ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2010, p. 1785–1792 Vol. 54, No. 5
0066-4804/10/$12.00 doi:10.1128/AAC.01513-09

Immunotherapy Markedly Increases the Effectiveness of Antimicrobial Therapy for Treatment of *Burkholderia pseudomallei* Infection▽†

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Received 22 October 2009/Returned for modification 7 December 2009/Accepted 11 February 2010

***Burkholderia pseudomallei* is a soil bacterium that is endemic in southeast Asia and northern Australia and that can cause both acutely lethal pneumonia and chronic systemic infections in humans. The effective treatment of infection with *B. pseudomallei* requires rapid diagnosis and prolonged treatment with high doses of antimicrobials, and even with appropriate antibiotic therapy, patient relapses are common. Thus, new approaches to the treatment of *B. pseudomallei* infections are needed. In the present study, we asked whether active immunotherapy with gamma interferon (IFN-γ), a key cytokine regulating the intracellular replication of *B. pseudomallei*, could increase the effectiveness of conventional antimicrobial therapy for *B. pseudomallei* infection. Macrophage infection assays and *in vivo* pulmonary challenge models were used to assess the inhibitory effects of combined treatment with IFN-γ and ceftazidime on *B. pseudomallei* infection. We found that treatment with even very low doses of IFN-γ and ceftazidime elicited strong synergistic inhibition of *B. pseudomallei* growth within infected macrophages. *In vivo*, active immunotherapy markedly potentiated the effectiveness of low-dose ceftazidime therapy for the treatment of infected mice in a pulmonary challenge model of *B. pseudomallei*. Combined treatment was associated with a significant reduction in the bacterial burden and a significant lessening of bacterial dissemination. We concluded, therefore, that immunotherapy with either endogenous or exogenous IFN-γ could significantly increase the effectiveness of conventional antimicrobial therapy for the treatment of acute *B. pseudomallei* infection.**

Burkholderia pseudomallei is a soil bacterium that is endemic in southeast Asia and northern Australia (2) as well as other tropical and subtropical regions of the world (6) and that causes several thousand human cases of melioidosis every year (7, 16, 23, 32). Without prompt antimicrobial therapy, infection with *B. pseudomallei* is often fatal, as illustrated by the high mortality rates among untreated cases (23). Even after initial appropriate therapy, many patients are still susceptible to relapse or reinfection with *B. pseudomallei* (7, 17, 25). Because *B. pseudomallei* infection is difficult to eradicate, prolonged antimicrobial therapy (months) is often prescribed for infected persons (24). *B. pseudomallei* also displays high levels of intrinsic resistance to many commonly used antibiotics. Although *B. pseudomallei* is not endemic in North America, there is still considerable concern over this organism by the Centers for Disease Control and Prevention because of its high potential for use as a bioweapon. *B. pseudomallei* is currently classified as a category B select agent. In addition, no vaccine for the prevention of infection with *Burkholderia* is currently available.

For these reasons, new approaches to improve the effectiveness of antimicrobial therapy for *B. pseudomallei* infection are urgently needed. Previous studies have shown that nonspecific activation of the innate immunity by the systemic (intraperitoneal [i.p.]) administration of CpG oligonucleotides prior to infection could provide protection against systemic challenge with *B. pseudomallei* (33). We recently reported that mucosally delivered cationic liposome-DNA complex (CLDC) immunotherapy is particularly effective in protecting mice from inhalational challenge with both *B. mallei* and *B. pseudomallei* (12). In that study, gamma interferon (IFN-γ) was identified as the key cytokine mediating the protection afforded by CLDC immunotherapy.

We therefore investigated whether active immunotherapy could be combined with conventional antimicrobial therapy to increase the effectiveness of treatment of *B. pseudomallei* infections. Such an approach was previously evaluated by using the cytokine granulocyte colony-stimulating factor (G-CSF) in combination with ceftazidime on the basis of the findings of studies that showed that neutrophils are key effector cells for controlling *B. pseudomallei* infection (3, 4, 11). However, subsequent studies with mouse infection models revealed that G-CSF was not effective when it was combined with ceftazidime for the treatment of *B. pseudomallei* infection (26).

In several other infection models, IFN-γ has been combined with antimicrobial agents to increase the effectiveness of treatment. For example, the combination of IFN-γ with the antibiotics gentamicin and vancomycin enhanced the clearance of *Enterococcus faecalis* in an *in vitro* neutrophil infection model (21). However, in a mouse model of *E. faecalis* infection, only low doses of IFN-γ increased the effectiveness of antimicrobial therapy, while high IFN-γ doses were ineffective or deleterious (20). In a *Francisella novicida* infection model, the intranasal administration of recombinant interleukin-12 (rIL-12; which induced IFN-γ production) increased the effectiveness of an-

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† Supplemental material for this article may be found at http://aac.asm.org/.

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▽ Published ahead of print on 22 February 2010.

timicrobial therapy (22). Similarly, treatment of *Mycobacterium avium*-infected mice with rIL-12 was found to increase the effectiveness of antimicrobial therapy in clearing the bacterial infection (9).

Thus, there was reason to believe that immunotherapy, particularly with an immunotherapeutic capable of stimulating the potent release of IFN-γ, might be effective when it was combined with antimicrobial therapy for the treatment of *B. pseudomallei* infection. Currently, the first-line antimicrobials used for the treatment of acute *B. pseudomallei* infection include ceftazidime, amoxicillin-clavulanic acid, carbapenems (meropenem or imipenem), and trimethoprim-sulfamethoxazole (23, 24). Of these, ceftazidime has been evaluated the most extensively in mouse infection models and was therefore selected for use in the studies reported here (26, 29, 30). For the stimulation of IFN-γ release *in vivo*, CLDCs were used, as our prior studies had shown that CLDCs are potent inducers of IFN-γ release (10, 13).

Therefore, we designed experiments to investigate the interactions between IFN-γ immunotherapy and ceftazidime for the control of intracellular infection with *B. pseudomallei*. Our results indicate that IFN-γ may be uniquely effective as an immunotherapeutic for increasing the susceptibility of intracellular *Burkholderia* organisms to killing by certain classes of antimicrobials. Thus, there is reason to believe that immunoantimicrobial therapy is a promising new approach to improving the effectiveness of current antimicrobial drugs for the treatment of *B. pseudomallei* infections.

MATERIALS AND METHODS

Bacteria. *B. pseudomallei* strain 1026b was used for these studies (8). This strain was inoculated in Luria broth (LB), grown at 37°C with shaking for 16 h, and then stored at −80°C in 15% glycerol. Each vial was thawed just before use, and the bacteria were diluted to the desired concentration with sterile phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO). The 50% lethal dose (LD_{50}) of *B. pseudomallei* inoculated into BALB/c mice by the intranasal (i.n.) route was determined by the Reed-Muench method to be 900 CFU (12). The challenge dose used for the *in vivo* studies was 8× LD_{50} (approximately 7,500 CFU).

Mice. Female BALB/c mice were used for these studies (Jackson Laboratories, Bar Harbor, ME). All mice were 6 to 12 weeks of age at the time of infection and were housed under pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Preparation and administration of CLDCs. CLDCs were prepared as described previously (10, 12). Briefly, sterile complexes of cationic liposomes were prepared with equimolar amounts of octadecanoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride (DOTIM) and cholesterol. Noncoding ultra-low-level endotoxin plasmid DNA was then added to the liposomes at a final concentration of 0.1 mg/ml to form the CLDCs. The preformed CLDCs were diluted in Tris-buffered 5% dextrose water (pH 7.4) and were administered to mice i.p. in a total volume of 200 μl. CLDC treatment was administered once i.p. 6 h after infection.

rIFN-γ. Recombinant murine IFN-γ (rIFN-γ) was purchased from Peprotech (Rocky Hill, NJ) and was reconstituted in PBS with 0.1% bovine serum albumin. Aliquots were frozen at −80°C until use. For the *in vitro* studies, rIFN-γ was added at the indicated concentrations after bacterial infection and the elimination of extracellular bacteria with kanamycin. For *in vivo* treatment, rIFN-γ was diluted in PBS with 0.1% bovine serum albumin and was administered i.p. 6 h and 18 h after infection in a total volume of 200 μl.

Ceftazidime. Ceftazidime was purchased from Sigma-Aldrich and diluted in PBS with 0.1% bovine serum albumin. Aliquots of the desired concentration were frozen at −20°C until use. The concentration of ceftazidime used for *in vivo* mouse treatments was 25 mg/kg of body weight, which was administered 6 h after infection and continued every 12 h for a total of six treatments (administered at 6, 18, 30, 42, 54, and 66 h after infection).

***In vitro* macrophage infection assay to assess *in vitro* interaction between cytokines and ceftazidime.** The AMJ.2 mouse alveolar macrophage cell line (American Type Tissue Collection, Manassas, VA) was used to investigate the ability of CLDC-elicited cytokines to enhance the activities of antimicrobial drugs. AMJ.2 cells were cultured in complete medium, which consisted of minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Invitrogen), 1× nonessential amino acids (Invitrogen), and 0.075% sodium bicarbonate (EMD Science, Gibbstown, NJ). No antibiotics were added to the complete medium. *B. pseudomallei* was added to 2×10^5 AMJ.2 cells per well of a 24-well plate at a multiplicity of infection of 5 CFU per cell in 500 μl antibiotic-free medium. The plates were centrifuged at 2,400 × *g* for 2 min and then incubated for 1 h at 37°C in 5% CO_2. The extracellular bacteria were then removed by washing the macrophages three times with 2 ml PBS, followed by treatment with medium plus 350 μg/ml kanamycin (Sigma-Aldrich) for 1 h. (The MIC for kanamycin against strain 1026b was 32 μg/ml.) After incubation with kanamycin, the macrophages were washed three times with PBS.

Ceftazidime, the CLDC supernatants, or rIFN-γ, alone or in combination, were then added to the cultures and the macrophages were cultured for an additional 24 h. (The CLDC-stimulated supernatants were prepared as described in the next paragraph.) The treatment medium was then removed, and the cells were washed three times with 2 ml sterile PBS and then lysed with 1 ml sterile distilled H_2O in order to quantitate the intracellular bacteria. Serial dilutions of the lysates were plated on LB agar plates, and the plates were incubated at 37°C for 48 h before the colonies were counted. In several experiments, the bacteria present in the last PBS wash were plated and counted, and then the counts were subtracted from the cell lysate counts to control for any extracellular bacteria not removed by thorough washing. In all treatment groups, the amount of bacteria present in the last PBS wash was negligible compared to the amount of bacteria present in the cell lysates. Thus, the lysate counts reflected the number of intracellular bacteria.

The ability of cytokines elicited by CLDC immunotherapy to inhibit the intracellular growth of *B. pseudomallei* was assessed by adding diluted supernatants prepared from overnight cultures of spleen cells from mice treated with CLDCs, as described previously (12). The concentration of IFN-γ in the CLDC supernatants used in these studies was found to be 1,813 pg/ml by use of a cytokine bead array (BD Biosciences, San Jose, CA), while the concentration of tumor necrosis factor alpha (TNF-α) determined by the cytokine bead array was 160 pg/ml. Control supernatants were prepared from the spleens of untreated mice. Neutralizing antibodies were used to determine whether IFN-γ or TNF-α was responsible for generating antibacterial activity in the *in vitro* macrophage infection assay. For this experiment, supernatants from CLDC-stimulated spleen cells were treated with 10 μg/ml anti-IFN-γ antibody (clone R4.6A2; eBioscience, San Diego, CA) or 10 μg/ml of anti-TNF-α antibody (clone TN3-19.12; eBioscience) for 30 min prior to the addition of the supernatants to the cells. Isotype antibodies for anti-IFN-γ (clone eBRG1) and anti-TNF-γ (clone eBio299Arm) were used as controls (eBioscience). Infected AMJ.2 cells were incubated with the supernatants for 24 h, and then the intracellular bacterial concentrations were determined as described above.

Pulmonary challenge model. All infections with *B. pseudomallei* were established by i.n. inoculation. The animals were anesthetized with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH) administered by the i.p. route. The desired challenge dose of *B. pseudomallei* was suspended in PBS, and 20 μl was delivered i.n. in alternating nostrils. The challenge dose was confirmed by retrospective plating on LB agar. For all survival studies, the animals were monitored for disease symptoms twice daily and were euthanized according to predetermined humane end points. All procedures were performed in a biosafety level 3 (BSL-3) facility, in accordance with approved BSL-3 and select agent protocols.

Determination of bacterial burden. The numbers of viable *B. pseudomallei* cells in the lung, liver, and spleen tissues were quantified 48 h after infection. The lungs, livers, and spleens were removed aseptically and homogenized in 5 ml sterile phosphate-buffered saline with a Biomaster 80 stomacher (Seward, Bohemia, NY). Viable bacterial counts were determined for each organ by plating serial 10-fold dilutions of the organ homogenates on LB agar. The plates were incubated at 37°C for 48 h, and the numbers of colonies on the appropriate plate were scored visually. The organ burden of the bacteria was expressed as the numbers of CFU per organ. The limit of detection for determination of the bacterial burden in the organ homogenates was 50 CFU/organ.

Statistical analyses. Data were analyzed by using Prism (version 5.0) software (Graph Pad, San Diego, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. For comparisons of the survival times of more than one group in an experiment, the Bonferroni corrected threshold was

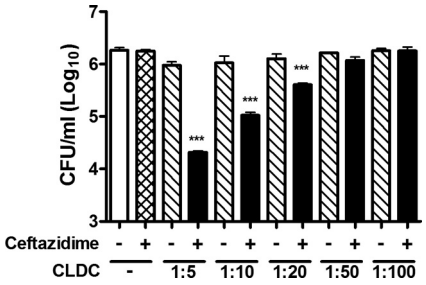

FIG. 1. CLDC supernatants synergize with ceftazidime to inhibit the intracellular replication of *B. pseudomallei*. AMJ.2 macrophages were infected in triplicate wells with *B. pseudomallei* strain 1026b for 1 h and were then treated with ceftazidime and/or CLDC spleen supernatants, alone or in combination, for 24 h, as described in Materials and Methods. The concentrations of intracellular bacteria were quantitated by serial dilution plating of macrophage lysates. The effects of the CLDC supernatants at the dilutions indicated, alone or in combination with 10 μg/ml of ceftazidime, on the intracellular replication of *B. pseudomallei* were assessed. The mean bacterial concentrations in each treatment group were plotted, and bars represent means ± standard deviations. Synergistic interactions were assessed statistically by two-way ANOVA (***, $P < 0.001$). The data presented here are representative of those from two independent experiments.

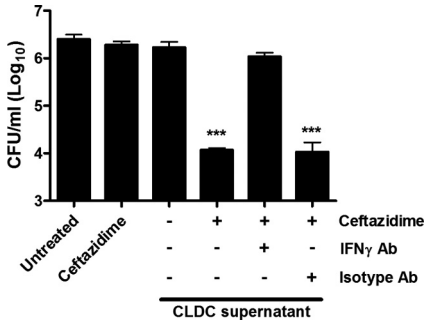

FIG. 2. Synergistic inhibitory activity of CLDC-stimulated supernatants is mediated by IFN-γ. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h and were then treated for 24 h with CLDC-stimulated spleen supernatants (1:5 dilution) alone or in combination with ceftazidime at 10 μg/ml. The CLDC supernatants were untreated or were treated with an IFN-γ-neutralizing antibody (Ab) or isotype control antibody for 30 min before they were added to the infected macrophages. The intracellular bacterial numbers were determined 24 h after infection, and the mean bacterial numbers (± standard deviations) were plotted. Assessments for synergistic interactions between ceftazidime and IFN-γ were performed statistically by two-way ANOVA (***, $P < 0.001$), as described previously (27). The data presented here are representative of those from two independent experiments.

applied to correct for multiple comparisons. Other analyses were performed by use of the Mann-Whitney test (for comparisons of two groups) or one-way analysis of variance (ANOVA), followed by Tukey's multiple-means comparison test (for comparisons of more than two groups). Possible synergistic interactions between the antimicrobials and cytokines, as detected by the *in vitro* assays, were assessed by two-way ANOVA, as described previously (27). Differences were considered statistically significant if the *P* values were <0.05.

RESULTS

CLDC-elicited cytokines synergize with ceftazidime to inhibit intracellular replication of *B. pseudomallei* in macrophages *in vitro*. An *in vitro* macrophage infection assay was used to determine whether immunotherapy could increase the effectiveness of ceftazidime therapy at inhibiting the intracellular replication of *B. pseudomallei*, since macrophages represent major target cells for *B. pseudomallei* infection *in vivo* (1, 15, 18, 31). The supernatants generated from the spleens of mice treated *in vivo* with CLDCs were used as a source of CLDC-stimulated cytokines.

Dose-titration studies demonstrated that treatment with 10 μg/ml ceftazidime did not significantly inhibit *B. pseudomallei* replication in infected macrophages (see Fig. S1 in the supplemental material). This concentration of ceftazidime is well within the range of ceftazidime concentrations clinically achievable *in vivo* (19). Similar titrations were performed with the CLDC supernatants, and we found that a 1:5 dilution was effective at eliciting a small reduction in the level of *B. pseudomallei* replication in infected macrophages (see Fig. S1 in the supplemental material).

Subtherapeutic concentrations of ceftazidime and CLDC supernatants were then combined to treat infected macrophages, and the interaction between the two drugs was assessed statistically by two-way ANOVA, as described previously (27). Marked, synergistic inhibition of the intracellular replication of *B. pseudomallei* was observed when cells were treated with the two agents in combination (Fig. 1). For example, treatment with the combination reduced the intracellular *B. pseudomallei* concentrations by almost 2 $\log_{10}$ units, from 1.9×10^6 CFU to 2.0×10^4 CFU. This effect was not observed when supernatants from nonstimulated spleen cells were used with ceftazidime (see Fig. S2 in the supplemental material). Synergistic inhibition of *B. pseudomallei* was observed at CLDC supernatant dilutions of up to 1:20, when they were combined with 10 μg/ml ceftazidime (Fig. 1). Thus, the cytokines elicited by CLDC immunotherapy were capable of synergistically inhibiting intracellular *B. pseudomallei* replication when they were combined with a low dose of ceftazidime.

IFN-γ is responsible for synergistic inhibition of *B. pseudomallei* replication. Experiments were next conducted to identify the cytokines present in the CLDC supernatants that mediated the synergistic interaction with ceftazidime. Previous studies had identified IFN-γ as the most likely candidate cytokine (10, 12, 28), so the *in vitro* infection assay was repeated with CLDC supernatants that had been pretreated with a neutralizing antibody to IFN-γ, as described in Materials and Methods. The effects of neutralizing TNF-α activity were also evaluated. Neutralization of the IFN-γ activity in the CLDC supernatants eliminated nearly all of the synergistic antibacterial activity, thus identifying IFN-γ as the cytokine primarily responsible for the interaction with ceftazidime (Fig. 2). Neutralization of the TNF-α activity had essentially no effect on the interaction of the CLDC supernatants with ceftazidime (data not shown).

Synergistic inhibition of *B. pseudomallei* replication when IFN-γ is combined with ceftazidime. The experiments described above identified IFN-γ as the primary cytokine mediating the synergistic activity of the CLDCs and ceftazidime. Therefore, we next determined whether recombinant murine IFN-γ could reproduce the effects of the CLDC supernatants in the *in vitro* infection assay. Infected macrophages were treated with ceftazidime (10 μg/ml) and a range of rIFN-γ concentrations from 1,000 to 0.1 U/ml, and the effects on

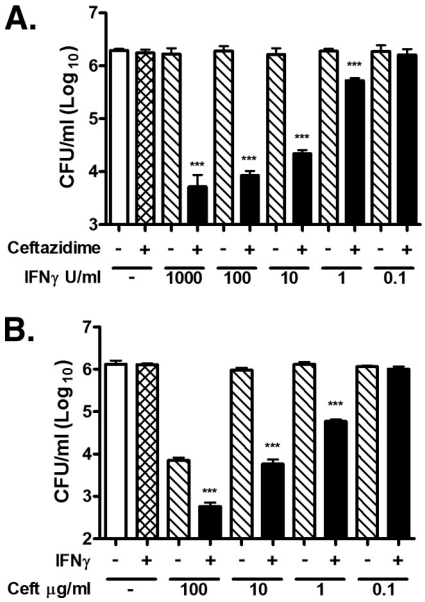

FIG. 3. rIFN-γ and ceftazidime reciprocally increase inhibition of intracellular replication of *B. pseudomallei in vitro*. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h and were then treated with rIFN-γ and ceftazidime for 24 h, and the intracellular bacterial numbers were determined. (A) Decreasing concentrations of rIFN-γ were added to infected macrophages treated with 10 μg/ml of ceftazidime, and the effects on intracellular *B. pseudomallei* replication were assessed. For each treatment, the mean bacterial numbers were plotted, and the bars represent group means ± standard deviations. (B) Decreasing concentrations of ceftazidime (Ceft) were added to macrophages treated with a fixed concentration of rIFN-γ (100 U/ml), and the effects on intracellular *B. pseudomallei* replication were assessed. Synergistic interactions between ceftazidime and IFN-γ were assessed statistically by two-way ANOVA (***, $P < 0.001$), as described previously (27). The data presented here are representative of those from two independent experiments.

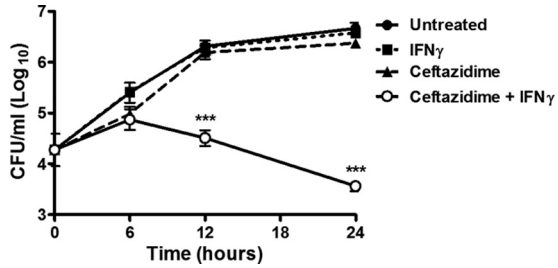

FIG. 4. Time course of intracellular *B. pseudomallei* replication and killing following treatment with IFN-γ and ceftazidime. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h and were then treated with rIFN-γ (100 U/ml) or ceftazidime (10 μg/ml), or with both agents in combination, for the indicated times, and the intracellular bacterial numbers were determined. The mean bacterial concentrations (± standard deviations) in the individual treatment groups were compared over time by repeated-measures ANOVA with the Bonferroni posttests (***, $P < 0.001$ for the group treated with ceftazidime plus IFN-γ combined versus all other groups). Similar results were obtained in one additional experiment.

intracellular *B. pseudomallei* infection were assessed (Fig. 3). We observed that the combination of rIFN-γ and ceftazidime elicited the strong synergistic inhibition of *B. pseudomallei* replication, reducing the bacterial counts from 1.9×10^6 CFU/ml in untreated cultures to 5.7×10^3 CFU/ml in cultures treated with 1,000 U/ml rIFN-γ and ceftazidime. Concentrations of rIFN-γ as low as 1 U/ml elicited the synergistic inhibition of *B. pseudomallei* intracellular growth (Fig. 3). Titration of ceftazidime in combination with 100 U/ml rIFN-γ demonstrated that ceftazidime concentrations as low as 1 μg/ml could elicit the synergistic inhibition of *B. pseudomallei* (Fig. 3).

Combined treatment with IFN-γ and ceftazidime rapidly suppresses intracellular replication and induces killing of *B. pseudomallei* in infected macrophages. Experiments were next conducted to characterize the *B. pseudomallei* inhibition kinetics following combined immunoantibiotic treatment *in vitro*. Infected macrophages were treated with ceftazidime (10 μg/ml) and rIFN-γ (100 U/ml). The effects on intracellular *B. pseudomallei* infection were assessed at the time of treatment initiation and at 6, 12, and 24 h after treatment (Fig. 4). Following *B. pseudomallei* infection, the bacterial counts were approximately 2×10^4 CFU/ml. In untreated cultures and cultures treated with ceftazidime alone or IFN-γ alone, the intracellular *B. pseudomallei* counts rose continuously over time to $>1 \times 10^6$ CFU/ml at 24 h postinfection. However, in cultures treated with both ceftazidime and IFN-γ, the bacterial counts initially increased during the first 6 h of treatment and then subsequently decreased by 12 h and further decreased to approximately 4×10^3 CFU/ml by 24 h. Thus, at 12 h macrophages treated with ceftazidime and IFN-γ had significantly lower bacterial counts than macrophages treated with ceftazidime or IFN-γ alone. In addition, the progressive decrease in the intracellular bacterial counts in the cultures treated with ceftazidime plus IFN-γ demonstrated that combined immunoantibiotic therapy resulted in the killing of *B. pseudomallei* in infected macrophages.

***In vivo* treatment with CLDCs and ceftazidime generates significant protection from lethal pneumonic *B. pseudomallei* challenge.** A murine model of acute pulmonary *B. pseudomallei* infection was used to determine whether the combination of immunotherapy plus ceftazidime treatment was also effective *in vivo*. Mice were infected with $8\times$ LD_{50} *B. pseudomallei* 1026b and treated 6 h after challenge with ceftazidime, administered i.p. This treatment was repeated every 12 h for a total of six treatments administered over 3 days. The dose of ceftazidime that consistently protected 20% of the acutely infected mice or less (when it was administered as a single agent, without immunotherapy) was 25 mg/kg. For the *in vivo* studies, a single dose of CLDCs was administered i.p. 6 h after infection, as previous studies have found that the immune-stimulatory effects of CLDCs are prolonged (12, 13). A single dose of 20 μl CLDCs administered i.p. was found to protect 20% of the *B. pseudomallei*-infected mice or less (when it was injected as a single treatment without ceftazidime), although it should be noted that higher doses of CLDCs alone were capable of protecting most mice from lethal infection (data not shown). Therefore, these subtherapeutic doses of ceftazidime and CLDCs were selected for use in the combination therapy studies.

For the combination therapy studies, the mice were challenged i.n. with *B. pseudomallei* and 6 h later were treated with CLDCs alone, ceftazidime alone, or both agents together. We observed that 90% of the mice treated with the combination of

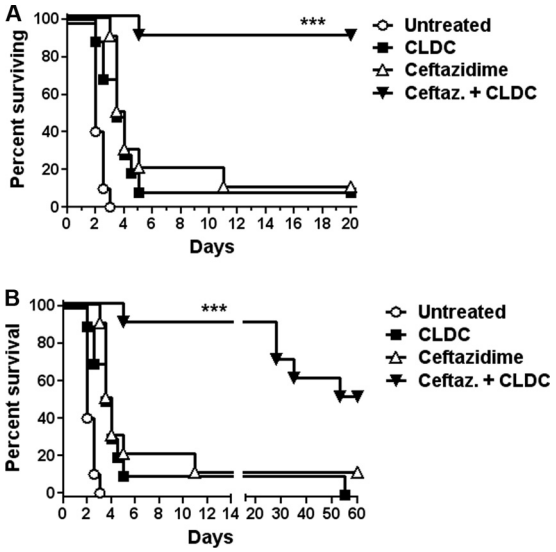

FIG. 5. Low-dose ceftazidime (Ceftaz.) plus CLDC immunotherapy effectively protects mice from acute and chronic infection with *B. pseudomallei*. (A) BALB/c mice ($n = 10$ mice per group) were challenged i.n. with 7.5×10^3 CFU of *B. pseudomallei*, as described in Materials and Methods. Six hours later the mice were treated i.p. with 25 mg/kg ceftazidime or 20 μl CLDC, or with both agents in combination. The ceftazidime treatments were continued every 12 h for a total of six treatments over 3 days, while CLDCs were administered only once. Short-term (20-day) survival times were assessed. (B) Mice ($n = 10$ per group) that initially survived the 20-day short-term period were followed for an additional 40 days to assess the effects of treatment on chronic infection. At the end of the 60-day observation period, any surviving animals were euthanized and their organs (lung, spleen, liver) were cultured for *B. pseudomallei*, as described in Materials and Methods. Survival times were assessed as described in Materials and Methods. Statistical differences in survival times were determined by the use of Kaplan-Meier curves, followed by the log-rank test. The Bonferroni corrected threshold was applied for comparison of multiple survival curves, such that a P value of <0.02 was considered significant for these analyses (***, $P < 0.001$ for combination therapy versus CLDC treatment and for combination therapy versus ceftazidime treatment). The survival curves represent pooled data from two independent experiments.

CLDCs and ceftazidime survived acute pulmonary challenge with *B. pseudomallei* (Fig. 5). In contrast, only 10% of the mice treated with ceftazidime or CLDCs alone survived until day 20 after challenge. Therefore, we concluded that a short course of combined treatment with a potent IFN-γ-inducing immunotherapeutic (i.e., CLDCs) significantly enhanced the efficacy of ceftazidime treatment against lethal *B. pseudomallei* infection.

Next, we also assessed the effects of the brief course of combined, low-dose therapy on long-term survival following high-dose *B. pseudomallei* pulmonary challenge. Notably, five of the nine mice in the combination treatment group that survived for the first 20 days after challenge (Fig. 5A) survived for an additional 40 days (Fig. 5B). Moreover, when these animals were euthanized and their organs cultured, four of the five mice were found to be free of culturable *B. pseudomallei* (data not shown). In contrast, the one surviving CLDC-treated mouse died on day 55 due to *B. pseudomallei* splenic infection and the one surviving ceftazidime-treated mouse euthanized at day 65 had *B. pseudomallei* detected within the lungs (data not shown). Thus, a brief 3-day course of treatment with combined low-dose immunoantimicrobial therapy produced long-term cures in 40% of the treated animals. Therefore, it is reasonable to expect that longer treatment with full-dose therapy should be capable of generating even greater protection from chronic *B. pseudomallei* infection.

Combined immunotherapy and antimicrobial therapy suppresses *B. pseudomallei* replication and dissemination *in vivo*. The effects of combined CLDC and ceftazidime treatment on the bacterial burdens in infected mice were assessed. The mice were killed 48 h after pulmonary *B. pseudomallei* challenge; and the bacterial burdens in lung, spleen, and liver tissues were quantified. Mice treated with ceftazidime and CLDCs in combination had significant decreases in bacterial burdens in their lungs, spleen, and liver compared to those in the organs of mice treated with either ceftazidime or CLDC alone or compared to those in the organs of untreated mice (Fig. 6). Treatment with each of the agents alone also significantly suppressed the bacterial counts in the lung, liver, and spleen compared to those in the untreated animals. These results therefore indicate that combined therapy with rIFN-γ and ceftazidime effectively suppresses bacterial replication in the lungs and significantly inhibits the dissemination of bacteria to the spleen and liver during short-term infection.

Treatment of mice with rIFN-γ significantly increases the effectiveness of ceftazidime therapy. Next, we investigated whether rIFN-γ could be substituted for CLDC and combined with low-dose ceftazidime treatment to generate increased *in vivo* protection from *B. pseudomallei* challenge. In dose-titration studies performed *in vivo*, high doses of rIFN-γ ($>1 \times 10^4$ U rIFN-γ per mouse) administered i.p. at 6 and 18 h after infection significantly protected the mice from lethal inhalational challenge with *B. pseudomallei* (data not shown). However, lower doses of rIFN-γ ($<5 \times 10^3$ U) did not protect the mice from lethal *B. pseudomallei* challenge (data not shown). Thus, a subtherapeutic dose of 3×10^3 U IFN-γ per mouse, administered 6 and 18 h after infection, was selected for use in the subsequent combination treatment studies. This dose consistently protected 20% of the mice or less when it was administered without ceftazidime (data not shown).

Seventy percent of the mice treated with the combination of low-dose rIFN-γ and low-dose ceftazidime survived for 20 days following inhalational challenge with *B. pseudomallei* (Fig. 7). In contrast, only 10% of the mice treated with rIFN-γ alone survived the challenge and none of the mice treated with low-dose ceftazidime alone survived until day 20 postinfection. Therefore, we concluded that treatment with rIFN-γ significantly enhances the effectiveness of low-dose ceftazidime treatment for the short-term control of *B. pseudomallei* infection.

We also examined the effects of rIFN-γ and ceftazidime combination treatment on long-term infection with *B. pseudomallei*. When mice that survived the initial 20-day period (Fig. 7A) were followed for an additional 40 days after the initial infection, we observed that six of the seven surviving mice treated with combination therapy eventually succumbed to disseminated infection (Fig. 7B). In addition, at 65 days, *B. pseudomallei* was present in the spleen of the one surviving combination-treated mouse (data not shown). The long-term survival times of the combination-treated mice were significantly ($P < 0.001$) greater than those of the mice treated with ceftazidime but were not significantly different than the rIFN-

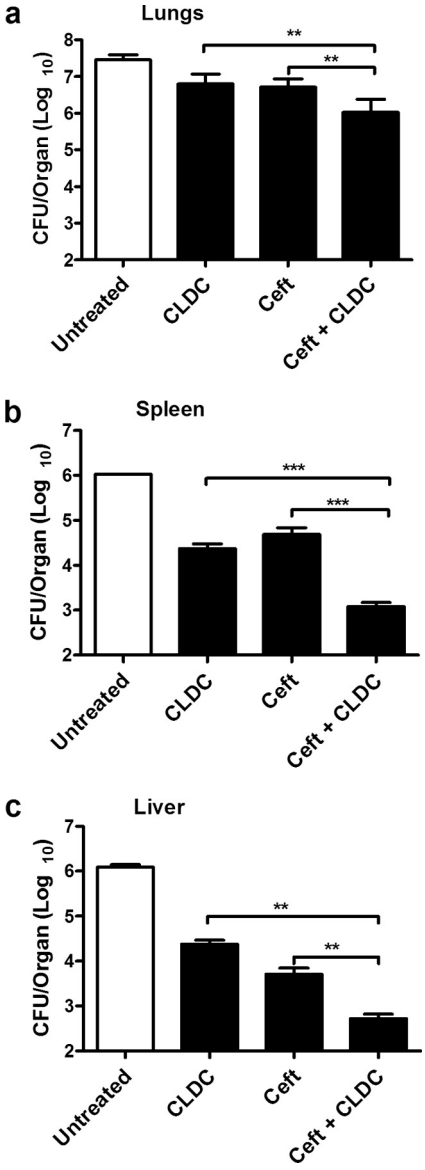

FIG. 6. Combined treatment with CLDCs and ceftazidime (Ceft.) significantly decreases the bacterial burden. BALB/c mice ($n = 5$ mice per group) were challenged i.n. with 8×10^3 CFU of *B. pseudomallei*. Six hours later the mice were treated i.p. with 25 mg/kg ceftazidime or 20 μl CLDC, or with both agents in combination. The ceftazidime treatments were continued every 12 h for a total of four treatments, and the CLDCs were administered once. The mice were euthanized 48 h after the bacterial challenge; and the bacterial burdens in the lungs (a), spleen (b), and liver (c) were quantified. Organ bacterial burdens between combination treatments and single-agent treatments were compared statistically by one-way ANOVA and the Tukey multiple-means-comparison test (***, $P < 0.001$; **, $P < 0.01$). Significant reductions ($P < 0.01$) in bacterial counts in the lungs, livers, and spleens of single-drug-treated animals compared with those in the organs of untreated control animals were also observed but are not noted here.

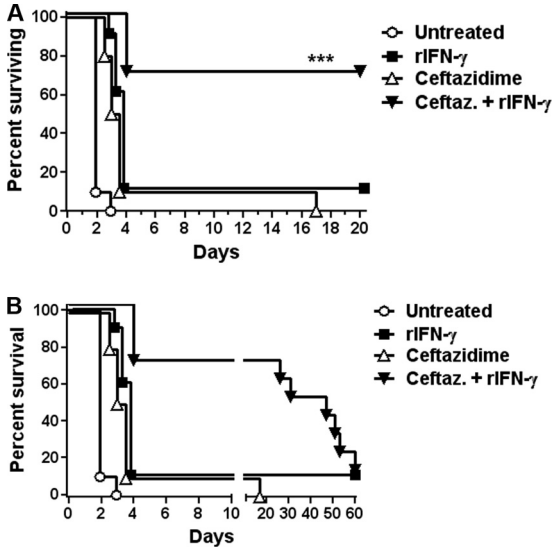

FIG. 7. Treatment with low-dose ceftazidime plus rIFN-γ protects mice from acute but not chronic *B. pseudomallei* infection. (A) BALB/c mice ($n = 10$ mice per group) were challenged with 7.5×10^3 CFU of *B. pseudomallei* administered by the i.n. route. Six hours later the mice were treated i.p. with 25 mg/kg ceftazidime (Ceftaz.) or 3×10^3 U rIFN-γ, or with both in combination. Ceftazidime treatments were continued every 12 h for a total of six treatments over 3 days. Treatment with rIFN-γ was administered twice, at 6 and 18 h postinfection. (B) Mice ($n = 10$ per group) that initially survived the 20-day short-term period were followed for an additional 40 days to assess the effects of the treatment on chronic infection. At the end of the 60-day observation period, any surviving animals were euthanized and their organs (lung, spleen, liver) were cultured for *B. pseudomallei*, as described in Materials and Methods. The survival times of the treated and the control mice were determined, and statistical differences in the survival times were determined by the use of Kaplan-Meier curves followed by the log-rank test. The Bonferroni corrected threshold was applied for comparison of multiple survival curves, such that a P value of <0.02 was considered significant for this analysis (***, $P < 0.001$ for combination therapy versus rIFN-γ treatment and for combination therapy versus ceftazidime treatment). The survival curves represent pooled data from two independent experiments.

γ-treated mice. Nonetheless, the trend apparent in these results was that the combination of rIFN-γ immunotherapy and antimicrobial therapy was more potent than either antimicrobial therapy alone or rIFN-γ immunotherapy alone. However, it was also apparent that combined short-term treatment with rIFN-γ and ceftazidime was not as potent as treatment with CLDCs plus ceftazidime for generating sustained protection from chronic *B. pseudomallei* infection. However, treatment with full doses of rIFN-γ and ceftazidime for longer periods of time might well be effective for the eradication of chronic infection with *B. pseudomallei*.

DISCUSSION

There is a clear need for new approaches to antimicrobial therapy for *B. pseudomallei* infection to increase the effectiveness of therapy, given the inherent antimicrobial resistance of *B. pseudomallei*. In the current study, we demonstrated that treatment with rIFN-γ or an immunotherapeutic that efficiently induces IFN-γ production *in vivo* significantly increases the effectiveness of conventional antimicrobial therapy for *B. pseudomallei* infection. These findings are important because they suggest a general strategy for improving the effectiveness of antimicrobial therapy for melioidosis.

Immunotherapy has previously been shown to increase the

effectiveness of antimicrobial therapy in mouse models of *Mycobacterium avium*, *Enterococcus faecalis*, and *Francisella novicida* infections (9, 20, 22). The effectiveness of combined therapy has also been demonstrated in a *Cryptococcus* infection model (5, 14). However, to our knowledge, this is the first report of a study that demonstrated the marked enhancement of antimicrobial therapy by immunotherapy for the treatment of *B. pseudomallei* infection by use of an acute lethal challenge model.

Previous studies suggested that treatment with recombinant granulocyte colony-stimulating factor (rG-CSF) might be used to augment the effectiveness of antimicrobial therapy for the treatment of *B. pseudomallei* infections (4). Those studies were based on the protective role of neutrophils in *B. pseudomallei* infection (11). However, studies failed to confirm an *in vivo* role for treatment with rG-CSF in augmenting the effectiveness of ceftazidime for the treatment of *B. pseudomallei* infection (26). Our studies indicated that immunoantimicrobial therapy could be used effectively for the treatment of *B. pseudomallei* infection but that IFN-γ would be the preferred cytokine for this application. Moreover, these studies also suggest that combined immunoantimicrobial therapy may be particularly effective for eliminating the chronic stage of *B. pseudomallei* infection.

At present, the mechanism(s) by which IFN-γ interacts with ceftazidime to suppress the intracellular replication of *B. pseudomallei* remains undetermined. Possible mechanisms include the induction of reactive nitrogen or reactive oxygen species by IFN-γ, which then subsequently increase the susceptibility of *B. pseudomallei* to killing by ceftazidime. However, the results of preliminary experiments (R. Troyer et al., unpublished data) suggest that this is not the case. It is also possible that IFN-γ treatment could increase the permeability of macrophages to ceftazidime, but again, preliminary experiments (Troyer et al., unpublished) suggest that this is not the mechanism. Our preliminary experiments suggest that the induction of additional intracellular antibacterial mechanisms by IFN-γ treatment may account for the synergistic interaction between immunotherapy and ceftazidime therapy. Therefore, at present, the mechanisms by which IFN-γ and certain antibiotics interact to generate the synergistic killing of intracellular *Burkholderia* remain an area of active investigation.

In summary, we report here a strong synergistic interaction between IFN-γ and ceftazidime that results in the suppression of *B. pseudomallei* replication in both acute and chronic infection models. The effect was demonstrated by using an *in vitro* macrophage infection model and was confirmed *in vivo* by using a lethal bacterial challenge model. The results of these experiments also suggest that immunotherapeutics capable of eliciting the more sustained release of IFN-γ may be more effective than treatment with rIFN-γ. The combined immunoantimicrobial treatment approach may be especially useful for reducing the duration of antimicrobial treatment and reducing the chance for patient relapse following antibiotic therapy.

ACKNOWLEDGMENTS

We acknowledge the assistance of Scott Hafeman (CSU) for preparation of the CLDC used in these studies.

S.W.D. and H.P.S. were supported by NIAID, NIH, grant U54 AI065357.

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