Published in final edited form as:

Trans R Soc Trop Med Hyg. 2008 December ; 102(0 1): S119–S126. doi:10.1016/S0035-9203(08)70028-2.

Inhalation of *Burkholderia thailandensis* results in lethal necrotizing pneumonia in mice: a surrogate model for pneumonic melioidosis

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Summary

Burkholderia thailandensis is closely related to *Burkholderia pseudomallei*, the causative agent of melioidosis, a lethal tropical disease. *B. thailandensis* is largely avirulent in humans and unlike *B. pseudomallei*, does not require strict biocontainment conditions. Because it may be a useful research surrogate for *B. pseudomallei*, we developed a murine model of airborne *B. thailandensis* infection. In both C57BL/6 and BALB/c mice, deposition of 10^3 CFU/lung or less of *B. thailandensis* was non-lethal and infection was readily controlled. Compared to C57BL6 mice, BALB/c mice exhibited modest resistance to infection after deposition of 10^4 CFU/lung. Deposition of 10^5 CFU/lung resulted in disseminated infection and was universally fatal by three days. This dose induced robust pulmonary neutrophilia, production of inflammatory cytokines, and elevated serum markers of distant organ injury. Histology demonstrated multiple small foci of necrotizing pneumonia but lung architecture was otherwise preserved, suggesting that respiratory failure is not the cause of death. These findings demonstrate that airborne *B. thailandensis* infection in mice provides an accessible surrogate model of melioidosis.

Keywords

Burkholderia; melioidosis; pneumonia; lung; aerosol

1. Introduction

Burkholderia pseudomallei is a Gram-negative soil saprophyte and the causative agent of melioidosis, an endemic disease throughout southeast Asia and northern Australia.

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Authors' contributions

TEW and SJS designed the experiments, and TEW performed the experiments, analyzed and interpreted the data, and drafted the manuscript. HDL provided guidance in interpreting data, CF acquired histology images, and HDL and CF interpreted the histology. All authors read and approved the final manuscript. TEW and SJS are guarantors of the paper.

Inhalation or transcutaneous inoculation are the most likely routes of infection and pneumonia is the most common manifestation (White, 2003). Despite antibiotic therapy, the infection is frequently fatal (White et al., 1989). *B. pseudomallei* also is considered a potential biowarfare agent (Bossi et al., 2004) and as a CDC B category pathogen can be studied only in Biosafety Level (BSL)-3 facilities in many countries. *B. thailandensis* is a closely related bacterium with a similar environmental distribution that is morphologically indistinguishable from *B. pseudomallei* (Wuthiekanun et al., 1996). *B. thailandensis* shares extensive genomic similarity and the majority of virulence factors with *B. pseudomallei* (Yu et al., 2006), but chemically may be differentiated by its ability to assimilate L-arabinose (Smith et al., 1997). Importantly, *B. thailandensis* rarely causes human disease (Glass et al., 2006, Lertpatanasuwan et al., 1999). Because of its relative avirulence, containment conditions for *B. thailandensis* are much less stringent than for *B. pseudomallei*, rendering it more accessible to researchers. *B. thailandensis* is therefore a useful surrogate organism for investigators studying the pathogenicity of melioidosis (Yu et al., 2006).

A number of murine models of *B. pseudomallei* infection have been described. BALB/c mice are markedly more susceptible than C57BL/6 mice to *B. pseudomallei* when infection is generated by intranasal, oral, or systemic routes (Barnes and Ketheesan, 2005, Hoppe et al., 1999, Leakey et al., 1998, Liu et al., 2002). In these models, BALB/c and C57BL/6 strains of mice may represent acute and chronic presentations of melioidosis, respectively. However, the natural route of airborne infection is best mimicked by inhalation of aerosolized bacteria. A recent study of aerosolized *B. pseudomallei* did not reveal differences in mouse strain susceptibility, a finding that was attributed to the route of infection (Jeddeloh et al., 2003). This suggests that airborne models may not be comparable to intranasal models of *B. pseudomallei* infection.

Little is known about the virulence of *B. thailandensis* in animals, however. High doses are lethal to Syrian golden hamsters (Brett et al., 1997) and to BALB/c mice after intraperitoneal infection (Smith et al., 1997) and to C57BL/6 mice after intranasal inoculation (Wiersinga et al., 2007). However, virulence in intraperitoneal infection of Syrian hamsters and BALB/c mice is dependent on the bacterial strain (Deshazer, 2007). Airborne infection has not been described. Therefore, we describe here a novel model of murine pneumonic infection with aerosolized *B. thailandensis*. In order to investigate differential mouse strain susceptibilities, we compare airborne infection in C57BL/6 and BALB/c mice. We characterize survival, bacterial replication and dissemination, pulmonary and systemic inflammatory responses, markers of distant organ injury, and lung histology.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Burkholderia thailandensis E264 was kindly provided by Dr. Donald Woods at the University of Calgary. Bacteria were grown from frozen glycerol stock in LB broth at 37 °C for 18 hours, isolated by centrifugation, washed twice, and suspended in Dulbecco's PBS to the desired concentration. An optical density of 0.20 at 540 nm yielded approximately 5×10^6 CFU/mL.

2.2. Animals

Specific-pathogen-free BALB/c and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were housed in laminar flow cages and were permitted ad lib access to sterile food and water. Euthanasia was accomplished with intraperitoneal pentobarbital followed by exsanguination from cardiac puncture. The Institutional Animal Care and Use Committee of the University of Washington approved all experimental procedures.

2.3. Murine model of pneumonia and bacterial quantification

Mice were exposed to aerosolized bacteria using a snout-only inhalation system (In-Tox Products, Moriarty, NM). Aerosols were generated from a MiniHEART hi-flo nebulizer (Westmed, Tucson, AZ) driven at 40 psi. Airflow through the system was maintained for 10 minutes at 24 L/min followed by five minutes purge with air. Bacterial deposition in each experiment was determined from quantitative culture of lung tissue from sentinel mice sacrificed immediately after infection. Animals were examined daily for illness or death and abdominal surface temperatures were measured using a Ranger MX4P digital infrared thermometer (Raytek, Santa Cruz, CA). Ill animals with temperatures < 21.5 °C, ruffled fur, eye crusting, hunched posture, and lack of resistance to handling were deemed terminal and euthanized (naturally occurring death was not required as an endpoint). At specific time points after infection mice were sacrificed; the left lung, median hepatic lobe, and spleen each were homogenized in 1 mL sterile Dulbecco's PBS and serial dilutions plated on LB agar. Colonies were counted after 2–4 days of incubation at 37 °C in humid air with 5% CO_2.

2.4. Bronchoalveolar lavage cell counts and lung histopathology

At serial time points after infection, C57BL/6 mice were euthanized, the left hilum tied off, and a 20 gauge catheter inserted endotracheally. The right lung was lavaged with four 0.5-ml aliquots of 0.9% NaCl supplemented with 0.6 mM EDTA. The right lung was then inflated to 15-cm pressure with 4% paraformaldehyde and immersed in the same fixative. Cell counts in bronchoalveolar lavage specimens were measured in a hemocytometer. Differentials were determined from examination of cytocentrifuge slides (Thermo Shandon, Pittsburgh, PA) that were stained with a modified Wright-Giemsa technique (Diff-Quik; Dade Behring, Dudingen, Switzerland). Lung tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were examined by a veterinary pathologist who was blinded to animal group assignments.

2.5. Measurements of cytokines and chemistries

C57BL/6 lung homogenates in Dulbecco's PBS were diluted 1:1 in lysis buffer containing 2× protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), incubated on ice for 30 min, and then centrifuged at 1,500 g. Supernatants were harvested and stored at −80 °C until assayed for cytokines. Whole blood was centrifuged, serum removed and stored at −80 °C until assayed. TNF-α, IL-1β, IL-6, IFN-γ, KC, MCP-1, MIP-2, and GM-CSF in lung homogenates and serum were measured using a multiplex bead assay (Luminex, Austin, TX), using reagents purchased from R&D Systems (Minneapolis, MN). Glucose, blood urea

nitrogen (BUN), creatinine, and alanine aminotransferase were measured in serum by a clinical laboratory after blood collection in a Microtainer Serum Separator Tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and centrifugation.

2.6. Statistical analyses

Combined data that follow a normal distribution are reported as mean ± standard deviation. Comparisons between two groups of normally distributed data were made using the t-test. Survival analyses were performed with the log rank test. All statistics were performed with Stata 9.0 (College Station, TX) or GraphPad Prism 4.0 (San Diego, CA). A p value ≤ 0.05 was considered significant.

3. Results

3.1. Survival

To determine whether inhaled *B. thailandensis* was virulent in mice we aerosolized approximately 3.5 mL of 2×10^5, 2×10^6, 1×10^7, and 2×10^8 CFU/mL bacteria in order to deposit 87, 2×10^3, 3×10^4, and 3×10^5 CFU/lung, respectively, in C57BL/6 and BALB/c mice (Fig. 1). These strains of mice were chosen because of differential C57BL/6 and BALB/c mice susceptibility to intranasal but not to aerosolized *B. pseudomallei* infection. Deposition of 3×10^5 CFU/lung was uniformly and rapidly fatal. All animals challenged with up to 2×10^3 CFU/lung survived for at least three weeks and appeared well for the entire duration of observation. BALB/c mice appeared modestly more resistant than C57BL/6 mice after deposition of 3×10^4 CFU/lung but infection was lethal in the majority of animals. Measurements of abdominal surface temperature correlated well with clinical outcome; all surviving mice maintained temperatures greater than 27 °C whereas lower temperatures were inevitably associated with progressive clinical deterioration, worsening hypothermia, and a moribund state requiring euthanasia.

3.2. Bacterial replication, dissemination, or clearance

To characterize infection further, we assessed bacterial burdens in lung, liver, and spleen of C57BL/6 and BALB/c mice infected with varying doses of *B. thailandensis* by aerosol (Fig. 2). At doses of 2×10^3 CFU/lung or less, mice contained the infection, whereas at higher doses there was aggressive pulmonary replication accompanied by dissemination to liver and spleen. There were no differences in bacterial replication or dissemination between C57BL/6 and BALB/c mice 72 hours after deposition with 2×10^3 CFU/lung or less, or with 3×10^5 CFU/lung. However, 72 hours after inoculation of 3×10^4 CFU/lung, BALB/c mice had significantly lower bacterial burdens in lung and liver compared to C57BL/6 mice.

The time course of sub-lethal infection was characterized in C57BL/6 mice by depositing 2×10^3 CFU/lung *B. thailandensis* by aerosol (Fig. 3A). Dissemination of infection to liver and spleen was evident one day after infection but containment was apparent in all organs of three of four animals at three days. Net bacterial clearance in all organs of all animals was established by seven days after infection.

The pattern of bacterial replication in lethal infection of C57BL/6 mice was determined by depositing 4×10^5 CFU/lung *B. thailandensis* by aerosol (Fig. 3B). Bacteria were identified in the liver four hours later and in the spleen at 24 hours. In these mice, bacterial burdens in lung, liver, and spleen at 24 hours were only about a half a log higher than in the respective organs of mice sub-lethally infected with 2×10^3 CFU/lung at the same time point. However, in contrast to the lower dose group, progressive replication was observed at 72 hours in all organs of high dose animals.

3.3. Pulmonary and systemic inflammation

To evaluate the host response to *B. thailandensis* infection, we performed bronchoalveolar lavage (BAL) and quantified cell counts in C57BL/6 mice that were uninfected or at four, 24, and 72 hours after deposition of 10^5 CFU/lung (Fig. 4A). Neutrophils were evident in BAL fluid by four hours after infection and increased significantly by 24 hours. At 72 hours, this pulmonary BAL neutrophilia had declined substantially.

We also measured inflammatory cytokine levels in lung homogenates and serum of C57BL/6 mice that were uninfected or at four, 24, and 72 hours after deposition of 10^5 CFU/lung (Fig. 4B). TNF-α, IL-1β, MIP-2, KC, IL-6, MCP-1, and G-CSF all increased in the lung four hours after infection and remained elevated until death at 72 hours. KC, MIP-2, and IL-6 were elevated in serum by four hours after infection but serum levels of other cytokines were not increased until 24 hours or 72 hours after infection. Notably, heightened IFN-γ levels were not detected at all in the lung or serum.

To determine the degree of distant organ injury in this lethal model of infection, serum glucose, BUN, creatinine, and liver transaminase ALT were measured in C57BL/6 mice that were naïve or four, 24, and 72 hours after deposition of 10^5 CFU/lung (Fig. 5). Mice developed progressive hypoglycemia over the course of infection and azotemia was apparent at 72 hours after infection. Transaminase measurements were variable in naïve mice or at four hours after infection but were uniformly greater than 1,000 U/L at 72 hours after infection.

3.4. Lung histology

To examine lung histology in the aerosol model of infection, sections of lungs harvested from C57BL/6 mice four, 24, and 72 hours after deposition of 10^5 CFU/lung *B. thailandensis* were stained with hematoxylin and eosin (Fig. 6). Consistent with the BAL findings, little inflammation was observed at four hours but there was a mild perivascular neutrophilic infiltrate. Multiple small necrotizing foci comprised mostly of normal to degenerate neutrophils centered on the alveoli were apparent at 24 hours. At 72 hours, the predominant cell types in the alveolar inflammatory foci were histiocytes with some admixture of neutrophils. Although the mice were near death at this time point, lung architecture beyond the foci of infection was well preserved.

4. Discussion

We describe here a murine model of airborne infection with *B. thailandensis* that results in multifocal pneumonia, systemic dissemination, and inoculum-dependent mortality. We

found that both C57BL/6 and BALB/c mice were susceptible to pulmonary infection with *B. thailandensis* although BALB/c mice exhibited a modest advantage in survival and bacterial containment after an intermediate inoculum.

Little is known about *B. thailandensis* infection in mammals. It is generally avirulent in humans, although two case reports describe a soft tissue infection and pneumonia with sepsis, respectively (Glass et al., 2006, Lertpatanasuwan et al., 1999). An LD_{50} at 48 hours after intraperitoneal infection of 1.8×10^6 CFU has been estimated in Syrian golden hamsters (Brett et al., 1997). More recently, intranasal infection with 1×10^6 CFU caused 100% mortality in C57BL/6 mice (Wiersinga et al., 2007). We determined that deposition of 10^4 CFU/lung by aerosol is an approximate mortality threshold in C57BL/6 and BALB/c mice. Deposition of 10^5 CFU/lung is predictably fatal in both strains.

Others have described differences between mouse strains in intranasal, intravenous, subcutaneous, and oral infection of C57BL/6 and BALB/c mice with *B. pseudomallei* (Barnes and Ketheesan, 2005, Hoppe et al., 1999, Leakey et al., 1998, Liu et al., 2002). C57Bl/6 mice appear more resistant to infection, resulting in chronic disease lasting weeks. There is hyperproduction of IFN-γ in BALB/c mice (Liu et al., 2002) and they develop fulminant, rapidly lethal disease. However, our findings using aerosol administration of *B. thailandensis* largely mirror a study of aerosolized *B. pseudomallei* that did not show a difference in survival between C57BL/6 and BALB/c mice (Jeddeloh et al., 2003). We did not detect a significant difference between strains in survival or bacterial burdens except at doses of 10^4 CFU/lung. Furthermore, the detected differences at this inoculum, while statistically significant, were clinically modest, and probably of limited relevance. In all cases we observed only acute, transient infection that was either contained or that led to uncontrolled bacterial replication and rapid death. We did not detect any evidence of chronic infection with *B. thailandensis*, unlike many models of *B. pseudomallei* infection (Barnes and Ketheesan, 2005, Hoppe et al., 1999, Leakey et al., 1998, Liu et al., 2002).

In our model of infection with *B. thailandensis*, we observed early dissemination to liver and spleen, even after administering sub-lethal infectious doses. The liver was consistently the site of greater bacterial replication than the spleen. Both splenic and liver abscesses have been described in human *B. pseudomallei* infection but splenic abscesses may be more common (Wibulpolprasert and Dhiensiri, 1999). In contrast with others' observations in intranasal *B. pseudomallei* infection (Barnes and Ketheesan, 2005), in our model, bacterial loads in liver and spleen did not exceed the burden in the lung 24 hours after aerosolization of *B. thailandensis*. This may be due to a different pattern of deposition of organisms in the lung by aerosol than by intranasal methods, or because *B. pseudomallei* is simply more virulent than *B. thailandensis* in mice.

We observed modest pulmonary neutrophilia in BAL fluid at four hours after infection that was amplified by 24 hours. By 72 hours, however, this had abated. This may be due to a number of factors such as neutrophil apoptosis, neutrophil destruction by proteases produced by *B. thailandensis*, migration of neutrophils to regions of necrotizing alveolitis, or exhaustion of host defenses. Inflammatory cytokine production in the lung also was clearly evident by four hours. However, despite early bacterial dissemination, inflammatory

markers in the serum were largely absent until the animals were near death. Strikingly, while IFN-γ is required for host control of intraperitoneal *B. pseudomallei* infection (Haque et al., 2006), IFN-γ levels were not increased above baseline at all in the lung or serum.

The histological findings were notable for a predominantly perivascular distribution of inflammatory cells early in the time course of high dose infection. Bronchioles adjacent to vessels had minimal involvement. At 24 hours, however, this shifted to alveolar-based neutrophilic inflammation. Paralleling our observations of a reduction in BAL neutrophils at 72 hours, the alveolar lesions at this timepoint were not dominated by neutrophils but instead were largely comprised of histiocytes. We also found that extensive parenchymal lung damage, even in mice dying from high dose infection, was absent. This suggests that respiratory failure is not the cause of death in these animals. Rather, in the setting of aggressive dissemination of the pathogen to other organs and evidence of inflammatory markers in the serum at 72 hours, it is likely that an exuberant systemic inflammatory response is responsible. This is supported by our findings of relative hypoglycemia, azotemia, and transaminase elevations indicating kidney and liver dysfunction in moribund mice. This is consistent with clinical melioidosis in humans, which frequently manifests as pneumonia and sepsis. The variable transaminase elevations observed in naïve mice or in mice four hours after infection may be partly attributable to handling (Swaim et al., 1985).

Overall, these experiments confirm that *B. thailandensis* is a virulent pathogen in mice when administered by aerosol. Infection is acute with few differences between BALB/c and C57BL/6 mice. Our findings provide further evidence that *B. thailandensis* may be a useful organism to model melioidosis without requiring BSL-3 biocontainment facilities.

Acknowledgments

We recognize the expert technical assistance of Ravi Iyer and Malinka Jansson-Hutson. This work was supported by NIH 5U54AI057141 (TEW and SJS).

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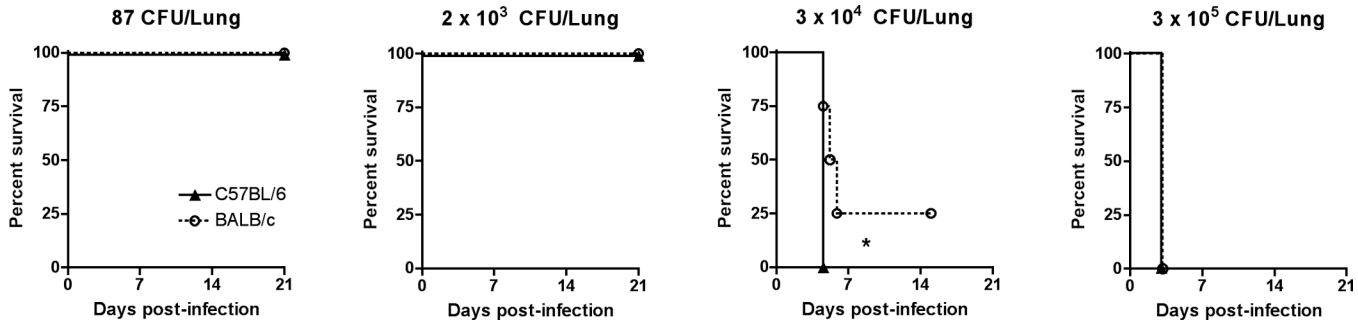

Fig. 1. Deposition of ≥3 × 10^4 CFU/lung *B. thailandensis* causes lethal infection in both C57BL/6 and BALB/c mice

Varying doses of *B. thailandensis* were aerosolized to C57BL/6 and BALB/c mice and survival was recorded. Each condition comprises four mice. Each experiment is representative of two comparable experiments performed independently. Curve comparisons are made using the log rank test and * indicates $p<0.05$.

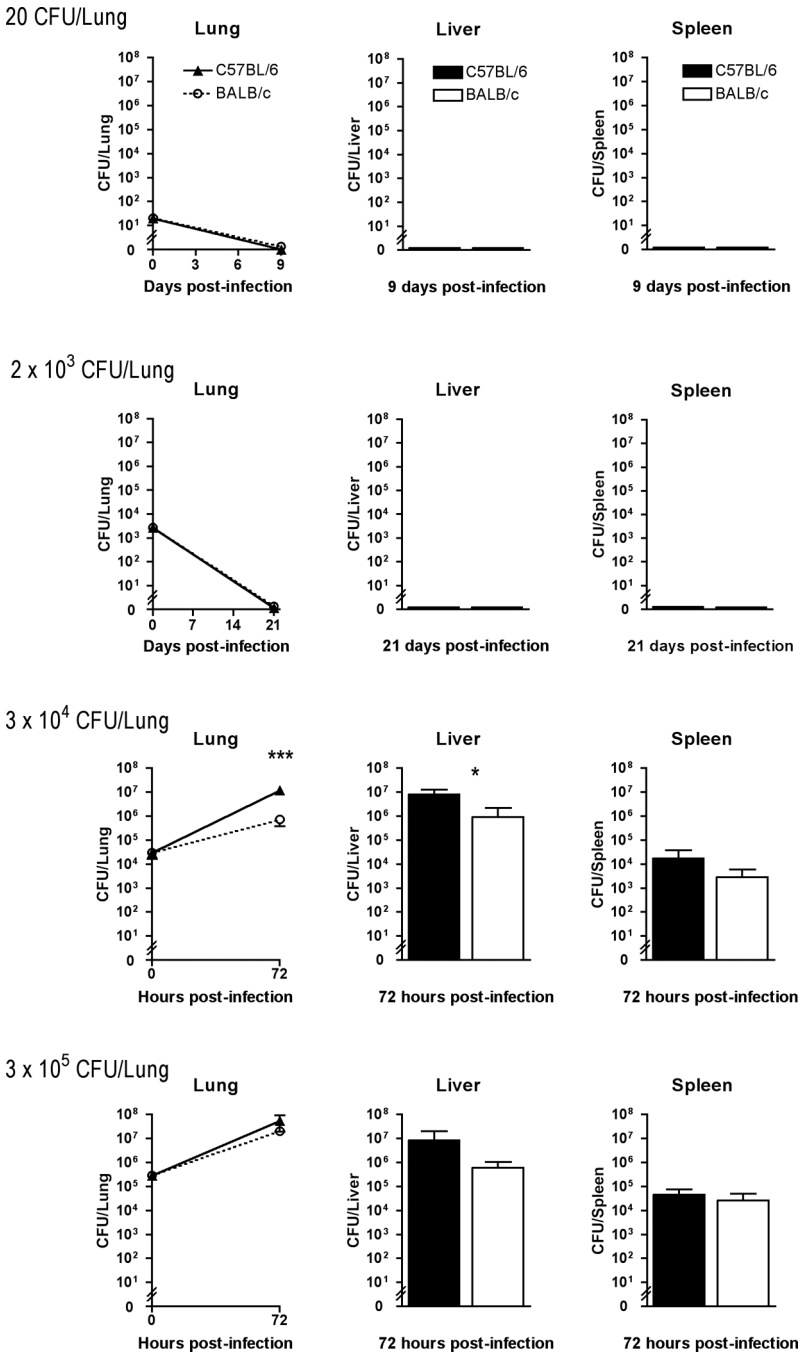

Fig. 2. Deposition of ≤2 × 10^3 CFU/lung *B. thailandensis* is cleared whereas depositions ≥3 × 10^4 CFU/lung result in aggressive replication and dissemination in both C57BL/6 and BALB/c mice

Varying doses of *B. thailandensis* were aerosolized to C57BL/6 and BALB/c mice. Lung, liver, and spleen were harvested and quantitatively cultured at 72 hours, 9 days, or 21 days after infection. Each condition comprises four mice. Each experiment is representative of two comparable experiments performed independently. Data are displayed as mean ± standard deviation and comparisons are made using the t-test. * indicates $p<0.05$ and *** indicates $p<0.001$.

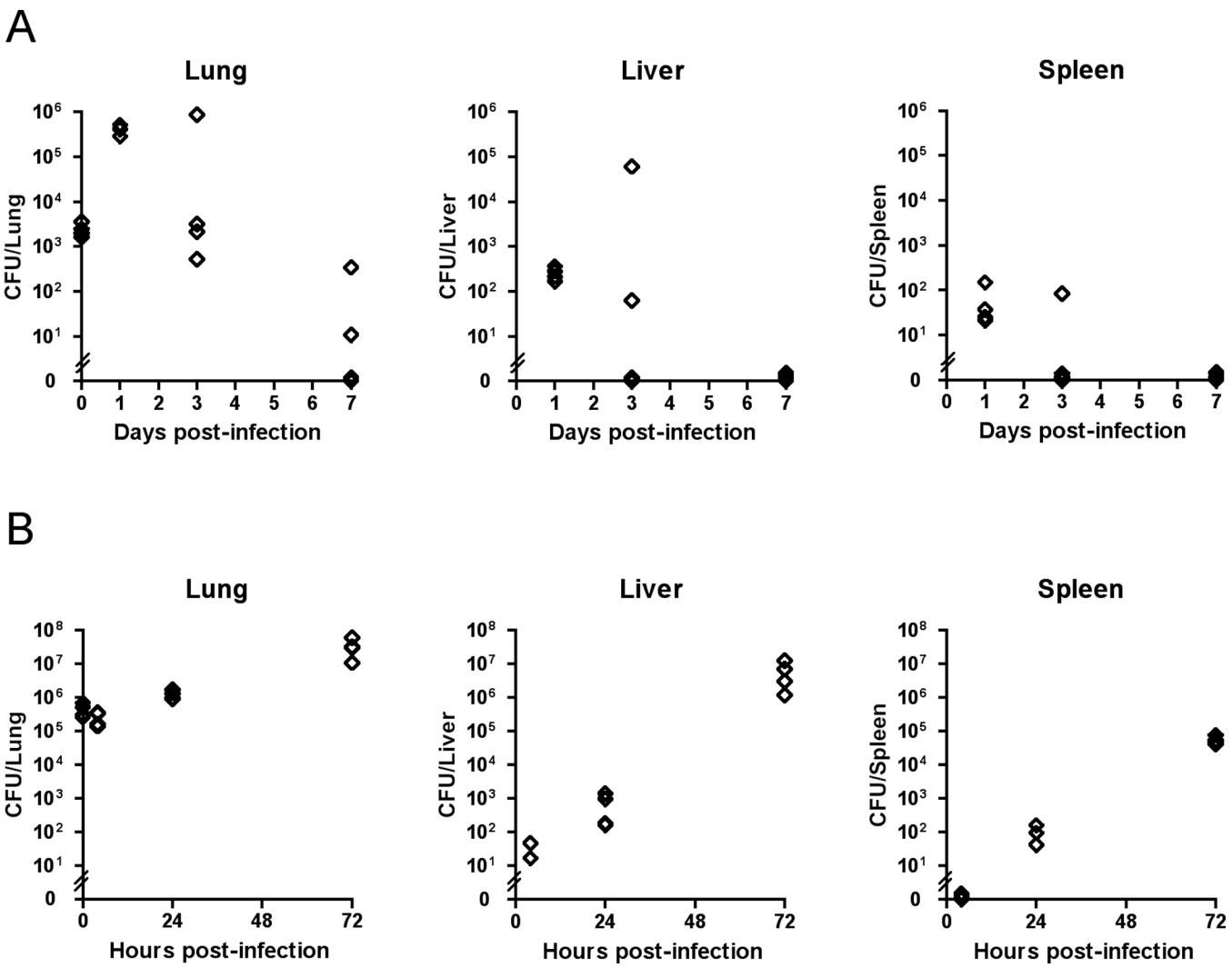

Fig. 3. Aerosol infection of *B. thailandensis* in C57BL/6 mice is characterized by early replication and distant dissemination

A: A sub-lethal dose (2×10^3 CFU/lung) of *B. thailandensis* was deposited in the lungs of C57BL/6 mice. Lung, liver, and spleen were harvested and quantitatively cultured at one, three, and seven days after infection. **B**: A lethal dose (4×10^5 CFU/lung) of *B. thailandensis* was deposited in the lungs of C57BL/6 mice. Lung, liver, and spleen were harvested and quantitatively cultured at four, 24, and 72 hours after infection. Each condition comprises four mice. Each experiment is representative of two comparable experiments performed independently.

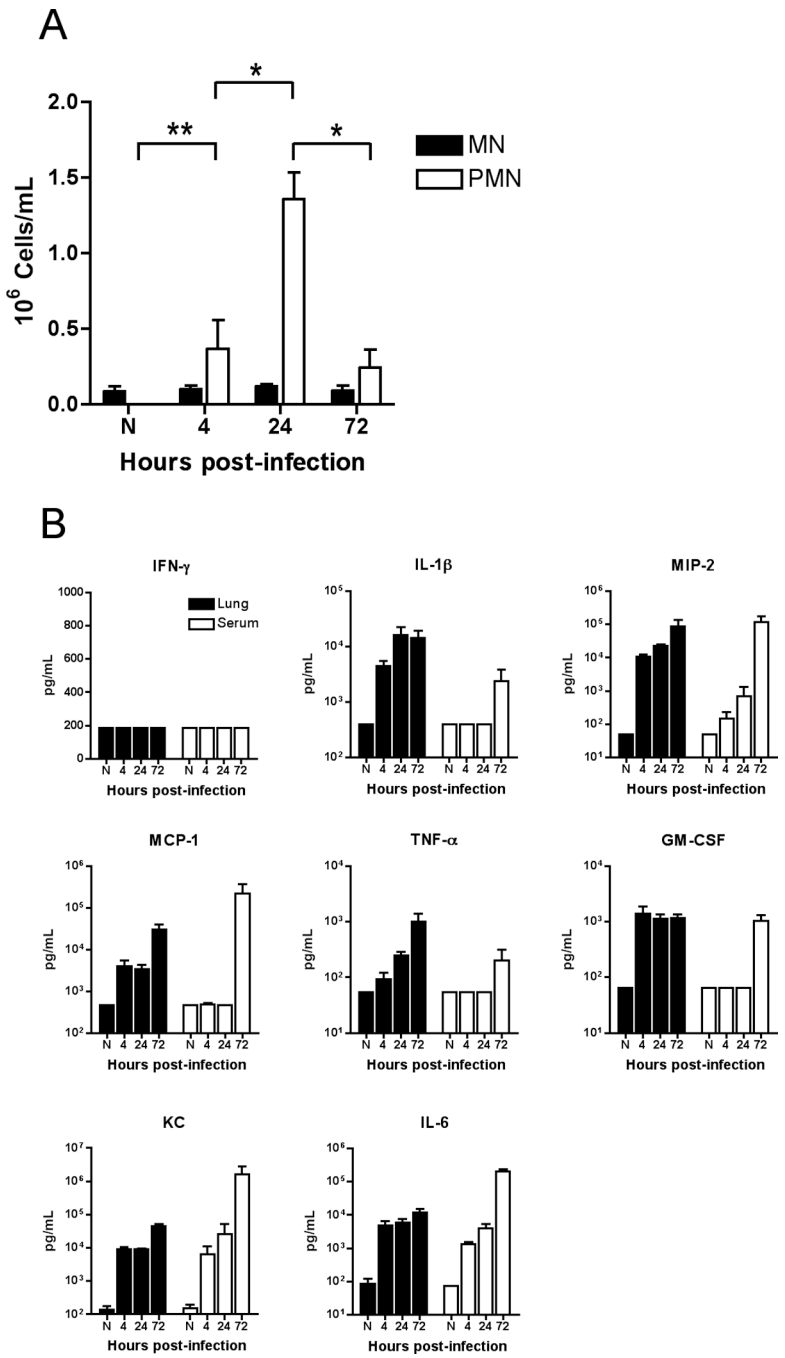

Fig. 4. Pulmonary and serum inflammatory markers after inhalation of high dose *B. thailandensis*

10^5 CFU/lung *B. thailandensis* was deposited in the lungs of C57BL/6 mice. **A**: BAL was performed in naïve mice or in mice at four, 24, and 72 hours after infection. After performing cytospins and staining using a modified Wright-Giemsa technique, polymorphonuclear leukocytes (PMN) and mononuclear cells (MN) were enumerated in the BAL fluid. Data are displayed as mean ± standard deviation and comparisons between PMNs at different time points are each made with the t-test. * indicates $p<0.05$ and **

indicates $p<0.01$. **B**: Lung homogenate and serum cytokines were measured in duplicate by multiplex bead assay in naïve mice or in mice at four, 24, and 72 hours after infection. N indicates naïve mice. Each condition comprises four mice. These experiments are representative of two comparable experiments performed independently.

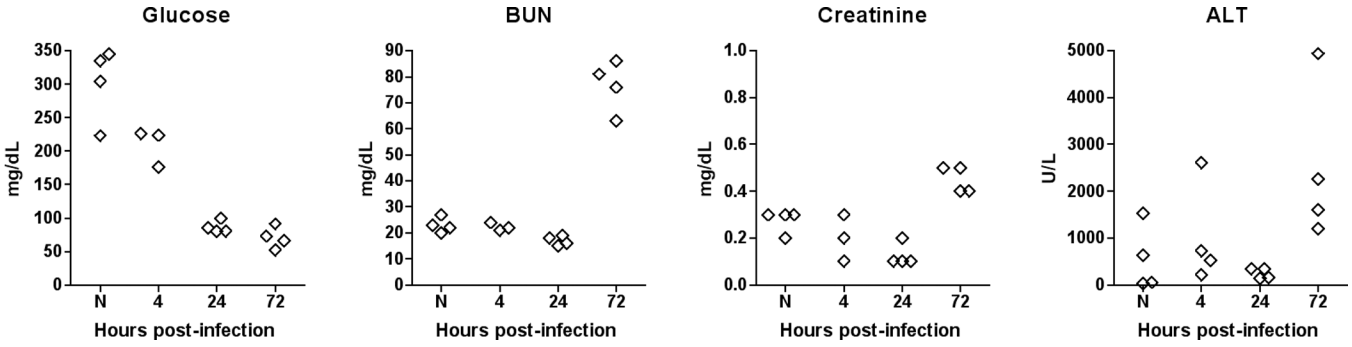

Fig. 5. Serum markers indicate end-organ injury after inhalation of high dose *B. thailandensis*

10^5 CFU/lung *B. thailandensis* was deposited in the lungs of C57BL/6 mice. Cardiac puncture and phlebotomy was performed in naïve mice (indicated by N) or in mice at four, 24, and 72 hours after infection. Chemistry measurements were performed in a clinical laboratory. Each condition comprises four mice and this experiment is representative of two comparable experiments performed independently.

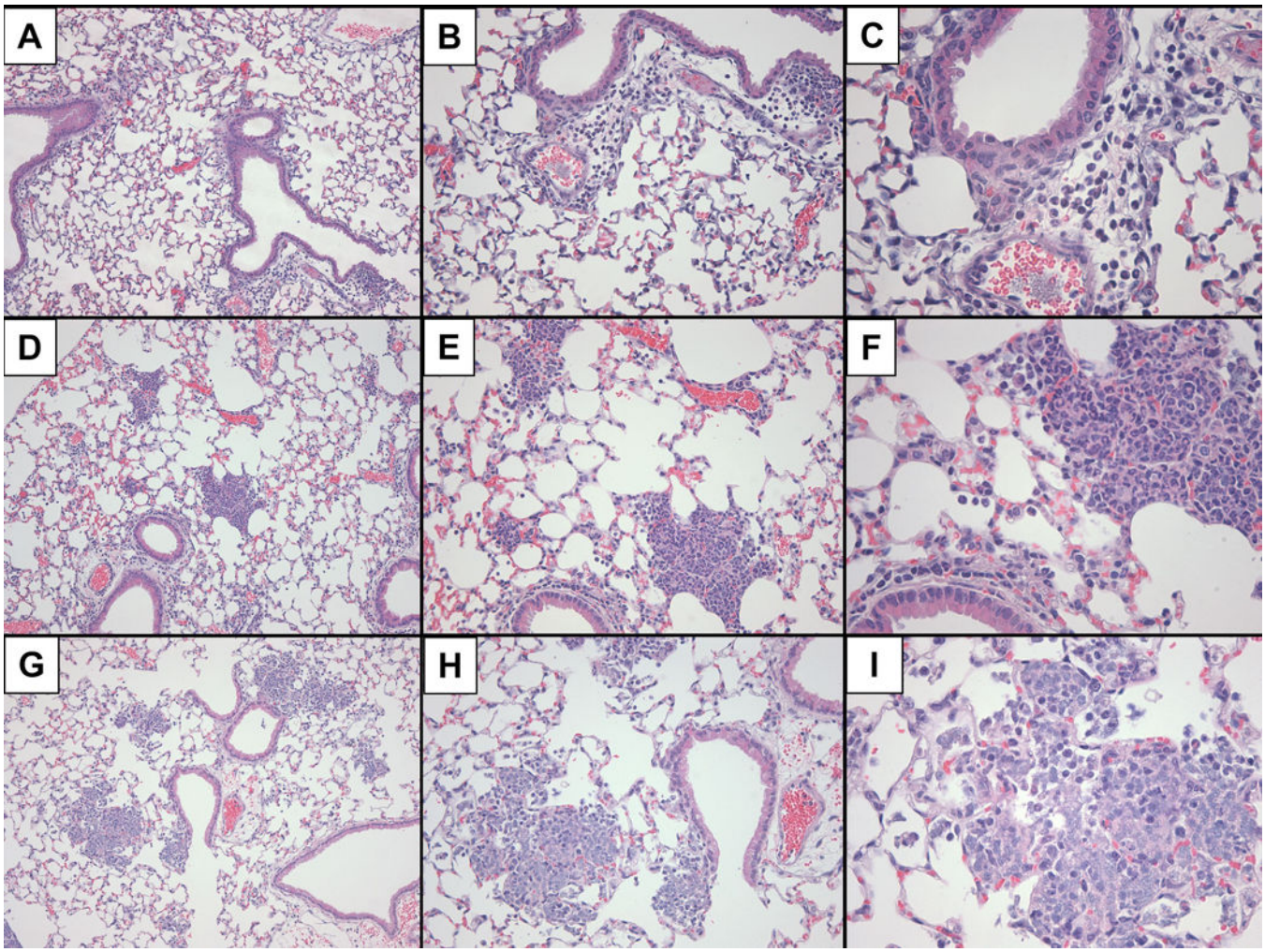

Fig. 6. Pulmonary histopathologic characteristics of high dose *B. thailandensis* aerosol infection

10^5 CFU/lung of *B. thailandensis* was deposited in the lungs of C57BL/6 mice. At four, 24, and 72 hours after infection, lungs were inflated with and fixed in 4% paraformaldehyde before being embedded in paraffin. Sections were stained with hematoxylin and eosin. Representative low and high power fields (10×, 20×, and 40×) are shown from mice four (**A, B, C**), 24 (**D, E, F**), and 72 hours (**G, H, I**) after infection. At four hours a mild neutrophilic perivasculitis was noted with minimal alveolar abnormalities. By 24 hours, multifocal necrotizing lesions composed of normal to degenerate neutrophils were observed in the alveolar spaces. At 72 hours these alveolar lesions had progressed but were largely histiocytic admixed with neutrophils and inflammatory debris. Each timepoint comprised four mice and this experiment is representative of two comparable experiments performed independently.