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Protection from Pneumonic Infection with *Burkholderia* Species by Inhalational Immunotherapy▽

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***Burkholderia mallei* and *B. pseudomallei* are important human pathogens and cause the diseases glanders and melioidosis, respectively. Both organisms are highly infectious when inhaled and are inherently resistant to many antimicrobials, thus making it difficult to treat pneumonic *Burkholderia* infections. We investigated whether it was possible to achieve rapid protection against inhaled *Burkholderia* infection by using inhaled immunotherapy. For this purpose, cationic liposome DNA complexes (CLDC), which are potent activators of innate immunity, were used to elicit the activation of pulmonary innate immune responses. We found that mucosal CLDC administration before or shortly after bacterial challenge could generate complete or nearly complete protection from inhalational challenge with 100% lethal doses of *B. mallei* and *B. pseudomallei*. Protection was found to be dependent on the CLDC-mediated induction of gamma interferon responses in lung tissues and was partially dependent on the activation of NK cells. However, CLDC-mediated protection was not dependent on the induction of inducible nitric oxide synthase, as assessed by depletion studies. We concluded that the potent local activation of innate immune responses in the lung could be used to elicit rapid and nonspecific protection from aerosol exposure to both *B. mallei* and *B. pseudomallei*.**

Pathogenic *Burkholderia* species, including *B. mallei* and *B. pseudomallei*, are gram-negative facultative intracellular bacteria. *Burkholderia pseudomallei* is a soil bacterium that causes a disease known as melioidosis, while *B. mallei* is an obligate mammalian pathogen that causes glanders (26, 37). The primary host for *B. mallei* is equines, though the organism also can infect other mammals, including humans (21, 22). Without antimicrobial therapy, infection with *B. mallei* is nearly 100% fatal (37). Both *B. mallei* and *B. pseudomallei* are classified as category B select agents by the Centers for Disease Control and Prevention due to their high potential for use as bioweapons and their high resistance to antibiotics (1, 28, 33, 39). Currently, prolonged (up to 6 months) antimicrobial therapy of *Burkholderia* infection is required (31, 40). Currently there is no vaccine available for preventing infection with *Burkholderia* organisms.

Proinflammatory cytokines are critical for generating protective immunity to acute *Burkholderia* infection. For example, mice unable to produce interleukin-12 (IL-12) are highly susceptible to infection with *B. mallei*, and treatment with recombinant IL-12 provides partial protection against infection. (2, 29). Tumor necrosis factor α (TNF-α) also plays an important protective role in *B. pseudomallei* infection, as the antibody neutralization of TNF-α increases susceptibility to infection, and TNF-$\alpha^{-/-}$ and TNF-α-receptor$^{-/-}$ (TNF-α-R$^{-/-}$) mice are highly susceptible to lethal infection (4, 30). Gamma interferon (IFN-γ) also is critical in generating protective immunity to *Burkholderia* infection (29, 30). Indeed, even very low concentrations of IFN-γ are sufficient to generate protection against *B. pseudomallei* infection (14).

Given the inherent antimicrobial resistance of *Burkholderia* and the lack of effective vaccines, there is a need for alternative approaches to rapidly protect from aerosol infection. The nonspecific activation of innate immunity by the administration of immunotherapeutics represents one such approach. For example, it was shown previously that the systemic (intraperitoneal [i.p.]) administration of CpG oligonucleotides prior to infection provided protection against low-dose aerosol *B. mallei* challenge (38). A similar CpG oligonucleotide treatment approach also was shown to be effective against systemic challenge with *B. pseudomallei* (41). However, an inhaled immunotherapeutic could be more rapidly administered and generate protective immunity more rapidly than a parenterally administered agent. Therefore, we evaluated the potential effectiveness of a mucosally administered immunotherapeutic for protection from inhaled *Burkholderia* infection. The studies described here used cationic liposome-DNA complexes (CLDC) as immunotherapeutics, as our previous investigations have shown CLDC to be potent inducers of innate immunity (11).

We investigated the effects of the timing of mucosal CLDC administration on the induction of protective immunity and whether CLDC immunotherapy could protect against both *B. mallei* and *B. pseudomallei* infection. These studies also used a rigorous high-dose inhalational *Burkholderia* challenge model and investigated immunological mechanisms of protection. We report here that the intranasal (i.n.) administration of a liposome-based immunotherapeutic was capable of eliciting significant protection from pneumonic *Burkholderia* infec-

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tion. These results suggest that this approach is an effective strategy for generating rapid and nonspecific protection from the inhalation of acutely pathogenic bacteria.

MATERIALS AND METHODS

Mice. Female BALB/c mice and IFN-$\gamma^{-/-}$ mice on the BALB/c background were used in this study (Jackson Laboratories, Bar Harbor, ME). All mice were 6 to 8 weeks of age at the time of infection and were housed under pathogen-free conditions. We determined that BALB/c mice were more susceptible to *B. mallei* infection by the i.n. route than C57BL/6 mice, which is consistent with results obtained by other investigators in prior studies (12, 37).

Preparation and administration of CLDC. Sterile complexes of cationic liposomes were prepared using equimolar amounts of DOTIM [octadecanoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride] and cholesterol, which were prepared as described previously, except that the liposomes were extruded through a final filter diameter of 200 nm rather than 100 nm (32). Liposome-DNA complexes were formed just prior to injection by gently mixing cationic liposomes with plasmid DNA in 5% dextrose in water at room temperature. The final plasmid DNA concentration in the complexes was 200 μg DNA per ml. Plasmid DNA was isolated from *Escherichia coli* DH5α using the Qiagen Endo-free Giga kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The plasmid used for these experiments was a eukaryotic expression plasmid that contained no transgene insert, as described previously (11). CLDC were administered to mice i.n. in a 20-μl volume (10 μl per nostril).

Preparation of *Burkholderia mallei* and *B. pseudomallei* stocks and animal infections. *Burkholderia mallei* (strain ATCC 23344) and *Burkholderia pseudomallei* (strain 1026b) both were kindly provided by Herbert Schweizer, Colorado State University. All animal procedures were approved by the Animal Care and Use Committee at Colorado State University. All procedures involving *Burkholderia* were performed in a biosafety level 3 facility, in accordance with select agent regulations and with Institutional Biosafety Committee oversight at Colorado State University.

For *B. mallei* studies, the ATCC 23344 *B. mallei* strain first was serially passaged three times by animal infection in mice. The use of animal passage has been reported previously to increase the virulence of *B. mallei*, and we observed the same phenomenon in our studies (27, 37). In addition, prior to each in vivo or in vitro infection with *B. mallei*, fresh broth cultures were grown in brucella broth supplemented with 4% glycerol (BB4G) (Remel, Lenexa, KS). Bacteria were harvested during the log phase of growth, titers were determined based on optical density values, and bacterial dilutions were prepared in sterile phosphate-buffered saline (PBS). Bacterial titers (in CFU) of each inocula were determined by plating serial dilutions of the inocula on BB4G agar plates (Remel).

Prior to infection, mice were anesthetized by the i.p. injection of ketamine (Fort Dodge Animal Health, Overland Park, KS) and xylazine (Ben Venue Laboratories, Bedford, OH) solution prepared in sterile water. Mice were infected with *B. mallei* i.n. using a total volume of 20 μl of inoculum (10 μl per nostril). Animal challenge studies were conducted to determine the 50% lethal dose (LD_{50}) and LD_{100} of *B. mallei* in BALB/c mice. The LD_{50} of *B. mallei* in BALB/c mice by the i.n. route was determined by the Reed-Meunch method to be 820 CFU, and the experimental LD_{100} was 4.2×10^3 CFU. For in vitro experiments with *B. mallei*, frozen stocks of known titers of the animal-passaged *B. mallei* were diluted in cell culture media, added to cells, and incubated at 37°C for the indicated amount of time and at the indicated MOI.

Frozen stocks of *B. pseudomallei* of known titers were prepared from cultures grown in Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA) by freezing the cultures in LB medium containing 20% glycerol. Inocula for in vivo infection with *B. pseudomallei* were prepared by thawing and diluting frozen stocks in sterile PBS. The LD_{50} of *B. pseudomallei* in BALB/c mice by the i.n. route was determined by the Reed-Meunch method to be 900 CFU, and the experimental LD_{100} was 4.5×10^3 CFU.

Determination of bacterial burden in tissues. To determine bacterial burdens in lung, liver, and spleen tissues of infected mice, the mice were humanely euthanized, and organs from each mouse were harvested and placed in 5 ml sterile PBS. Organs were homogenized using a Stomacher 80 Biomaster (Seward, Bohemia, NY). Serial 10-fold dilutions of supernatants then were prepared in sterile saline and plated on BB4G agar plates (Remel). Agar plates were incubated at 37°C for 48 h, and colonies were counted. The limit of detection for the determination of the bacterial burden was either 50 or 100 CFU/organ, depending on the experiment.

Histological analyses. Lung, liver, and spleen tissues were harvested and placed in 10% formalin for 24 h. In the case of lung tissues, the lungs were inflated with formalin via tracheal injection prior to being removed and then were placed in formalin solution for 24 h. After 24 h, organs were transferred into 70% ethanol for another 7 days. Tissues then were sectioned and stained with hematoxylin and eosin. Organ pathology was assessed by an experienced veterinary pathologist (H. Bielefeldt-Ohmann).

Cytokine quantitation. To assess the effects of immunotherapy on cytokine production in the lungs, CLDC (or diluent) was administered i.n. to mice (n = 4 per group), which were euthanized 6 or 24 h later. Lungs were harvested and homogenized using a Stomacher 80 Biomaster (Seward) in 4 ml sterile saline. The lung homogenate was clarified by centrifugation, and the supernatants were frozen at −80°C prior to cytokine analysis. Concentrations of IL-12p40 and IFN-γ were measured by commercial enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

In vitro infection of macrophages with *B. mallei*. The mouse alveolar macrophage cell line (AMJ2; American Type Tissue Collection, Manassas, VA) was used to investigate the ability of CLDC supernatants to enhance bactericidal activity in vitro. Cells were cultured in 24-well plates in complete medium without antibiotics. Cells were cultured in complete medium that 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). Adherent cells were infected with *B. mallei* at a multiplicity of infection (MOI) of 2 for 1 h in 250 μl medium at 37°C and 5% CO_2. Extracellular bacteria were removed by washing the cells three times with PBS, followed by treatment with medium plus 350 μg/ml kanamycin (Sigma-Aldrich) for 1 h. After incubation with kanamycin for 1 h, the cells were washed and then cultured in complete medium with 10 μg/ml kanamycin for an additional 24 h. To quantitate intracellular bacterial numbers, the cells were washed three times with PBS and then lysed with sterile double-distilled water containing 0.01% Triton X-100. Serial dilutions of lysate were plated on BB4G agar plates, and plates were incubated at 37°C for 48 h, at which time colonies were counted.

Cytokine inhibition of macrophage infection with *B. mallei*. The ability of cytokines elicited by CLDC immunotherapy to inhibit the intracellular replication of *B. mallei* in infected macrophages was assessed using AMJ2 alveolar macrophages. AMJ2 cells (1×10^5 per well in triplicate wells of 24-well plates) were pretreated for 24 h before infection with diluted (1:10 or 1:100) supernatants from spleen cells of mice treated in vivo with CLDC or with supernatants from control spleen cells. Briefly, supernatants were generated as described previously by using overnight cultures of spleen cells prepared from mice injected intravenously (i.v.) with 200 μl CLDC 3 h prior to sacrifice (11). It was determined by ELISA that the supernatants from spleen cells of CLDC-injected mice contained 3 ng/ml IFN-γ and 140 pg/ml TNF-α (data not shown).

Neutralizing antibodies were used to identify key cytokines that may have been responsible for generating antibacterial activity in supernatants from spleen cells. Thus, supernatants were treated with 10 μg/ml anti-IFN-γ antibody (clone R4.6A2) (eBioscience, San Diego, CA), 10 μg/ml of anti-TNF-α antibody (clone TN3-19.12) (eBioscience), or a mixture of equal concentrations of both antibodies. Controls included incubating the supernatants with equivalent amounts of irrelevant isotype-matched antibodies (clones eBRG1 and eBio299Arm; eBiosciences) at 37°C for 30 min prior to the addition of supernatants to cells. Cells were incubated with supernatants for 24 h and then infected with *B. mallei*, and intracellular bacterial concentrations were determined as noted above. The effects of individual cytokines on inhibiting the *B. mallei* infection of AMJ2 cells also were evaluated. AMJ2 cells were incubated with recombinant murine IFN-γ (10 ng/ml; Preprotec, Rocky Hill, NJ) or murine TNF-α (10 ng/ml; Preprotec) for 24 h prior to *B. mallei* infection. Twenty-four hours after infection, intracellular concentrations of bacteria were determined as indicated above.

NK cell depletion. NK cells were depleted systemically in mice (n = 5 per group) by the injection of anti-asialo GM1 antibody (Wako Chemicals, Richmond, VA), as described previously (9, 11, 13). Briefly, mice were injected i.p. with 50 μg of anti-asialo GM1 antibody 24 h prior to CLDC treatment. This treatment was found to decrease the number of splenic and lung NK cells by more than 75% (data not shown). The mice then were infected i.n. with *B. mallei* 24 h after that (48 h after the injection of asialo-GM1 antibody). Controls included mice injected i.p. with an equivalent amount of rabbit immunoglobulin G antibody (Jackson Immunoresearch, West Grove, PA).

Inhibition of nitric oxide production in vivo. Inducible nitric oxide synthase (iNOS) activity was inhibited by the treatment of mice with aminoguanidine (AG) (Sigma-Aldrich). Mice were injected i.p twice daily with 0.2 ml of a 50-mg/ml solution of AG prepared in PBS, starting 5 days prior to infection and

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continuing until 7 days post infection, using a previously described protocol (5, 7). Control mice were injected with an equal volume of sterile PBS.

Statistical analysis. Statistical analysis was performed using Prism 5.0 software (GraphPad, La Jolla, CA). For the comparison of two groups, two-tailed *t* tests were performed. For the comparison of more than two groups, one-way analysis of variance (ANOVA) was done, followed by Tukey's multiple means comparison test. Survival times were compared using Kaplan-Meier curves and the log-rank test. Data were considered statistically significant for $P < 0.05$.

RESULTS

Mucosal administration of CLCD immunotherapy protects mice from acute *B. mallei* pneumonic infection. Previous studies demonstrated that systemic immunotherapy using CpG oligonucleotides could provide protection from chronic infection following low-dose *B. mallei* pneumonia (38). However, we wished to determine whether mucosal immunotherapy with a liposome-based immunotherapeutic was effective in an acutely lethal *B. mallei* pneumonia model. Therefore, we conducted protection studies in mice challenged with a high dose (10× LD_{50}) of *B. mallei* by the i.n. route.

In the first studies, we assessed the effects of the timing of CLDC administration on the induction of protective immunity. BALB/c mice ($n = 5$ per group) were administered 20 μl of CLDC by the i.n. route, either 24 h prior to infection, concurrently with infection, or 24 h after infection. Mice then were infected i.n. with 10× LD_{50} (approximately 1×10^4 CFU) of *B. mallei* and monitored for effects on survival. We observed that the administration of CLDC 24 h prior to infection generated complete protection from lethal *B. mallei* infection (Fig. 1). Concurrent i.n. administration of CLDC immunotherapy provided partial protection from lethal infection, whereas treatment 24 h after infection did not generate significant protection (Fig. 1). In addition, the administration of CLDC 6 h after infection also failed to elicit significant protection (data not shown). We also noted that protection elicited by mucosal CLDC immunotherapy prevented lethal acute infection but failed to completely protect from chronic infection, as approximately 50% of treated mice developed chronic infection by day 60 after challenge (data not shown). Thus, pretreatment by the i.n. administration of liposome-based immunotherapy was sufficient to generate significant protection from high-dose inhalational challenge with *B. mallei*.

Experiments then were conducted to assess the dose responsiveness of CLDC protection from *B. mallei* challenge by using the 24 h of pretreatment model. Mice ($n = 5$ per group) were pretreated with the standard concentration of CLDC or with CLDC diluted 1:2 or 1:20 in diluent (Fig. 1). We found that the administration of CLDC diluted 1:2 still provided significant ($P < 0.01$) protection, whereas CLDC diluted 1:20 failed to elicit protection.

We next assessed the ability of CLDC immunotherapy to protect from a higher *B. mallei* challenge dose. Mice were subjected to challenge with 50× LD_{50} (5×10^4 CFU), and the effects on survival time were assessed. We found that CLDC immunotherapy still provided significant protection in this higher-dose challenge model. Though 80% of the mice succumbed to this high-dose challenge, the median survival time for CLDC-treated mice was 4 days, whereas it was 2 days for untreated mice ($P = 0.003$) (data not shown). We also assessed whether increasing the CLDC dose increased protection. Increasing the dose of CLDC administered by doubling the concentration of liposomes and plasmid DNA (and still administering the same i.n. volume of CLDC) did not, however, increase survival significantly over that elicited by the administration of the standard amount of CLDC (data not shown).

Burkholderia mallei and *B. pseudomallei* are related pathogens, but they also possess some key differences with respect to genomic complexity and the numbers of potential virulence factors (17, 25). Therefore, we conducted challenge studies with *B. pseudomallei* to determine whether CLDC immunotherapy also was capable of eliciting protective immunity against pneumonic infection with this organism. BALB/c mice ($n = 5$ per group) were treated with CLDC i.n. 24 h prior to infection and then were subject to i.n. infection with 10× LD_{50} (1×10^4 CFU) of *B. pseudomallei*, and the effects on survival times were assessed (Fig. 1). We found that CLDC immunotherapy was effective in completely protecting mice from lethal pneumonic infection with *B. pseudomallei*. Thus, CLDC immunotherapy was capable of eliciting broadly protective immunity against two species of *Burkholderia*.

Reduced bacterial burden and organ pathology in mice treated with CLDC immunotherapy. The preceding studies demonstrated that CLDC immunotherapy could protect against lethal respiratory *Burkholderia* infection in a dose-dependent fashion. Therefore, studies were conducted next to elucidate the mechanisms of protection. First, the effects of CLDC treatment on bacterial burdens in the lungs, spleen, and liver were assessed. BALB/c mice ($n = 5$ per group) were sham treated or were treated with CLDC i.n. 24 h prior to infection and then were subjected to infection with approximately 1×10^4 CFU *B. mallei* i.n. The mice were euthanized 3 days after infection and bacterial were burdens determined, as described in Materials and Methods. We found that there were significant ($P < 0.0001$) reductions in the bacterial burdens in lung, liver, and spleen in CLDC-treated mice compared to that in untreated mice (Fig. 2).

CLDC immunotherapy did not, however, generate complete sterilizing immunity. For example, nearly all CLDC-treated mice, though they survived acute infection, eventually developed chronic disseminated *B. mallei* infection in the liver and spleen. Bacterial burdens in the lung remained below the limit of detection (50 CFU) on day 21, 30, 45, or 60 after infection. However, increasing bacterial burdens were noted in the liver and especially the spleen at later time points following CLDC treatment and *B. mallei* challenge (data not shown).

Pretreatment with CLDC immunotherapy also substantially reduced acute organ pathology in *B. mallei*-infected mice. In untreated mice ($n = 4$ per group) euthanized 3 days after *B. mallei* challenge, there was marked neutrophil infiltration in the lungs, along with hemorrhage from pulmonary vessels, the necrosis of respiratory epithelium, and the apoptosis of leukocytes and lung parenchyma (Fig. 3). In contrast, in CLDC-treated mice the lung neutrophil infiltrate was reduced and hemorrhage was not observed in pulmonary vessels.

In untreated *B. mallei*-infected mice, hepatic lesions consisted of infiltrates of neutrophils and monocytes. In the livers of untreated mice there were large areas of necrosis, and in some mice hepatic lesions coalesced, whereas hepatic lesions in CLDC treated mice showed no evidence of necrosis and generally were much smaller (Fig. 3). Important changes in

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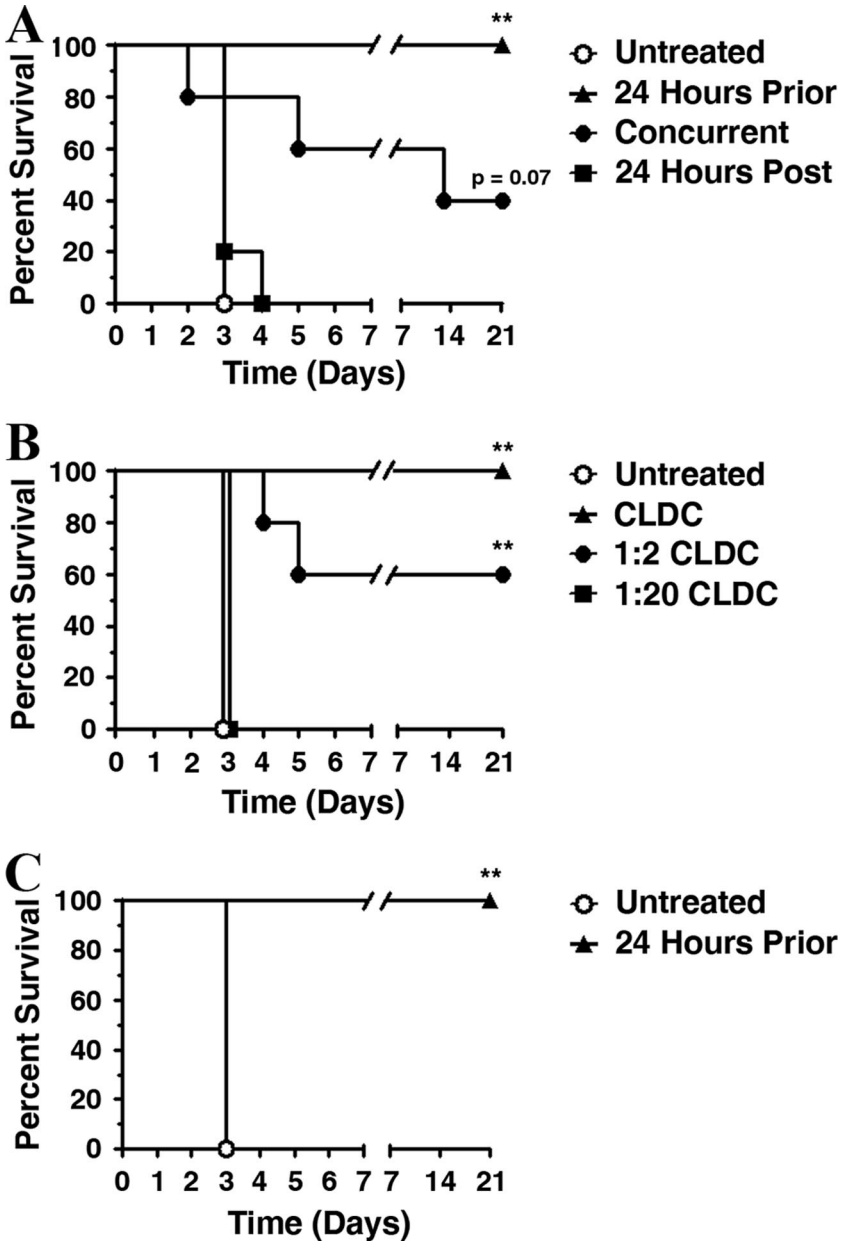

FIG. 1. Protective effects of CLDC against pneumonic *Burkholderia* infection are dependent on the timing and dose of CLDC administered. (A) BALB/c mice ($n = 5$ animals per group) were challenged i.n. with 1×10^4 CFU *B. mallei*, and the effects of the timing of the administration of CLDC immunotherapy on survival were assessed. Mice were untreated or were treated with CLDC 24 h prior to infection, at the time of infection, or 24 h after infection, and survival times were determined as described in Materials and Methods. (B) The effects of the CLDC dose on the induction of protective immunity were assessed. Mice ($n = 5$ animals per group) were treated by the i.n. administration of standard CLDC or CLDC diluted 1:2 or 1:20 24 h before i.n. challenge with 1×10^4 CFU *B. mallei*, and survival times were determined. (C) BALB/c mice ($n = 5$ per group) were untreated or were treated with CLDC 24 h prior to i.n. infection with $10\times$ LD_{50} (1×10^4 CFU) of *B. pseudomallei*. Statistical differences for the data shown were determined by Kaplan-Meier analysis followed by a log-rank test (**, $P < 0.01$). Data shown in all three panels are representative of two independent experiments each.

spleen pathology were not observed between CLDC-treated and untreated mice. Thus, these studies demonstrated that CLDC immunotherapy significantly reduced bacterial replication and organ pathology in mice infected with *B. mallei* compared to those for infected mice not receiving immunotherapy.

CLDC-elicited cytokines block *B. mallei* infection of alveolar macrophages in vitro. The fact that the i.n. administration of CLDC elicited a significant decrease in bacterial replication in the lungs suggested that treatment triggered the release of cytokines that were capable of eliciting antibacterial activity in macrophages. For example, it is known that the delivery of liposome-DNA complexes to the lungs can elicit the local production of IFN-γ and TNF-α (6). To investigate this mechanism further and identify possible protective cytokines, we

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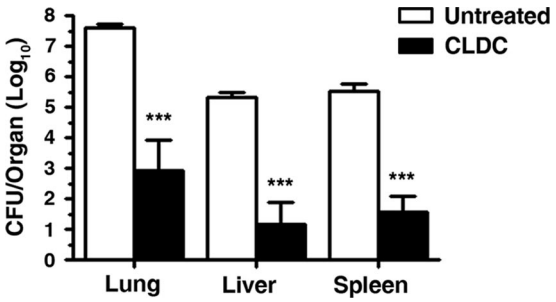

FIG. 2. Effects of CLDC immunotherapy on bacterial burden in the lung, liver, and spleen of mice following inhalational challenge with *B. mallei.* BALB/c mice ($n = 5$ per group) were treated with CLDC by i.n. administration 24 h prior to challenge and then challenge by the inhalation of 1×10^4 CFU *B. mallei.* Bacterial burdens (in CFU) were determined in each organ 72 h after *B. mallei* challenge, as described in Materials and Methods. Significant reductions in bacterial burdens were seen in mice treated with CLDC in all three organs analyzed, as assessed by a two-tailed Student's *t* test (***, $P < 0.001$). Data are representative of two independent experiments.

used an in vitro infection system consisting of AMJ2 cells (an alveolar macrophage line) and supernatants from spleens of mice that had been injected i.v. with CLDC. In a previous study, we found that spleen cells were a major source of cytokine production in CLDC-treated mice (11). AMJ2 cells were treated with CLDC supernatants (or supernatants from untreated control mice) for 24 h. The cells then were infected with *B. mallei* at an MOI of 2, and 24 h later the numbers of intracellular bacteria were determined.

The preincubation of macrophages with supernatants from CLDC-treated mice, but not supernatants from untreated control mice, resulted in significant reduction in the numbers of viable intracellular *B. mallei* (Fig. 4). We also observed the inhibition of *B. mallei* infection in macrophage cultures with supernatants from CLDC-stimulated lungs, but the effect was much less potent than that with spleen supernatants (data not shown). Significant antibacterial activity was observed when supernatants were diluted 1:1 (not shown) or 1:10, but it was lost when supernatants were diluted 1:100. In previous studies, CLDC were shown to elicit large amounts of IFN-γ and smaller amounts of TNF-α, both of which have antibacterial activity against *Burkholderia* (4, 29, 30). Therefore, we used neutralization experiments to assess the relative contribution of each cytokine to antibacterial activity. The neutralization of IFN-γ activity resulted in the significant abrogation of the macrophage antibacterial activity of CLDC supernatants (Fig. 4). In contrast, the neutralization of TNF-α activity had a much smaller effect. However, the neutralization of both cytokines resulted in the greater inhibition of antibacterial activity, suggesting that TNF-α production contributed to the effectiveness of IFN-γ in suppressing *B. mallei* replication in infected macrophages. Finally, the pretreatment of AMJ2 cells with 10, 1, or

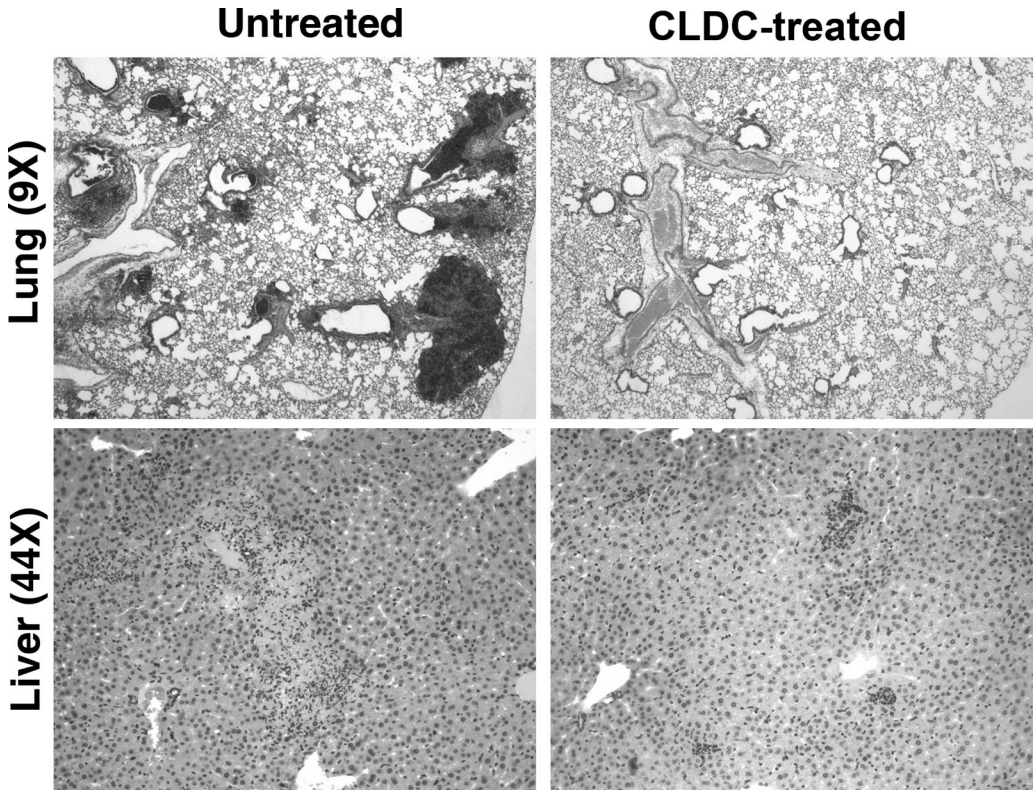

FIG. 3. Comparison of lung and liver pathology in untreated and CLDC-treated mice following lethal *B. mallei* challenge. BALB/c mice ($n = 5$ per group) were untreated or were treated with CLDC 24 h prior to i.n. infection with 10^4 CFU *B. mallei*. On day 3 after infection, mice were euthanized and lung and liver tissues were collected and processed for histopathological evaluation, as described in Materials and Methods. Representative sections from lung (×2 magnification) and liver (×20 magnification) from untreated and CLDC-treated mice were photographed. Images are representative of those obtained in two independent experiments.

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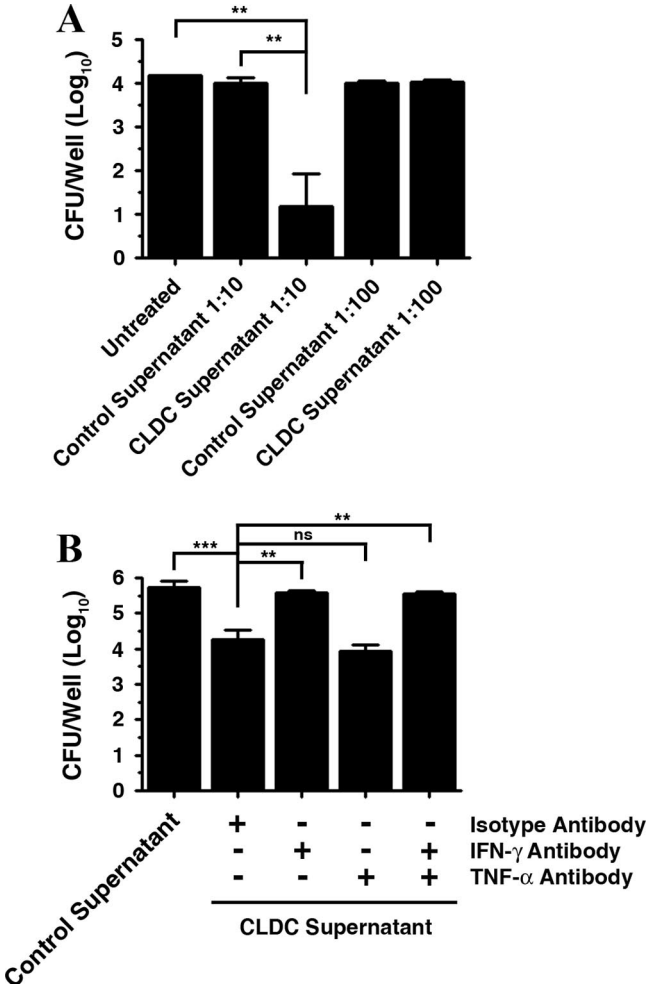

FIG. 4. IFN-γ elicited by CLDC treatment inhibits the *B. mallei* infection of alveolar macrophages in vitro. (A) AMJ2 (an alveolar macrophage cell line) cells in triplicate wells of 24-well plates were treated 24 h prior to infection with supernatants generated from CLDC-treated or untreated mouse spleen cells, as described in Materials and Methods. The cells then were infected with *B. mallei*, and 24 h later the numbers of intracellular *B. mallei* organisms were determined. The treatment of AMJ2 cells with CLDC supernatant resulted in a significant reduction ($P < 0.01$) in the numbers of intracellular *B. mallei* organisms. (B) Neutralizing antibodies to IFN-γ and TNF-α were utilized to determine the key cytokines present in CLDC-stimulated spleen supernatants that were responsible for the inhibition of *B. mallei* infection in macrophages, as described in Materials and Methods. The neutralization of IFN-γ resulted in the significant ($P < 0.05$) inhibition of the antibacterial activity of CLDC supernatants against the *B. mallei* infection of AMJ2 cells. In contrast, the inhibition of TNF-α alone did not significantly inhibit CLDC activity. Statistical significance was assessed using ANOVA, followed by Tukey's multiple means comparison test. These results are representative of those obtained in three independent experiments.

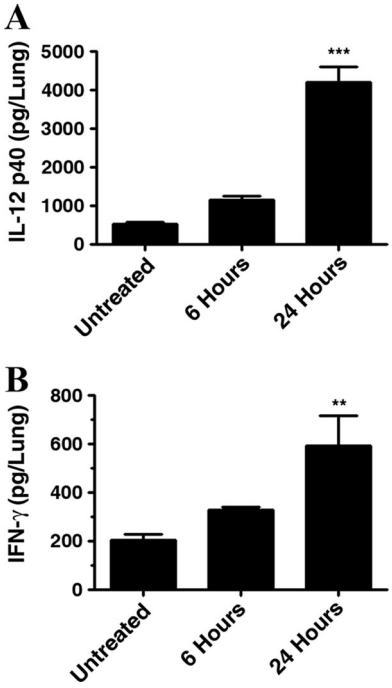

FIG. 5. Induction of IL-12p40 and IFN-γ production in the lungs following the i.n. administration of CLDC. BALB/c mice ($n = 5$ per group) were treated with CLDC i.n. and then were euthanized 6 or 24 h after treatment. Concentrations of IL-12p40 and IFN-γ were determined in homogenized lung tissues by ELISA, as described in Materials and Methods. A one-way ANOVA followed by Tukey's multiple means comparison test was used to determine significant differences between the three treatment groups (**, $P < 0.01$; ***, $P < 0.001$; compared to results for untreated samples). Significant increases in both IL-12 and IFN-γ were observed 24 h after the administration of CLDC. Data are representative of two independent experiments.

0.1 ng/ml recombinant IFN-γ resulted in 100, 98, and 98% inhibition of bacterial growth, respectively (data not shown). These results indicated that the CLDC-induced production of IFN-γ mediated many of the protective effects of CLDC immunotherapy noted in vivo, particularly since we have observed that alveolar macrophages are one of the primary early target cells for *Burkholderia* infection in the lungs (data not shown).

i.n. administration of CLDC elicits production of IL-12 and IFN-γ in the lungs. Both IL-12 and IFN-γ are known to play critical roles in protective immunity to *Burkholderia* infection (2, 18–20, 29, 30). In addition, the preceding in vitro studies indicated that IFN-γ could control *Burkholderia* replication in alveolar macrophages, a likely target cell for the inhalational route of infection. Therefore, we assessed the ability of the inhalational delivery of CLDC to elicit IL-12 and IFN-γ production in the lungs. Six and 24 h after the i.n. administration of CLDC, IL-12 and IFN-γ concentrations in lung tissues were determined by ELISA (Fig. 5). Significant increases in the concentrations of both IL-12 and IFN-γ were observed in lung tissues 24 h following the i.n. delivery of CLDC, with smaller increases noted at 6 h after administration. These results indicated that the inhalational delivery of relatively low doses of CLDC to the lung could trigger the significant production of a key cytokine (IFN-γ) responsible for suppressing the intracellular replication of *B. mallei*. The kinetics of cytokine induction in the lung in response to CLDC immunotherapy also may explain why pretreatment with CLDC 24 h before challenge did elicit protective immunity, whereas treatment 6 h after challenge did not.

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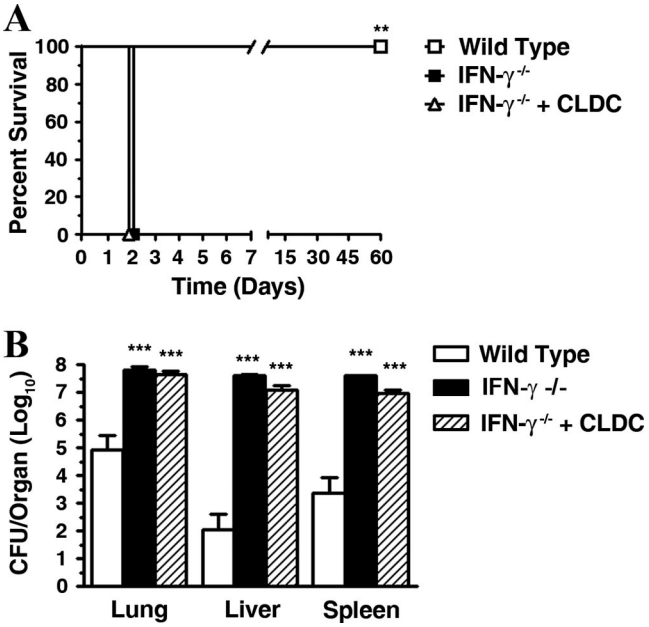

FIG. 6. IFN-γ is necessary for the in vivo protective of CLDC immunotherapy. (A) BALB/c wild-type mice ($n = 5$ per group) or IFN-$\gamma^{-/-}$ mice ($n = 5$) were treated with CLDC 24 h prior to infection or were left untreated. Mice were infected by low-dose challenge using the i.n. administration of 5×10^2 CFU *B. mallei*, and survival was assessed. Survival times in the IFN-$\gamma^{-/-}$ mice treated with i.n.-administered CLDC were not significantly different in terms of infection than untreated IFN-$\gamma^{-/-}$ mice. Statistical differences were determined by Kaplan-Meier analysis using a log-rank test (**, $P < 0.01$ compared to results for the wild type). Similar results were obtained in a second experiment done in IFN-$\gamma^{-/-}$ mice on a C57BL/6 background. (B) Wild-type BALB/c mice ($n = 5$) or IFN-$\gamma^{-/-}$ mice ($n = 5$) were treated with i.n.-administered CLDC 24 h prior to infection or were left untreated. Mice were infected with 5×10^2 CFU *B. mallei*, and on day 2 after infection mice were euthanized and bacterial burdens in the lungs, liver, and spleen were determined, as described in Materials and Methods. The bacterial burdens in all three organs of IFN-$\gamma^{-/-}$ mice treated with CLDC were not significantly different from those of untreated IFN-$\gamma^{-/-}$ mice. Similar results were obtained in a second experiment done in IFN-$\gamma^{-/-}$ mice on a C57BL/6 background. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple means comparison test (***, $P < 0.001$ compared to results for the wild type).

IFN-γ is necessary for CLDC-mediated protection in vivo. Experiments next were conducted to directly elucidate the in vivo role of IFN-γ in CLDC-mediated protection from lethal challenge with *B. mallei*. For these experiments, we used IFN-$\gamma^{-/-}$ mice on the BALB/c background. In addition, a much lower challenge dose of *B. mallei* was administered (0.5 LD_{50} or 500 CFU), since IFN-$\gamma^{-/-}$ mice are known to be exquisitely sensitive to *Burkholderia* infection (29, 30). Untreated IFN-$\gamma^{-/-}$ mice, IFN-$\gamma^{-/-}$ mice treated with CLDC 24 h prior to infection (IFN-$\gamma^{-/-}$ plus CLDC), and wild-type BALB/c mice were infected with 0.5 LD_{50} (500 CFU) of *B. mallei* i.n. (Fig. 6). We observed that the CLDC pretreatment of IFN-$\gamma^{-/-}$ mice failed to elicit protective immunity, indicating that IFN-γ was a key component of the protective immunity elicited by CLDC immunotherapy.

The role of IFN-γ in suppressing *B. mallei* replication in vivo also was assessed. Bacterial burdens in the lung, liver, and spleen of IFN-$\gamma^{-/-}$ mice treated with CLDC were compared to those of untreated IFN-$\gamma^{-/-}$ mice and wild-type mice on day 2 after low-dose i.n. challenge with *B. mallei*. As expected, the numbers of bacteria in all three organs from IFN-$\gamma^{-/-}$ mice were significantly higher than those in organs from wild-type mice (Fig. 6). However, significant differences were not observed when the numbers of bacteria in untreated IFN-$\gamma^{-/-}$ mice and CLDC-treated IFN-$\gamma^{-/-}$ mice were compared (Fig. 6). Thus, these data are consistent with the conclusion that IFN-γ was necessary for CLDC immunotherapy to effectively suppress *B. mallei* replication and to increase survival following pulmonary challenge.

Role of NK cells in CLDC-induced protection from *B. mallei* challenge. Previous studies have indicated that NK cells were the major source of IFN-γ production following systemic (i.v.) treatment with CLDC (10, 34). However, the role of NK cells in mediating CLDC-induced pulmonary immunity to bacterial challenge has not been explored previously. Prior studies have revealed that multiple cell types, including macrophages, $CD8^+$ T cells, and NK cells, produce IFN-γ in response to *Burkholderia* infection (14, 29). To elucidate the role of NK cells in CLDC protection, NK cells were depleted systemically using the asialo GM1 antibody (9, 11, 13, 16). The antibody was administered 48 h prior to infection, and then the mice were treated 24 h prior to infection by the i.n. administration of CLDC. Mice then were infected with 10× LD_{50} of *B. mallei* and monitored for survival.

There was a significant reduction in survival ($P < 0.01$) of CLDC-treated mice depleted of NK cells compared to that of CLDC-treated mice not depleted of NK cells (Fig. 7). However, CLDC-treated and NK-depleted mice still had a significant improvement in survival compared to that of untreated mice (Fig. 7). Thus, these results indicated that NK cells were necessary for full CLDC-mediated protection. However, partial protection still was observed in NK cell-depleted mice, suggesting either that other cell types besides NK cells also contributed to the protective effects of CLDC therapy or that the residual NK cells not depleted by the administration of anti-asialo GM1 antibody were sufficient to elicit partial protection.

The role of NK cells in regulating the bacterial burden also was assessed. Bacterial burdens in the lung, liver, and spleen of mice depleted of NK cells and treated with CLDC were determined 3 days after infection. Bacterial burdens in NK cell-depleted and CLDC-treated mice were significantly increased in all three organs compared to those of mice treated with CLDC immunotherapy alone (Fig. 7). In addition, significant reductions in bacterial burdens also were observed in NK cell-depleted and CLDC-treated mice compared to those of untreated mice. These results are consistent with the idea that NK cells activated by CLDC immunotherapy contribute to antibacterial activity, most likely by the secretion of IFN-γ.

Nitric oxide production is not necessary for CLDC-mediated protection. The preceding in vitro and in vivo experiments demonstrated that IFN-γ was critical to the therapeutic effectiveness of CLDC immunotherapy. IFN-γ also is known to be a potent inducer of NOS2 (iNOS) activity, with the subsequent production of nitric oxide (24). Several in vitro studies utilizing both *B. pseudomallei* and *B. mallei* have demonstrated that the preactivation of cells with IFN-γ results in nitric oxide produc-

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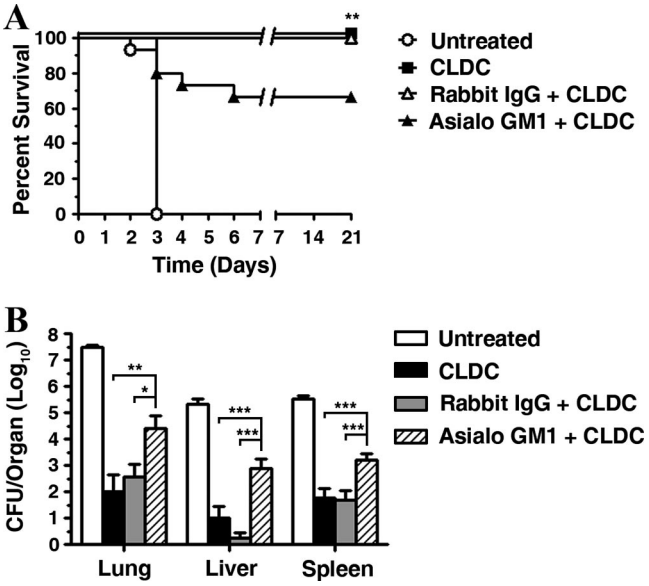

FIG. 7. Natural killer cells play an important role in the protective effects of CLDC immunotherapy. (A) BALB/c mice ($n = 10$ per group) were depleted of NK cells by the i.p. administration of an NK cell-depleting antibody 48 h prior to infection, as described in Materials and Methods. Control mice were treated with an irrelevant control rabbit antibody. Twenty-four hours after NK cell depletion, one group of mice was treated by the i.n. administration of CLDC immunotherapy, and 24 h later the mice were subjected to infection with 10^4 CFU *B. mallei* and survival times were assessed. The third group of NK cell-depleted mice was not treated with CLDC but was subjected to *B. mallei* challenge 48 h later. Statistical differences were determined by Kaplan-Meier analysis using a log-rank test (*, $P < 0.05$). (B) The effects of NK cell depletion and CLDC treatment on bacterial burdens in lung, liver, and spleen tissues were assessed 72 h after i.n. infection with *B. mallei*. Treatment groups are the same as those noted for panel A. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple means comparison test. (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

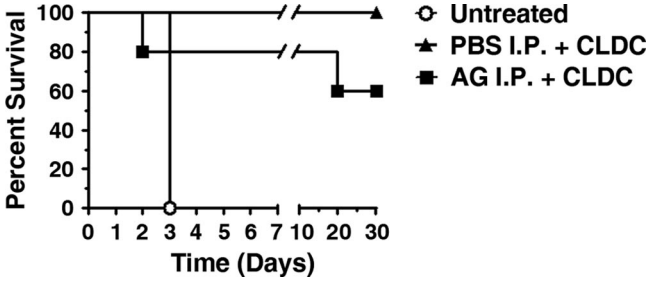

FIG. 8. Nitric oxide production is not necessary for the protective effects of CLDC immunotherapy. BALB/c mice ($n = 5$ per group) were treated i.p. twice daily with AG (or PBS) starting 5 days prior to infection and continuing until 7 days postinfection, as described in Materials and Methods. Mice were treated i.n. with CLDC 24 h prior to infection with 15× LD_{50} (1.5×10^4 CFU) of *B. mallei* i.n., and survival times were determined. Statistical differences were determined by Kaplan-Meier analysis and a log-rank test. Data are representative of two independent experiments.

tion, which in turn mediates bacterial killing. Other in vitro studies have shown that *Burkholderia* species may downmodulate nitric oxide production in order to enhance its survival (3, 15, 23, 35, 36). In contrast to the results obtained in these in vitro studies, in vivo *B. pseudomallei* challenge studies using $NOS2^{-/-}$ mice have failed to demonstrate a role for nitric oxide in controlling infection (8).

Given the critical role of IFN-γ in mediating the protective effects of CLDC immunotherapy, we wished to determine whether the induction of iNOS activity and nitric oxide production was necessary for CLDC activity. To address this question, we used the iNOS inhibitor AG to block the production of nitric oxide in vivo. Mice were treated with AG beginning 5 days prior to infection. Mice then were treated with CLDC i.n. 24 h prior to infection and then were infected i.n. with 15× LD_{50} (1.5×10^4 CFU) of *B. mallei*. We observed that pretreatment with AG had no effect on CLDC protection (Fig. 8). To ensure that the AG treatment protocol we used effectively inhibited nitric oxide production, in a separate experiment we injected AG-treated mice with lipopolysaccharide and assessed nitric oxide production in serum 6 h later, and we found that AG treatment completely suppressed lipopolysaccharide-induced nitric oxide production (data not shown). Moreover, in naive mice not treated with AG, we also noted that CLDC treatment failed to induce detectable nitric oxide production in lung tissues or alveolar lavage fluid (data not shown). By real-time PCR analysis, we also failed to observe increased iNOS expression in lung tissues of mice treated with CLDC (data not shown). Thus, several lines of evidence indicated that the induction of nitric oxide production was not critical for CLDC-mediated protection from pneumonic infection with *B. mallei*, although we cannot completely rule out a minor role for NO in protection.

DISCUSSION

The results presented here indicated that the mucosal delivery of a potent activator of innate immunity could provide significant protection from lethal pneumonic infection with both *B. mallei* and *B. pseudomallei*. The most effective protection was achieved when mice were pretreated with CLDC 24 h prior to challenge, which suggested that critical immune protective mechanisms had to be active at the time of initial contact with the inhaled bacteria to be fully effective. The local, but not systemic, induction of IFN-γ release was required for protection, and protection appeared to be independent of nitric oxide production. Although previous studies found that the systemic administration of CpG-based immunotherapeutics could protect mice from the development of chronic pneumonia following low-dose *Burkholderia* challenge, these are the first studies to our knowledge to demonstrate effective protection from acutely lethal respiratory *Burkholderia* infection following the inhalational delivery of an immunotherapeutic (2, 41).

IFN-γ played a critical role in the protective effects elicited by CLDC immunotherapy. In support of this, we observed that IFN-$\gamma^{-/-}$ mice were completely unprotected by CLDC immunotherapy. It is likely that the primary source of IFN-γ production elicited by CLDC was from activated NK cells. The potent activation of NK cells and IFN-γ release from the lungs has been reported previously following CLDC immunotherapy (11). In the current study, we also demonstrated that NK cells played a role in the protective effects mediated by CLDC. However, the requirement for NK cells to mediate the protec-

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tive effects of CLDC was more apparent at lower challenge doses. For example, NK-depleted, CLDC-treated mice challenged with 8× LD_{50} (8×10^3 CFU) had a 60 to 80% survival rate, whereas mice infected with 12× LD_{50} (1.2×10^4 CFU) has only a 20% survival rate. Nevertheless, both of these challenge doses produced 100% mortality in untreated mice.

Despite a series of in vitro studies that have shown that nitric oxide is important for the bactericidal effects of IFN-γ against both *B. pseudomallei* and *B. mallei*, in our studies we failed to find a role for nitric oxide in CLDC-mediated protective activity (3, 15, 23, 35, 36). Our findings thus are in agreement with a previous in vivo study wherein mice lacking the iNOS gene ($NOS2^{-/-}$) did not exhibit increased susceptibility to *B. pseudomallei* infection (8). Thus, we concluded that CLDC-mediated protection from lethal *B. mallei* pneumonia was largely independent of nitric oxide production.

Although the mucosal administration of CLDC elicited complete protection against acute *B. mallei* infection, the infection was not completely eliminated. For example, when mice surviving the acute challenge were monitored out to 60 days, we observed the recrudescence of infection in the spleens and livers of 80 to 90% of mice. Thus, it would be important to follow up CLDC immunotherapy with conventional antimicrobial therapy to assure the complete eradication of *Burkholderia*. In summary, these studies indicate that appropriately timed mucosally administered immunotherapy may induce effective nonspecific protection against pneumonic intracellular bacterial pathogens such as *B. mallei* and *B. pseudomallei*.

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