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 250 μg of RB6 (open circles) or an equivalent amount of control rIgG (closed circles) at −18 and +24 h after i.n. challenge with 10^7 CFU of *A. baumannii*. Body weight, clinical scores, and survival were monitored for 7 days. The data are compiled from two independent experiments with similar results.

and used immediately. Unless otherwise specified, anesthetized mice were inoculated i.n. with approximately 10^7 *A. baumannii* organisms in 50 μl saline. Actual inoculum concentrations were determined by plating 10-fold serial dilutions on brain heart infusion agar supplemented with 50 μg/ml streptomycin. The clinical signs of mice were monitored and recorded with following scores: 0, no abnormal clinical sign; −1, ruffled fur but lively; −2, ruffled fur, moving slowly, down, and sick; −3, ruffled fur, squeezed eye, hunched, hardly moving, and very sick; −4, moribund; and −5, dead.

MAb treatment. For in vivo depletion of neutrophils, mice were treated intraperitoneally (i.p.) either with the rat anti-mouse monoclonal antibody (MAb) RB6-8C5 (RB6) (25 or 250 μg in 200 μl sterile saline), which recognizes the neutrophil surface marker Gr-1, or with an equivalent amount of purified rat immunoglobulin G (rIgG) (Sigma Chemical Co., St. Louis, MO) as described previously (6, 8, 28). Treatments were administered 18 h before and 1 day after initiation of *A. baumannii* infection. The specificity and efficacy of this MAb have been well established by us and others (3, 7, 8, 10, 23, 25, 30, 31). Treatment with 250 μg of this MAb depleted >95% of circulating neutrophils for at least 2 days as determined by total and differential blood leukocyte counts (7). In other experiments, mice were treated i.p. with hamster anti-mouse MAb 2E2 (200 μg; kindly supplied by the National Cancer Institute, Rockville, MD) (20) or rat anti-mouse MAb R46A2 (500 μg) (4) to neutralize endogenous tumor necrosis factor alpha (TNF-α) or gamma interferon (IFN-γ), respectively, or with MAb TIB210 (4) or 120G8 (29) to deplete $CD8^+$ T cells or plasmacytoid dendritic cells, respectively.

BAL and sample collections. Groups of five RB6- or rIgG-treated C57BL/6 mice were sacrificed at 0, 4, 24, and 72 h after i.n. inoculation with $\sim 10^7$ CFU *A. baumannii*. Blood samples were collected for serum separation. The trachea was exposed through a midline incision and cannulated with a plastic catheter. Lungs were lavaged five times with 1.0 ml phosphate-buffered saline supplemented with 3 mM EDTA as previously described (5). Cytospin slides of 2×10^4 bronchoalveolar lavage (BAL) fluid cells were prepared using a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) and stained with HemaStat 3 (Fisher, Pittsburgh, PA). Differential cell counts were determined by examining 200 cells, and the total numbers of neutrophils, lymphocytes, and macrophages were calculated. The lavage fluid was centrifuged at 2,450 × *g* for 7 min, and the supernatant was collected, filter sterilized, and stored at −80°C for cytokine analysis.

Quantitative bacteriology and histopathology. For bacterial kinetic analysis, lungs and spleen were aseptically removed and homogenized in sterile saline using aerosol-proof homogenizers. Aliquots (100 μl) of 10-fold serial dilutions of the homogenates were cultured, in duplicate, on plates of brain heart infusion agar supplemented with 50 μg/ml streptomycin to quantify the number of viable *A. baumannii* organisms in the respective organs. For histopathological examination, the lung and spleen were fixed immediately in 10% neutral buffered formalin and processed by standard paraffin embedding methods (Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Ontario). Sections were cut 4 μm thick, stained with hematoxylin-eosin, and examined by light microscopy (19).

Tissue RNA extraction and quantitative reverse transcription-PCR analysis. For cytokine/chemokine mRNA expression analysis, the lung and spleen were dissected, immersed immediately in RNAlater (QIAGEN, Germantown, MD), and stored at −20°C until extraction.

Relative amounts of cytokine and chemokine mRNAs in the lung and spleen over the course of infection in the two groups of mice were estimated using a real-time PCR-based method essentially as described elsewhere (13, 19). Briefly, total RNA was extracted from tissues, and cDNA was prepared, amplified, and quantified using primers and probes designed with the Primer3 program available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Mouse housekeeping gene β2 microglobulin mRNA was measured and used to calculate relative expression ($2^{-\Delta\Delta CT}$). Levels of cytokine and chemokine PCR products were normalized to β2 microglobulin, which was not differentially expressed over the course of infection in RB6- and rIgG-treated mice. Data are presented as the average of relative expression values compared to those in the corresponding tissues of mice killed immediately before infection (i.e., 0 h, uninfected) (19, 21). The differences in ΔC_T between RB6- and rIgG-treated mice were compared by the Mann-Whitney U test, based on the method of Yuan et al. (34).

Measurement of serum and BAL fluid cytokines and chemokines. Serum and BAL fluid levels of cytokines and chemokines were determined using the mouse panel of Fluorokine MAP Multiplex Kits (R & D Systems, Inc. Minneapolis,

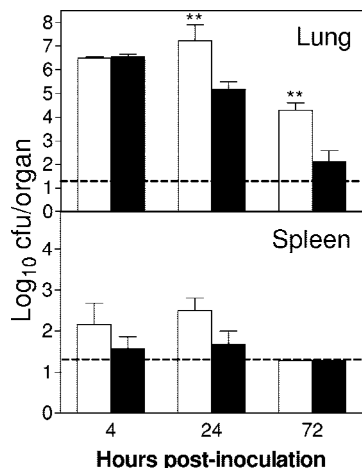


FIG. 3. Effect of RB6 treatment on bacterial burdens in the lung and spleen following i.n. inoculation with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 250 μg of RB6 (open bars) or an equivalent amount of control rIgG (solid bars) at −18 and +24 h after i.n. challenge with 10^7 CFU of *A. baumannii*. Bacterial burdens in the lung and spleen were determined by quantitative bacteriology. The data are presented as mean ± SD (n = 5) and represent one of at least two experiments with similar results. The detection limit (dashed lines) for the bacterial burdens was 1.3 $\log_{10}$ CFU/organ. **, $P < 0.01$ versus rIgG-treated mice.

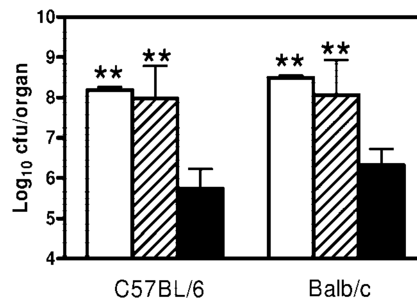


FIG. 4. Effect of RB6 treatment dosage on bacterial burdens in the lung following i.n. inoculation with *A. baumannii*. Groups of five C57BL/6 or BALB/c mice were treated by i.p. injection with either 250 μg (open bars) or 25 μg (hatched bars) of RB6 or an equivalent amount of control rIgG (solid bars) at 18 h before i.n. challenge with 2.6 × 10^7 CFU of *A. baumannii*. Bacterial burdens in the lung were determined by quantitative bacteriology. The data are presented as mean ± SD (n = 5), and the detection limit for the bacterial burdens was 1.3 $\log_{10}$ CFU/lung. **, $P < 0.01$ versus rIgG-treated mice.

MN) on a Luminex 100IS system (Luminex, Austin, TX). Undiluted BAL samples and 1:2 diluted serum samples (50 μl) were analyzed as specified by the manufacturer. The analysis was done in duplicate, and the cytokine/chemokine concentrations were calculated against the standards using Beadview software (version 1.03; Upstate) (19).

Statistical analysis. Data are presented as means ± standard deviations (SD) for each group, unless otherwise specified. Differences in quantitative measurements were assessed by Student's t test or two-way analysis of variance followed by Bonferroni's post hoc multiple-comparison tests when appropriate. The survival rates between groups were analyzed by the log rank test. Differences were considered significant when the P value was <0.05.

RESULTS

Susceptibility of C57BL/6 mice to i.n. *A. baumannii* inoculation and kinetics of bacterial replication and extrapulmonary dissemination. Groups of C57BL/6 mice were inoculated i.n. with 10^6, 10^7, or 10^8 CFU *A. baumannii* on day 0, and their clinical signs and outcome were monitored. Mice that received 10^6 CFU *A. baumannii* showed no clinical signs of infection throughout the experiment, whereas mice inoculated with 10^7 or 10^8 CFU *A. baumannii* developed mild to moderate clinical signs of infections (i.e., ruffled fur, lethargy, and hunched stature) as early as 24 h postinoculation (p.i.), remained symptomatic for the next 48 h, and appeared clinically healthy by 96 h. However, no mouse died of the infection at any challenge dose. Five mice from each group were sacrificed at 0, 4, 24, 48, or 96 h p.i. and used for the determination of bacterial burdens in the lungs and spleens. As can be seen in Fig. 1, the kinetics of the bacterial growth and subsequent clearance of infection in the lungs was related to the dose of initial inoculation. However, regardless of the number of bacteria administered, the number of *A. baumannii* organisms in the lungs transiently increased at 4 h and then gradually declined over the course of infection, although the rate of decline varied depending on the initial inoculum dosage. Mice that received 10^6 CFU *A. baumannii* cleared the infection almost completely by 48 h; the group of mice receiving 10^8 CFU showed little change in the bacterial burden in the lungs during the first 48 h after inoculation and retained moderate numbers of bacteria by 96 h, whereas the mice that received the lower doses (10^6 or 10^7 CFU) showed no cultivable bacteria by this time (Fig. 1). There was no extrapulmonary dissemination detected in the group receiving the lowest dose (10^6 CFU), but small numbers of viable *A. baumannii* organisms were cultured from the

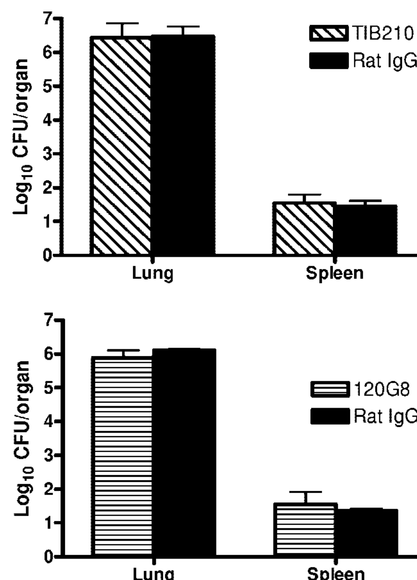
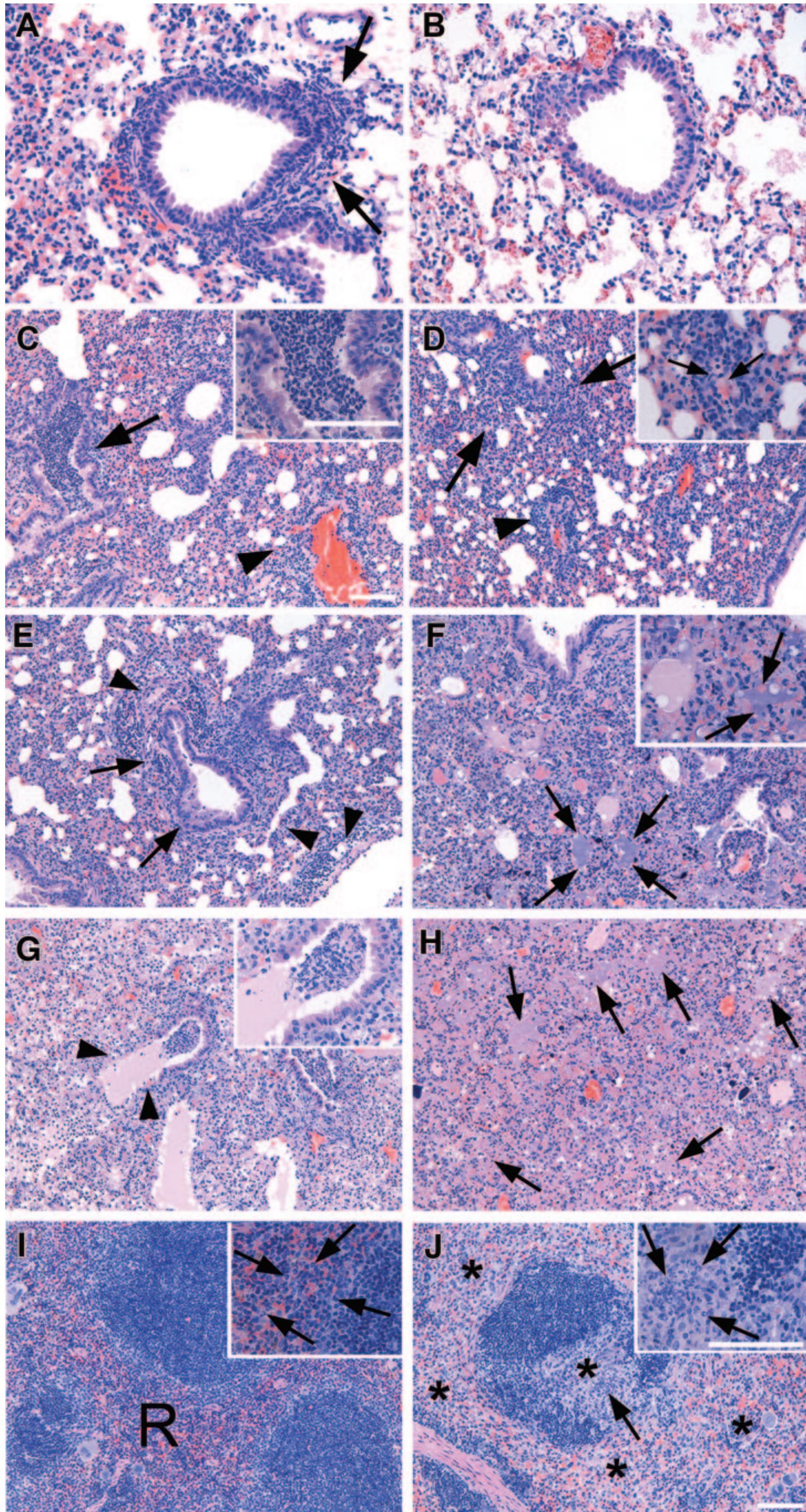


FIG. 5. Effect of depletion of $CD8^+$ T cells or plasmacytoid dendritic cells on bacterial burdens in the lung following i.n. inoculation with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 500 μg of MAb TIB210 (top), 150 μg of MAb 120G8 (bottom), or an equivalent amount of control rIgG at 18 h before i.n. challenge with 2.5 × 10^7 CFU of *A. baumannii*. Bacterial burdens in the lung were determined by quantitative bacteriology. The data are presented as mean ± SD (n = 5), and the detection limit for the bacterial burdens was 1.3 $\log_{10}$ CFU/lung.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spleens of the groups receiving the medium (10^7 CFU) and high (10^8 CFU) challenge doses for up to 48 h. No viable organisms were cultured from the spleens of any group at 96 h p.i. (Fig. 1). Upon necropsy at 7 days p.i., mice inoculated with the medium and high doses of *A. baumannii* showed patches or areas of lung consolidation, while the low-dose group showed no abnormal gross or microscopic pulmonary changes by this time (data not shown).

Depletion of neutrophils by RB6 MAb treatment exacerbates i.n. *A. baumannii* infection. We next determined the potential contribution of neutrophils to host resistance against i.n. *A. baumannii* infection by depleting these leukocytes in vivo with MAb RB6. Groups of five C57BL/6 mice were treated with 250 μg of RB6 or an equivalent amount of control rIgG 18 h before and 1 day after i.n. inoculation with 10^7 CFU *A. baumannii* and their clinical signs monitored. Treatment of uninfected mice with RB6 or rIgG showed no effect on the body weight, and the mice showed no clinical signs of infection throughout the experiment (data not shown). However, RB6-treated challenged mice showed significantly increased susceptibility to infection, with all animals showing more severe clinical signs and substantial loss of body weight, compared with rIgG-treated challenged mice (Fig. 2, top and middle). Moreover, 70% of RB6-treated mice died of infection between 72 and 96 h p.i., while all rIgG-treated mice survived the infection ($\chi^2 = 8.81$; $P < 0.005$) (Fig. 2, bottom). At day 7, the RB6-treated mice that survived the infection remained sick with mild signs of disease (i.e., slightly ruffled fur and moderately reduced activity), whereas all rIgG-treated mice were normal in appearance and activity.

Since neutrophils are phagocytic and potentially bactericidal effector cells, we next determined whether neutrophil depletion increases the bacterial burden in the lung and promotes extrapulmonary dissemination of the pathogen. As shown in Fig. 3, RB6-treated mice showed significantly higher bacterial burdens ($P < 0.01$) in the lungs than rIgG-treated mice following i.n. inoculation with 10^7 CFU of *A. baumannii*, with 100- and 1,000-fold increases at 24 and 72 h p.i., respectively. At day 7, no *A. baumannii* was isolated from the lungs of rIgG-treated mice, while $\log_{10}$ 5.0 CFU of *A. baumannii* were cultured from the lungs of the remaining RB6-treated mice that survived the infection. Moreover, there were substantially greater numbers of *A. baumannii* organisms in the spleens of RB6-treated mice than in rIgG-treated mice at 4 and 24 h p.i. (Fig. 3), although the differences did not reach statistical significance. Despite the frequent culture of low numbers of *A. baumannii* organisms from the spleens of RB6-treated, inoculated mice, only a few organisms were occasionally cultured in blood samples of the same mice (data not shown). The reason for this is not clear, and it could be because (i) the bacteriemia was only very transient, (ii) the level of bacteriemia was too low to be cultured by our method, or (iii) the route of the bacterial spread from lungs to spleens was not hematogenous.

Since it has recently been shown that in addition to neutrophils, other cells (such as a subset of $CD8^+$ T cells and plasmacytoid dendritic cells) also express Gr-1 markers (25, 32, 33), additional experiments were performed to confirm the importance of neutrophils in the observed effect on the enhanced susceptibility in RB6-treated mice. C57BL/6 mice were treated i.p. with low doses (25 μg) of MAb RB6 18 h before i.n. challenge with *A. baumannii*, since it has previously been shown that this treatment regimen has no adverse effect on other, nonneutrophil $Gr\text{-}1^+$ cell populations (25, 33). As can be seen in Fig. 4, the bacterial burdens in the lungs of low-dose-RB6-treated mice were almost identical to those in mice treated with the high dose (250 μg). Moreover, the effect of low and high doses of RB6 on the enhanced susceptibility of mice to i.n. *A. baumannii* infection was also observed in similarly treated BALB/c mice (Fig. 4). Furthermore, mice depleted of $CD8^+$ T cells or plasmacytoid dendritic cells showed similar susceptibility to i.n. *A. baumannii* infection as rIgG-treated mice (Fig. 5). These data suggest that depletion of neutrophils with MAb RB6 enhances local growth (in the lung) of *A. baumannii* with mild systemic dissemination (in the spleen) in mice.

Histopathological observation. To further elucidate the role of neutrophils in host resistance against i.n. *A. baumannii* infection, the lungs and spleens were collected from RB6- or rIgG-treated mice at various time points after i.n. challenge with 10^7 CFU *A. baumannii*. There was no remarkable change in the lungs or spleens from RB6- or rIgG-treated mice at 0 h. At 4 h, rIgG-treated mice showed neutrophil infiltration in the

FIG. 6. Histopathological findings for the lungs and spleens from control rIgG- and RB6-treated mice killed at 4 (A and B), 24 (C and D), and 72 (E to J) h after i.n. inoculation with 10^7 CFU *A. baumannii*. (A and B) The lung from an rIgG-treated mouse killed at 4 h (A) shows early neutrophil infiltration in the peribronchial areas (arrows), whereas the lung from a RB6-treated mouse killed at the same time (B) shows no remarkable change. (C) The lung from an rIgG-treated mouse killed at 24 h shows severe bronchopneumonia with the presence of large numbers of neutrophils in the lumen of bronchi (arrow) and large bronchioles and moderately severe infiltration of mixed neutrophils and mononuclear cells in the perivascular and peribronchial areas (arrowhead). Inset, higher magnification showing the presence of large numbers of neutrophils in the lumen of a bronchus. (D) The lung from a RB6-treated mouse killed at 24 h shows a predominantly interstitial pneumonitis (arrows) and perivascular accumulation of mononuclear cells (arrowhead). Inset, higher magnification showing the accumulation of mononuclear cells around a small blood vessel (arrows). (E and F) The lungs from rIgG-treated mice killed at 72 h show extensive perivascular (arrowheads) and moderate peribronchial (arrows) accumulation of lymphoid cells admixed with some neutrophils (E) and complete consolidation together with the presence of large numbers of neutrophils admixed with many mononuclear cells and large numbers of bacterial colonies (arrows) (F). Inset, higher magnification showing the presence of mixed inflammatory cells and bacterial colonies (arrows). (G and H) The lungs from RB6-treated mice killed at 72 h show severe acute bronchopneumonia with the erosion of the epithelial mucosa (arrowheads and inset) (G) and complete consolidation with the presence of large numbers of bacteria (arrows) and necrotic cellular debris but with little mononuclear cell response (H). (I) The spleen from an rIgG-treated mouse killed at 72 h shows moderate expansion of the red pulp (R) and the infiltration of small numbers of neutrophils in the interfollicular areas (arrows within inset). (J) The spleen from an RB6-treated mouse killed at 72 h shows a moderate degree of lymphocyte depletion (*) and infiltration of neutrophils in both the red pulp and the lymphoid follicles (arrow). Inset, higher magnification showing the infiltration of neutrophils in the lymphoid follicle (arrows) and the depletion of lymphocytes. Hematoxylin and eosin staining was used. Bars, 40 μm.

perivascular and peribronchial areas (Fig. 6A) and cellular exudates in the lumens of large airways. In contrast, RB6-treated mice showed no remarkable change at 4 h despite the presence of nearly 10^7 bacteria in the lungs (Fig. 6B). At 24 h, rIgG-treated mice showed severe bronchopneumonia consisting of large numbers of neutrophils in the lumens of bronchi and large bronchioles and moderately severe infiltration of mixed neutrophils and mononuclear cells in the perivascular and peribronchial areas (Fig. 6C). The lung lesions in the RB6-treated mice at this time point were mainly interstitial and consisted primarily of perivascular accumulations of mononuclear cells (Fig. 6D). In addition, by 24 h of infection, the RB6-treated mice showed the infiltration of small numbers of neutrophils in the red pulps and interfollicular areas of the spleen. By 72 h, the lesions seen in the lungs from rIgG-treated mice were much more variable than at earlier time points. Overall, the lungs were highly consolidated in many areas. In some areas, there was an extensive perivascular and, to a lesser extent, peribronchial accumulation of lymphoid cells admixed with some neutrophils (Fig. 6E) and small to moderate numbers of neutrophils in the lumens of associated airways. In other areas, the lungs showed complete consolidation associated with the presence of large numbers of neutrophils admixed with many mononuclear cells and large numbers of bacteria (Fig. 6F). The spleens from these mice showed a moderate expansion of the red pulp, due to the increase in the numbers of macrophages and reticuloendothelial cells, and the infiltration of small numbers of neutrophils in the interfollicular areas (Fig. 6I). The lungs from RB6-treated mice killed at 72 h showed extensive bronchopneumonia with the presence of large numbers of neutrophils, fibrinous exudates, and cellular debris in the lumens of many airways (Fig. 6G). Some affected airways showed the erosion of the epithelial mucosa. In the severely affected areas, the lungs were completely consolidated, with the presence of large numbers of bacteria and necrotic cellular debris (Fig. 6H) but with little mononuclear cell response. Many lymphoid follicles in the spleens of these mice showed a moderate degree of lymphocyte depletion and infiltration of neutrophils in both the red pulp and the lymphoid follicles (Fig. 6J).

Effect of depletion of neutrophils on the cellular composition in the lung after i.n. *A. baumannii* inoculation. To determine the kinetics of pulmonary neutrophil recruitment, groups of five RB6- and rIgG-treated mice were killed at 0, 4, 24, and 72 h after i.n. inoculation with 10^7 CFU *A. baumannii*. The lungs were lavaged and total and differential cell counts in the BAL fluid determined. There was no significant difference in the total BAL cells between RB6- and rIgG-treated mice at 0 h ($3.10 \times 10^5 \pm 0.49 \times 10^5$ cells versus $1.78 \times 10^5 \pm 0.59 \times 10^5$ cells), with 99% being macrophages. i.n. inoculation of rIgG-treated mice with 10^7 CFU *A. baumannii* induced a rapid recruitment of neutrophils into the lung, which was evident as early as 4 h, peaked at 24 h, and remained highly elevated at 72 h, with 67.3, 93.0, and 71.0% of BAL fluid cells, respectively, being neutrophils (Fig. 7). RB6 treatment remarkably reduced both the percentage and the absolute numbers of neutrophils in the bronchoalveolar space at 4 h p.i., although there was no substantial effect on the total number of BAL fluid cells. By 24 h, the RB6-treated mice had significantly (~10-fold) fewer total BAL fluid cells and neutrophils ($P < 0.005$), whereas the total number of alveolar macrophages was not significantly affected (Fig. 7). By 72 h, the total and differential BAL fluid cells were comparable for RB6- and rIgG-treated mice. The numbers of lymphocytes or eosinophils in the BAL fluid throughout the course of the experiment in both groups of mice were negligible.

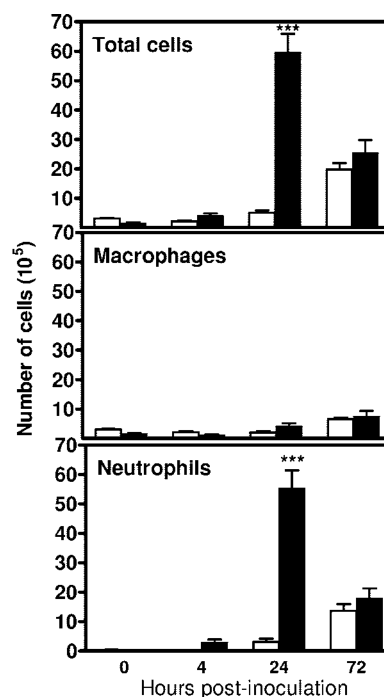

FIG. 7. Effect of RB6 treatment on the composition of cell populations in the BAL fluid from mice i.n. inoculated with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 250 μg of RB6 (open bars) or an equivalent amount of control rIgG (solid bars) at −18 and +24 h after i.n. challenge with 10^7 CFU of *A. baumannii*. At the indicated times, mice were exsanguinated, their lungs were lavaged, and total and differential cell counts were determined. Error bars indicate SD. ***, $P < 0.005$ versus control rIgG-treated mice.

Effect of depletion of neutrophils on pulmonary and systemic cytokine/chemokine responses to i.n. *A. baumannii* challenge. To examine the effect of neutrophil depletion on pulmonary and systemic cytokine/chemokine responses after i.n. *A. baumannii* infection, total RNA was extracted from lungs and spleens from RB6- or rIgG-treated mice over the course of infection and assessed for mRNA expression of a selected panel of proinflammatory cytokines/chemokines by real-time reverse transcription-PCR (Fig. 8). Among the tested cytokines and chemokines, there were substantial increases in interleukin-1β (IL-1β), IL-6, keratinocyte chemoattractant protein [KC], macrophage inflammatory protein 1 α [MIP-1α], MIP-2, monocyte chemoattractant protein 1 (MCP-1), and TNF-α mRNA expression and a moderate increase in IL-10 mRNA expression in the lungs of both RB6- and rIgG-treated mice over the course of infection, but with different kinetics (Fig. 8). The mRNA expression for most of these cytokines and chemokines peaked at 4 h and subsided substantially or returned to baseline by 72 h in the lungs of rIgG-treated mice. In contrast, there was a delay in the peak mRNA expression of

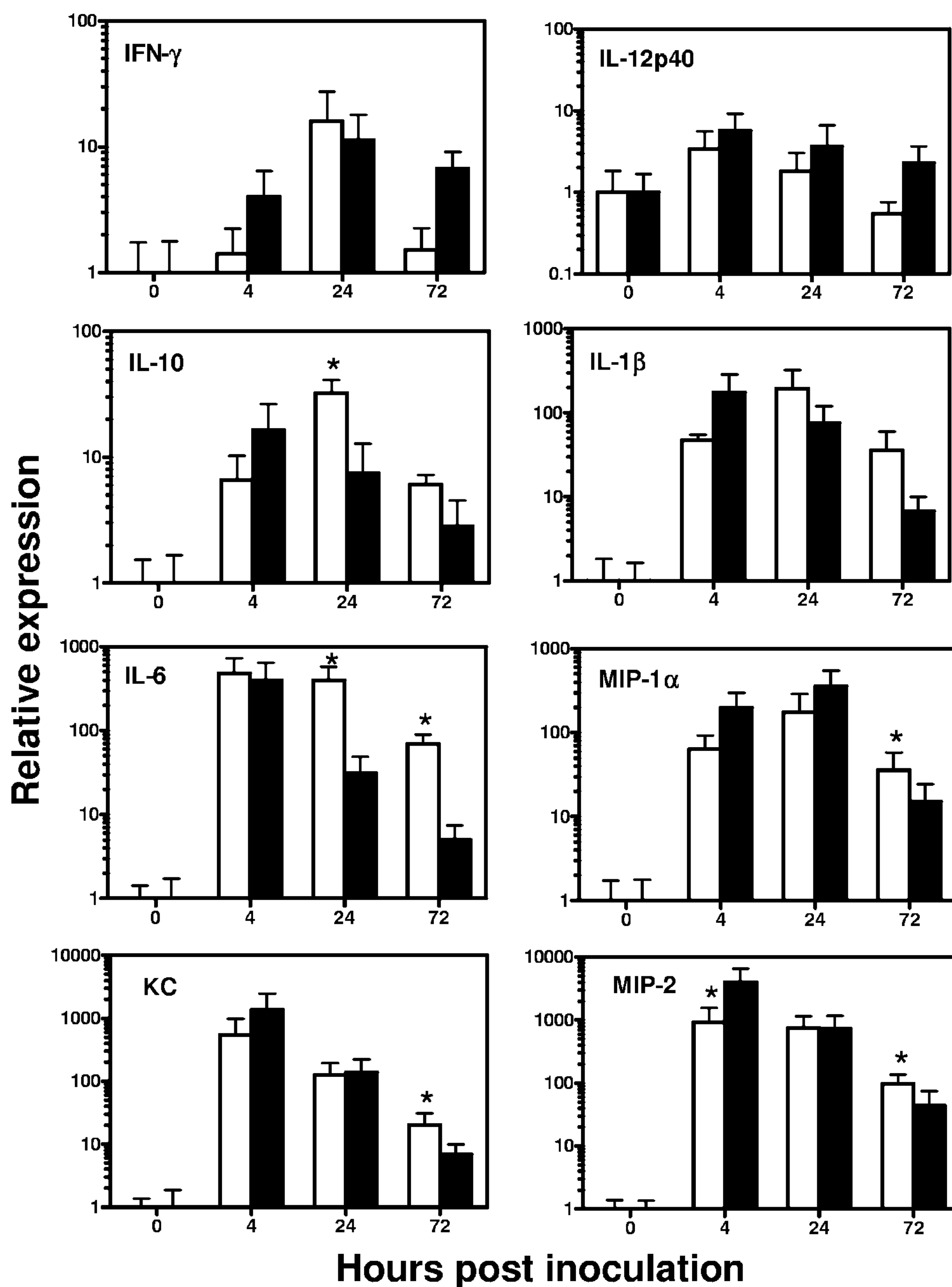

FIG. 8. Relative levels of proinflammatory cytokine and chemokine mRNA expression in the lungs of RB6 (open bars)- and control rIgG (solid bars)-treated mice i.n. inoculated with *A. baumannii*. Groups of C57BL/6 mice ($n = 5$) were treated by i.p. injection with 250 μg of MAb RB6 or an equivalent amount of control rIgG at −18 and 24 h after i.n. inoculation with 2×10^7 CFU of *A. baumannii*, and the lungs were collected at 0, 4, 24, and 72 h. Relative levels of cytokine and chemokine mRNA expression were determined by real-time PCR analysis as described in Materials and Methods. Results shown are the average of relative expression values with the 95% confidence interval determined using cDNA from five mice. *, $P < 0.05$ versus control rIgG-treated mice.

those cytokines and chemokines in the lungs of RB6-treated mice (Fig. 8). On the other hand, there was no change in the mRNA expression of IL-12p35 and p40 over the course of infection, and the IFN-γ mRNA expression was only transiently increased at 24 h by similar magnitudes in both the RB6- and rIgG-treated mice. There was very little change in the mRNA expression levels of the tested cytokines or chemokines in the spleens of RB6- or rIgG-treated mice over the course of infection except that the RB6-treated mice showed transient increases (~5-fold) in the expression of IL-6, IL-10, KC, MIP-2, and MCP-1 at 24 h only (data not shown).

In addition to tissue cytokine/chemokine mRNA levels, the serum and BAL fluid levels of the corresponding cytokine and chemokine proteins were determined over the course of the infection. There was no significant difference in the levels of all cytokine/chemokines examined in the BAL fluid between RB6-

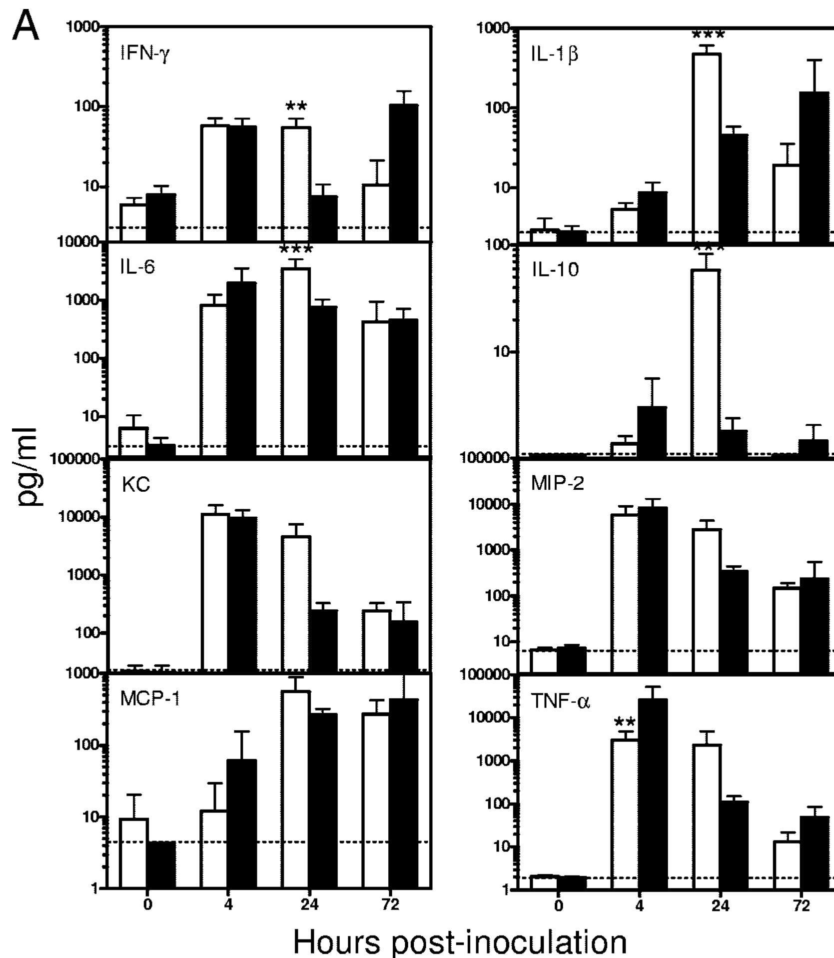

FIG. 9. Effect of RB6 treatment on cytokine and chemokine levels in sera (A) and BAL fluid (B) from mice i.n. inoculated with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 250 μg of RB6 (open bars) or an equivalent amount of control rIgG (solid bars) at −18 and +24 h after i.n. challenge with 10^7 CFU of *A. baumannii*. Blood and BAL samples were collected at 0, 4, 24, and 72 h. Cytokine and chemokine levels in the serum and BAL fluid were determined using the mouse panel of Fluorokine MAP Multiplex Kits (R & D Systems, Inc., Minneapolis, MN) on a Luminex 100 IS instrument. Data are expressed as mean ± SD for five mice at each time point. The detection limits of the assays were 2.5 to 15 pg/ml, as indicated by dotted lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ (versus control rIgG-treated mice).

and rIgG-treated mice at 0 h. Overall, the BAL fluid cytokine/chemokine levels in both RB6- and rIgG-treated mice increased substantially following the i.n. *A. baumannii* challenge, with the majority of the assayed cytokine/chemokines reaching their peak levels at 4 or 24 h p.i. (Fig. 9A). In agreement with the observed tissue mRNA expression patterns, the BAL fluid levels of IFN-γ, IL-1β, IL-6, IL-10, KC, MIP-2, MCP-1, and TNF-α in RB6-treated mice were either lower than or comparable to those in rIgG-treated mice at 4 h p.i. (Fig. 9A). By 24 h, most of these cytokines/chemokines were moderately or significantly higher in the BAL fluid of RB6-treated mice than in that of rIgG-treated mice (Fig. 9A). By 72 h p.i., the BAL fluid levels of most cytokines/chemokines of RB6-treated mice were once again lower than or comparable to those in rIgG-treated mice. On the other hand, the serum cytokine/chemokine levels in rIgG-treated mice changed little over the course of infection except that the levels of IL-6 and TNF-α were significantly increased at 4 h p.i. (Fig. 9B). In contrast, most of the cytokines and chemokines tested (IFN-γ, IL-6, IL-10, KC, MIP-2, MCP-1, and TNF-α) showed significantly higher increases at 24 h p.i. in RB6-treated than in rIgG-treated mice (overall, $P < 0.05$) (Fig. 9), probably reflecting the stimulation of transient extrapulmonary dissemination of this pathogen in RB6-treated mice.

i.n. MIP-2 treatment induces pulmonary neutrophil recruitment and enhances host resistance to i.n. *A. baumannii* inoculation. The preceding experiments demonstrated that neutrophil depletion increased *A. baumannii* burdens in the lungs and delayed local and systemic cytokine/chemokine responses (Fig. 2 to 9). We next examined whether or not enhanced early recruitment of neutrophils by i.n. administration of the neutrophil-inducing chemokine MIP-2 would decrease pulmonary *A. baumannii* growth in normal mice. As shown previously by others (10), a single i.n. administration to naive mice of 1 μg of recombinant murine MIP-2 induced a significant influx of neutrophils into the lungs at 24 h ($P < 0.005$) without significantly affecting the total numbers of lymphocytes or macrophages therein (Fig. 10A). To determine the effect of MIP-2 treatment on the pulmonary clearance of *A. baumannii*, groups of C57BL/6 mice were treated i.n. with either 2.5 μg MIP-2 or

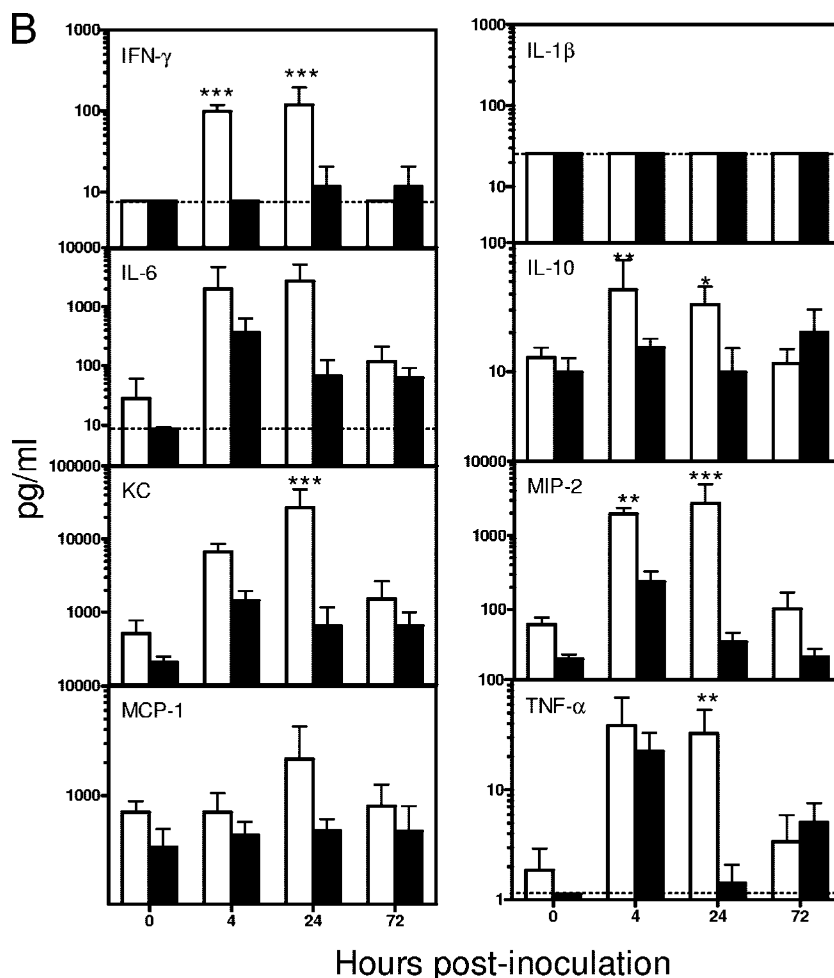

FIG. 9—*Continued*.

0.1% bovine serum albumin (BSA) diluent at −24 h and −4 h and then were challenged i.n. with 2.4×10^7 CFU of *A. baumannii* at 0 h. Both MIP-2- and diluent-treated mice were sacrificed at 24 h p.i., and the bacterial burdens in the lung and spleen were determined. Following i.n. challenge with *A. baumannii*, MIP-2-treated mice showed significantly reduced bacterial burdens in the lungs of the majority of mice at 24 h ($P < 0.01$) (Fig. 10B). As expected, 0.1% BSA diluent treatment did not alter the cellular composition of the BAL fluid and had no effect on bacterial burdens (Fig. 10).

DISCUSSION

A. baumannii is an emerging bacterial pathogen, which rapidly develops multiple drug resistance and is responsible for the majority of nosocomial pulmonary infections (9, 11, 14). Interactions between *A. baumannii* and the host innate immune system likely govern the extent of bacterial proliferation and local host tissue inflammatory response following pulmonary challenge with the pathogen. As demonstrated by others (17, 26), we found that normal C57BL/6 mice were capable of clearing *A. baumannii* within 72 h after i.n. inoculation with 10^7 CFU of the pathogen. This suggests that, under normal circumstances, innate immune defenses in the lungs can effectively control *A. baumannii* infection and prevent the development of severe disease. Moreover, we found that the recruitment of large numbers of neutrophils and the predominantly neutrophilic inflammatory response in the major airways and lung parenchyma of infected mice resembled the suppurative bronchopneumonia previously observed in human patients (1, 2) and correlated well with the control and eradication of *A. baumannii* growth in the lungs and spleens.

Neutrophils are crucial in host defense against a wide array of respiratory pathogens. However, their contributions are not the same for all infections or even for different routes of infection with the same pathogen (3, 7, 8, 10, 23–25, 30, 31). To our knowledge, there are no published reports on the in vivo contribution of neutrophils to *A. baumannii*-associated pneumonia. In this study, we used a mouse model of i.n. challenge, which mimics one of the natural routes of *A. baumannii* exposure in clinical settings (15), to examine the role of neutrophils in host defense against acute respiratory *A. baumannii* infection. Our results showed for the first time that neutrophils play an important role in host defense against respiratory infection with this pathogen. We found that neutrophils were normally rapidly recruited to the lungs after i.n. *A. baumannii* challenge.

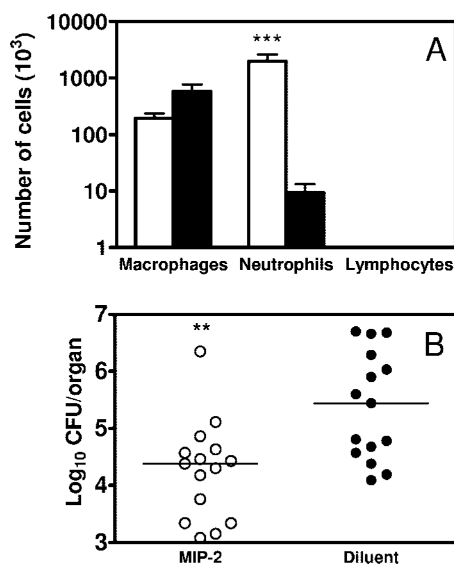

FIG. 10. (A) i.n. MIP-2 administration results in recruitment of neutrophils to bronchoalveolar spaces. Groups of C57BL/6 mice were instilled by the i.n. route with either 1.0 μg of recombinant murine MIP-2 (open bars) or 0.1% BSA diluent (solid bars). The mice were killed 24 h later, their lungs were lavaged, and total and differential cell counts were determined. The data are presented as mean ± SD (n = 5). ***, $P < 0.005$ versus mice treated with diluent. (B) Effect of i.n. MIP-2 administration on *A. baumannii* burden in the lungs. Groups of C57BL/6 mice were treated i.n. with either 2.5 μg of recombinant murine MIP-2 (open bars) or diluent (solid bars) in 50 μl at 24 and 4 h before i.n. challenge with 2×10^7 CFU *A. baumannii*. At 24 h, the mice were killed, lung homogenates were prepared from individual mice, and CFU were determined. The data are compiled from three independent experiments with similar results; each individual dot represents the value from a single mouse, and the horizontal line represents the median value for the group (n = 15). **, $P < 0.01$ versus diluent-treated mice.

Additionally, depletion of neutrophils prior to challenge converted a self-limiting infection into a rapidly lethal one that was associated with loss of control of bacterial replication at the primary site of implantation and subsequent, although mild, extrapulmonary dissemination of the pathogen to the spleen. Moreover, i.n. administration of the neutrophil-recruiting chemokine MIP-2 to immunocompetent mice enhanced the clearance of *A. baumannii* from the lungs and prevented its systemic dissemination.

The MAb RB6 used to deplete mice of neutrophils in this study recognizes the Gr-1 marker on cell surface of mature eosinophils and neutrophils and specifically depletes these granulocytes in vivo (32). Additionally, other investigators have reported intermediate expression of Gr-1 on other cells, such as a small population of $CD8^+$ cells and monocytes/dendritic cells (25, 33). However, we believe that the increased susceptibility to i.n. *A. baumannii* infection in RB6-treated mice in our study was most likely associated with the depletion of neutrophils rather than other Gr-1^+ cells because (i) low doses of RB6, which have no effect on nonneutrophil Gr-1^+ cells, resulted in a similar increase in the bacterial burden as high-dose treatment (Fig. 4); (ii) depletion of $CD8^+$ cells or plasmacytoid dendritic cells in mice by MAb treatments had no effect on the bacterial burden after i.n. *A. baumannii* challenge (Fig. 5); and (iii) the recruitment of alveolar macrophages was unaffected by treatment with 250 μg of RB6 in this study (Fig. 7).

Although our study has shown that neutrophils are important in host defense against respiratory *A. baumannii* infection, their precise mechanisms of action remain unknown. In this regard, neutrophils are potent effectors of the innate immune response and contribute to protection in other bacterial infections through their direct antimicrobial capacity and the production of cytokines and chemokines that instruct the recruitment and activation of other immune cells (24). In the present study, the differences in the bacterial burdens in control and neutropenic mice at the primary site of infection and in the extrapulmonary tissue were detected as early as 4 h p.i. and peaked at 24 h (Fig. 3). Previous studies have shown that i.n. *A. baumannii* infection induces local production of moderate amounts of TNF-α, IL-1β, IL-6, MCP-1, MIP-2, and KC at 4 and 24 h p.i. (17, 26). Here we show that, in addition to these cytokines, the mRNA expression and production of IFN-γ and IL-10 were also transiently induced in the lungs at 24 h after i.n. *A. baumannii* infection. Overall, the magnitude of mRNA expression in the lung and spleen and cytokine levels in the BAL fluid and serum were similar in pattern to the bacterial burdens in the tissue, which peaked at 4 h in rIgG-treated mice and at 24 h in RB6-treated mice (Fig. 3, 8, and 9). The transient changes in cytokine/chemokine mRNA expression and levels in the serum and spleen during respiratory *A. baumannii* infection reflect the nature of infection, since *A. baumannii* was only transiently present in the spleens in neutrophil-depleted mice (Fig. 3). However, as with other studies, the cellular sources of these cytokines and chemokines remain to be elucidated. Although NK cells are likely to be the major source of IFN-γ secretion seen in this study, other pulmonary cells, such as alveolar epithelial cells, may also secret this cytokine, as recently reported for *Mycobacterium tuberculosis* infection (27).

Depletion of neutrophils substantially decreased levels of TNF-α, IL-6, IL-10, and MCP-1 in the lung at 4 h p.i. (Fig. 9), implying that neutrophils play a critical role in the generation of the early proinflammatory cytokine responses in the lungs. In this regard, previous studies have demonstrated that neutrophils can produce many of these cytokines (3), and decreases in TNF-α, IL-6, and other cytokine levels after RB6-induced neutropenia have been reported in other models of infection (3, 23). The differences in cytokine levels in the lungs between RB6- and rIgG-treated mice over the course of the infection show a more complicated picture. At 24 h p.i., RB6-treated mice showed significantly stronger pulmonary cytokine/chemokine responses than rIgG-treated mice, probably reflecting the fact that the depletion of neutrophils at the beginning of infection exacerbated pulmonary bacterial burdens and low levels of extrapulmonary bacterial dissemination in neutropenic mice (Fig. 3), which might stimulate the production of proinflammatory cytokines in infected tissues. By 72 h p.i., most RB6-treated mice were in the terminal stage of the infection and consequently showed substantially lower cytokine/chemokine levels in the BAL fluid than rIgG-treated mice despite the presence of significantly higher numbers of *A. baumannii* organisms in their lungs. Nevertheless, the delayed or reduced cytokine response seen in RB6-treated mice does not

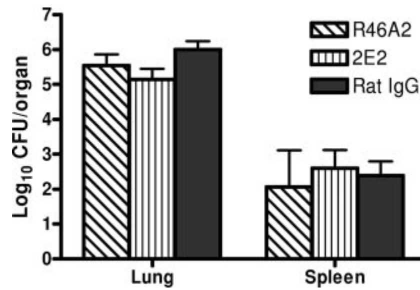

FIG. 11. Effect of neutralization of endogenous TNF-α or IFN-γ on bacterial burdens in the lung following i.n. inoculation with *A. baumannii*. Groups of five C57BL/6 mice were treated by i.p. injection with either 500 μg of IFN-γ neutralization MAb R46A2 or TNF-α neutralization MAb 2E2 or an equivalent amount of control rIgG at 18 h before i.n. challenge with 2.0×10^7 CFU of *A. baumannii*. Bacterial burdens in the lungs and spleens were determined by quantitative bacteriology. The data are presented as mean ± SD ($n = 5$), and the detection limit for the bacterial burdens was 1.3 $\log_{10}$ CFU/lung.

appear to directly contribute to the enhanced susceptibility to i.n. *A. baumannii* infection, since in vivo neutralization of endogenous TNF-α or IFN-γ in these mice failed to exacerbate the infection (Fig. 11). These data imply that an early direct bactericidal effect by the neutrophils may be primarily responsible for their role in host defense against this pathogen. In this regard, it has been previously shown that granule extracts from human and rat neutrophils and purified human defensin, a potent neutrophil antimicrobial peptide in vitro, are bactericidal to another *Acinetobacter* species, *A. calcoaceticus* (12, 22). This hypothesis is further supported by the finding that enhancement of pulmonary neutrophil recruitment with i.n. administration of neutrophil-inducing chemokine MIP-2 resulted in significant reductions in the bacterial burdens in the lung following i.n. *A. baumannii* inoculation (Fig. 10). Overall, these data demonstrate that neutrophils are an essential component of the protective innate immune response to respiratory *A. baumannii* infection. The identification of neutrophils as a key element of host defense against this pathogen is likely to have implications for the clinical management of this infection, since *Acinetobacter* is frequently isolated from neutropenic febrile patients in nosocomial settings (16).

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