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# Differential susceptibility of inbred mouse strains to *Burkholderia thailandensis* aerosol infection

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## Abstract

*Burkholderia pseudomallei* is the causative agent of melioidosis, an emerging bacterial disease that accounts for high rates of septicaemia and death in parts of Southeast Asia and Northern Australia. The closely related species *Burkholderia thailandensis* is considered avirulent in humans and has been used as a surrogate for *B. pseudomallei* in several studies. The pathogenesis of *B. pseudomallei* and the role of Toll-like receptors (TLRs) in host immunity to infection are not well-defined. In this study, we exposed four strains of inbred mice (BALB/c, C57BL/6, TLR4-deficient C3H/HeJ, and TLR4-competent C3H/HeN) to increasing doses of aerosolized *B. thailandensis* to determine strain susceptibility and the role of TLR4 during pulmonary infection. Our results indicate an increased susceptibility in the C57BL/6 and BALB/c strains, who displayed lethality, bacterial burden in organs, and pulmonary and systemic inflammation. C3H/HeJ were as resistant as C3H/HeN mice to *B. thailandensis* at the highest challenge dose examined, but TLR4-deficient animals exhibited a modest increase in chronic pulmonary inflammation. These results demonstrate that *B. thailandensis* can be used as a surrogate for experimental laboratory investigation of melioidosis in small animal models and that TLR4 may not play a prominent role during acute pneumonic melioidosis.

### Keywords

*Burkholderia*; Aerosol infection; TLR4

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**Author's contributions**

L.M. and C.R. designed the experiments, interpreted the data, and drafted the manuscript; J.H. and T.T. performed the experiments and analyzed the data; P.D. performed and interpreted the histology.

## 1. Introduction

Melioidosis is an endemic bacterial disease that affects populations in Southeast Asia and Northern Australia. The disease is caused by exposure and infection with *Burkholderia pseudomallei*, a Gram-negative facultative intracellular bacillus that is ubiquitous in the soil and water of endemic areas [1]. Melioidosis is thought to be contracted mainly through two routes of exposure: inhalation and subcutaneous inoculation. The former is considered a minor but none the less important route of infection [2]. Inhalation of the bacteria by aerosol can induce the acute pneumonic form of melioidosis which, like inhalational anthrax and plague, manifests as a more severe form of the disease [1,3]. *B. pseudomallei* is also considered a biological threat agent, and aerosol exposure to the bacteria in an intentional attack remains an ever present concern [4]. Considering that *B. pseudomallei* is an aerosol threat by natural infection in endemic areas and as a biological agent, studies that model the infection using inhalation as the modality of exposure are particularly important.

The closely related species, *Burkholderia thailandensis*, exhibits very high genomic similarity to *B. pseudomallei*. In fact, *B. thailandensis* strain E264 is designated as *B. pseudomallei* E264 in literature preceding 1998 [5,6]. Based upon differences in exoenzyme production, arabinose assimilation, hamster virulence, and 16S rRNA sequences, a new classification was proposed for the less virulent isolates, such as E264 [5,7]. Although *B. thailandensis* has been utilized to model *B. pseudomallei* infection [8–10], it is not considered pathogenic for humans with only rare isolated cases of clinical infection reported [11]. This may be attributed to the absence of capsule production by *B. thailandensis* [12], since acapsular mutants of *B. pseudomallei* are significantly reduced in virulence [13]. In the murine model of infection, the difference in $LD_{50}$ for *B. thailandensis* and *B. pseudomallei* is dependent upon the route of exposure, but is typically at least 2–3 logs higher for *B. thailandensis* by inhalation [8,14]. *B. thailandensis* is therefore much easier to work within the laboratory because handling and use do not require biosafety level three (BSL-3) containment.

Comparative studies demonstrate that *B. thailandensis*, like *B. pseudomallei*, can induce cell fusion and multinucleate giant cell formation after phagocytosis [15]. After invasion of A549 lung alveolar epithelial cells, *B. thailandensis* survived and replicated to the same extent as *B. pseudomallei* [16]. Furthermore, no differences in the responsiveness of murine alveolar macrophages, lung epithelial cell lines, or human peripheral blood mononuclear cells towards stimulation with *B. pseudomallei* or *B. thailandensis* were detected [14,17]. These observations were also confirmed *in vivo* in that similar acute pulmonary inflammation was observed in mice following intranasal infection with *B. pseudomallei* and *B. thailandensis* [14]. This suggests that innate immune responses to the two strains may be very similar. In support of this, lipopolysaccharide (LPS) from *B. pseudomallei* and *B. thailandensis* are antigenically indistinguishable and appear equally capable of activating macrophages *in vitro* [14,18,19]. However *B. pseudomallei* LPS does not appear to activate TLR4 to the magnitude that enterobacterial LPS does [20–22], and the importance of TLR4 in protection against this Gram-negative pulmonary pathogen is not well-defined [22,23]. A single study has examined the role of TLR2 and TLR4-mediated immunity to *B. pseudomallei* in the murine model. Intranasal inoculation with *B. pseudomallei* in $TLR^{-/-}$

and TLR4$^{-/-}$ mice revealed that TLR2 actually impairs host defense, while TLR4 did not appear to significantly impact protection [23]. An understanding of the role of TLR during *B. pseudomallei* infection is essential since individuals with impaired innate, but not cellular, immunity experience a higher incidence of melioidosis [24].

In this study we characterize the outcome of infection in mice challenged with increasing doses of aerosolized *B. thailandensis*. We chose to use strains of mice that are considered susceptible (BALB/c) and resistant (C57BL/6) to intracellular bacterial infection to identify potential differences in host immunity [25]. We also used CH3/HeJ mice that are deficient for TLR4 expression and control TLR4-competent mice, CH3/HeN, to assess the importance of this pattern recognition receptor in the pathogenesis of *Burkholderia*-related infection by the aerosol route.

## 2. Results

### 2.1. Differential survival of inbred mouse strains after aerosol challenge with B. thailandensis

*B. thailandensis* represents an attractive model for the study of the related and highly pathogenic *B. pseudomallei*. We therefore challenged four inbred strains of mice with increasing doses of aerosolized *B. thailandensis* to determine their susceptibility to inhalational infection. Two runs were required to expose all of the animals; even numbers of mice from each group were exposed in each run to ensure dosing was consistent among the groups. Aerosol doses and survival are shown in Fig. 1. No deaths were observed in any of the four strains at $1 \times 10^3$ colony-forming units (cfu) in pilot studies (not shown). The C3H/HeJ and C3H/HeN mice were resistant to all of the dosage levels attempted in this study (up to $1 \times 10^6$ cfu) (Fig. 1A–C). The C57BL/6 mice experienced a rapid decline in survival after the dose surpassed $1 \times 10^4$ cfu. At $1 \times 10^4$ cfu, 50% of the C57BL/6 succumbed by 72 h post-infection (p.i.) and displayed an overall survival of 30% (Fig. 1A). The BALB/c mice were not susceptible to $1 \times 10^4$ cfu (Fig. 1A) in two independent exposures. At $1 \times 10^5$ cfu, the C57BL/6 demonstrated 100% mortality by 72 h and were significantly more susceptible than the BALB/c and C3H strains ($P < 0.05$). The BALB/c displayed 16.7% mortality at this dose, however this was not statistically different than the C3H strains that demonstrated 100% survival ($P = 0.31$) (Fig. 1B). At $1 \times 10^6$ cfu, both the C57BL/6 and BALB/c mice demonstrated equal susceptibility ($P = 0.23$) with 100% mortality by 72 h in the BALB/c and 83.3% mortality by 48 h in the C57BL/6 (Fig. 1C) ($P < 0.01$ compared to C3H strains). Lethality of C57BL/6 mice exposed at $1 \times 10^5$ or $1 \times 10^6$ cfu was not significantly different ($P = 0.08$). In all experiments and independent of dose, mice that succumbed to aerosol challenge did so within 4 days of exposure, and mice that survived 5 days post-exposure eventually recovered as indicated by weight gain, normal activity, and bacterial clearance.

### 2.2. Recovery of B. thailandensis from lung, liver, and spleen

To further characterize strain susceptibility and infection outcome, tissues were harvested to determine bacterial burden in mice that succumbed early after challenge (<96 h) and in survivors (2 and 4 weeks p.i.). Mortality was only observed in the C57BL/6 mice after

challenge with $1 \times 10^4$ cfu and was associated with bacterial replication in the lungs and dissemination to the spleen and liver by 72 h p.i. (Fig. 2A). In a repeated experiment, BALB/c mice were challenged with $1 \times 10^4$ cfu and 3 asymptomatic animals were sacrificed at 72 h p.i. to ascertain bacterial loads. None of the BALB/c mice had detectable bacteria in the lungs, liver, spleen (Fig. 2A), or blood (not shown). This suggests that their tolerance of $1 \times 10^4$ cfu challenge dose correlates with an ability to eradicate bacteria within 3 days of exposure. At $1 \times 10^5$ cfu challenge dose, C57BL/6 also harbored bacteria in the lung, spleen, and liver at 72 h p.i. (not shown). We were not able to recover tissues from the one BALB/c mouse that succumbed within 24 h of challenge at this dose. At $1 \times 10^6$ cfu, C57BL/6 succumbed rapidly to challenge, but bacterial burdens in the lung, liver, and spleen were similar to those in mice challenged with lower doses (Fig. 2B). This may have resulted from an inability of the bacteria to replicate to higher numbers in the shorter time period to death (48 h). BALB/c mice also succumbed to challenge with $1 \times 10^6$ cfu, but the mean time to death was 24 h later than the C57BL/6. Bacterial burdens in the spleen and liver were similar for both strains of mice, but the BALB/c had nearly 2 logs higher bacteria in the lungs (Fig. 2B). In all mouse strains and independent of challenge dose, mice that survived had undetectable levels of bacteria at 2 and 4 weeks p.i. in any of the organs examined.

### 2.3. Production of pro-inflammatory cytokines in the pulmonary and systemic compartments

In order to characterize the mucosal and systemic immune responses in mice challenged with aerosolized *B. thailandensis*, we performed cytokine measurements in lung homogenates and sera from animals that succumbed early after challenge and in those that survived to the 4 week endpoint. We also analyzed the cytokine concentrations in uninfected mice as baseline controls. As seen in Fig. 3, the susceptible mouse strains, C57BL/6 and BALB/c, had significantly increased levels of GM-CSF, IFN-γ, IL-1β, IL-6, IL-12, MCP-1, and TNF-α in both the pulmonary and systemic compartments following challenge with 1.0E+6 cfu. We were unable to detect any IL-2, IL-4, IL-5, IL-7, IL-10, or IL-13 in either the lungs or sera of any mice examined. Uninfected control animals had undetectable levels of all tested cytokines in the lungs and sera (not shown). The levels of increased cytokines were very similar in both strains of susceptible mice with only a few exceptions. For example, IFN-γ, IL-12, and TNF-α levels were significantly higher in the sera of BALB/c mice that succumbed at 72 h p.i. compared to that of C57BL/6 mice at 48 h p.i., but this may simply be due to differences in cytokine kinetics rather than actual strain variability (Fig. 3).

In contrast to the mouse strains that succumbed to challenge, C3H/HeN and C3H/HeJ had minimal levels of GM-CSF, IFN-γ, IL-1β, and IL-12 in their lungs and sera at the 4-week study endpoint (Fig. 4). Low levels of MCP-1 were still detectable in the lungs of both strains, but not in the sera. Levels of the pro-inflammatory cytokines TNF-α and IL-6 were barely detectable in the lungs and sera of each strain (Fig. 4). Similar to the susceptible mouse strains, none of the other interleukins were detectable. There were no major differences in the cytokines measured in the lungs and sera of C3H/HeN versus C3H/HeJ mice at the 4-week endpoint. The one exception was the significantly increased (albeit low) level of IL-12 in the lungs of C3H/HeJ mice compared to C3H/HeN.

### 2.4. Histopathological changes in response to aerosol infection with B. thailandensis

The histological changes in all four strains of mice were examined for the $1 \times 10^4$ and $1 \times 10^6$ challenge doses in mice that succumbed to infection and in survivors. C57BL/6 mice that succumbed to $1 \times 10^6$ cfu in 48 h displayed moderate acute pneumonia characterized by multifocal suppurative alveolitis and bronchiolitis (Fig. 5A) as well as minimal suppurative hepatitis. At the same challenge dose, BALB/c succumbed 24 h later with more extensive pneumonia including large numbers of histiocytes surrounding suppurative foci (Fig. 5B) and minimal hepatitis.

In contrast to the mouse strains that succumbed to $1 \times 10^6$ cfu, the C3H strains that survived until the 4-week endpoint had little evidence of inflammation in their lungs, liver, or spleen at 2 and 4 weeks post-infection. The one exception is the TLR4-deficient C3H/HeJ mice that had evidence of antigenic stimulation in their lungs at 4 weeks post-infection. This appeared to intensify over time, despite bacterial clearance from the lungs. Minimal to mild lymphoid and epithelial hyperplasia was noted at 2 weeks p.i. in 2 out of 3 mice, while at 4 weeks, moderate lymphoid hyperplasia and mild epithelial hyperplasia was noted in 3 out of 3 C3H/HeJ mice (Fig. 5C). The C3H/HeN mice had minimal lymphoid hyperplasia at 2 weeks p.i. and normal tissue architecture by 4 weeks (Fig. 5D). At 4 weeks p.i., the sole survivor of the C57BL/6 strain challenged with $1 \times 10^6$ cfu had signs of chronic pneumonia and hepatitis, as well as mild hyperplasia in the spleen (not shown). No bacteria, however, were recovered from any of the tissues at the study endpoint.

## 3. Discussion

Various animal models of *B. pseudomallei* infection have been explored in recent years, including mice, diabetic rats, and Syrian hamsters [26–28]. The experimental outcome and similarity to clinical pathophysiology in human melioidosis are dependent upon the route of challenge, animal strain, bacterial strain, and infectious dose. *B. thailandensis* is an attractive surrogate for the study of *B. pseudomallei* due to the extreme genetic relatedness between the two organisms and ease of use. Several recent studies have established the utility of *B. thailandensis* in examining host immune responses and bacterial virulence factors that contribute to disease [8–10].

We initiated this study to determine if aerosol infection with *B. thailandensis* could reproduce the pulmonary infection established by *B. pseudomallei* in the murine model. Furthermore, we examined four different strains of inbred mice to identify potential differences in host immune responses and outcome to *B. thailandensis* lung infection. In this report we demonstrate significant differences in mouse strain susceptibility and inflammatory responses to aerosol challenge with *B. thailandensis*. We have shown that C57BL/6 and BALB/c mice are susceptible to challenge doses beginning at $1 \times 10^4$ and $1 \times 10^5$ cfu, respectively. In contrast, the TLR4-competent C3H/HeN and TLR4-deficient C3H/HeJ strains were observed to be highly resistant to aerosol infection with *B. thailandensis*. Our findings corroborate the recent demonstration by West et al. [8] who observed similar susceptibility in the BALB/c and C57BL/6 strains to aerosol challenge with *B. thailandensis*. This report is also consistent with that observed for *B. pseudomallei* in that both mouse strains appear equally susceptible to aerosol challenge [27]. Like *B.*

*pseudomallei*, *B. thailandensis* can invade and survive in both phagocytic and non-phagocytic cells [16]. It is therefore noteworthy that the C57BL/6 is not more resistant than the BALB/c to aerosol infection with either organism. During intranasal, oral, or systemic infection routes, C57BL/6 mice are markedly more resistant to *B. pseudomallei* than BALB/c [26,28,29]. Interestingly, the BALB/c mouse is more resistant than the C57BL/6 mouse to pulmonary infection with the extracellular pathogen, *Pseudomonas aeruginosa* – an organism that is very highly related to *B. thailandensis* and *B. pseudomallei*. The increased susceptibility of C57BL/6 mice to *P. aeruginosa* compared to BALB/c is attributed to the significant pro-inflammatory response observed in the lungs of C57BL/6 mice after pulmonary infection [30].

In our study, we noted significant inflammation in the lungs of both C57BL/6 and BALB/c mice that succumbed to aerosol challenge. Pro-inflammatory cytokines, such as IL-6, TNF-α, and MCP-1 are elevated in patients with melioidosis [1], and these were significantly elevated in the lungs and sera of both strains of mice that succumbed to *B. thailandensis*. This is also consistent with the detrimentally high levels of pro-inflammatory cytokines observed in BALB/c mice in response to aerosol challenge with *B. pseudomallei* [31]. These cytokines were undetectable in uninfected control mice and were barely detectable in resistant strains or susceptible strains that survived a particular challenge. For example, the BALB/c mice challenged with $1 \times 10^4$ cfu had undetectable levels of cytokines in their sera following sacrifice at 72 h. In addition, the sole C57BL/6 mouse that survived challenge with $1 \times 10^6$ cfu had cytokine levels near baseline 4 weeks after infection. The resistant C3H strains also demonstrated very minimal amounts of cytokine production in the lungs or sera at 4 weeks post challenge. These results indicate that systemic pro-inflammatory cytokine production, like that observed in melioidosis patients, is associated with disease in murine models of melioidosis [8,31] and additional study would be warranted to evaluate therapeutic interventions.

Histological examination of the lung, liver, and spleen of animals that succumbed to challenge demonstrated notable pulmonary lesions. C57BL/6 and BALB/c mice demonstrated moderate and severe acute pneumonia, respectively. A similar enhanced intensity was noted in BALB/c lungs compared to C57BL/6 mice that received a $1 \times 10^4$ cfu challenge dose, although only the latter strain succumbed. Our observations that BALB/c mice exhibit enhanced pulmonary inflammation compared to C57BL/6 mice could be an important difference in mouse strains that is linked to pathogenesis. Wiersinga et al. [32] demonstrated increased susceptibility of C57BL/6 IL-18 knockout (KO) mice to *B. pseudomallei*, although the difference in disease severity was measured by hepatocellular injury rather than the severity of pneumonia in wild type (WT) and KO strains. We observed only minimal acute hepatitis at the time of death in the C57BL/6 and BALB/c mice. These observations with *B. thailandensis* are in stark contrast to the pronounced splenomegaly and hepatic abscesses observed in mice infected with *B. pseudomallei* [14] and in human melioidosis patients [1].

Intranasal infection with low doses of *B. pseudomallei* yields a differential outcome in BALB/c and C57BL/6 mice. BALB/c mice rapidly succumb to challenge during the acute phase of infection, while C57BL/6 tolerate the infection for up to 6 weeks with apparent

bacterial growth in the liver and spleen [28]. The establishment of chronic infection by *B. thailandensis* would provide a great opportunity to study bacterial latency and reactivation disease. However, we failed to detect any evidence of persistence of *B. thailandensis* 4 weeks after challenge in any mouse strain or infectious dose examined. West et al. [8] also could not detect chronic carriage of *B. thailandensis* in the C57BL/6 or BALB/c strains following aerosol challenge. Taken together, these findings suggest that *B. thailandensis* is less able to efficiently colonize the spleen and liver of mice than the more virulent *B. pseudomallei*, particularly after aerosol challenge.

Interestingly, both C3H strains were highly resistant to infection with *B. thailandensis* and remained completely asymptomatic following aerosol challenge with a very high dose ($1 \times 10^6$ cfu). Therefore, we surmise that TLR4 plays no role in protection against acute lung infection with *B. thailandensis*. Nonetheless, the TLR4-deficient C3H/HeJ displayed persistent epithelial and lymphoid hyperplasia in the lungs compared to C3H/HeN mice 2 and 4 weeks after challenge – suggesting prolonged immune responses in the C3H/HeJ. It is difficult to comment on the significance of this finding since we could not detect bacterial persistence in the C3H/HeJ or C3H/HeN mice after 4 weeks. In similar studies, there were no remarkable differences in outcome or severity of tissue inflammation in C3H/HeJ and C3H/HeOuJ (WT) mice challenged with low-dose aerosolized *Francisella tularensis*; however, the histology was performed very early during the course of disease (2 and 4 days p.i.) [33]. In contrast, C3H/HeJ and WT mice infected with *Mycobacterium bovis* BCG were both able to control bacterial replication for up to 8 months, but C3H/HeJ mice demonstrated enhanced tissue inflammation that only became evident at 90 days p.i. [34]. Intranasal challenge of C57BL/6 mice with 500 cfu of *B. pseudomallei* caused similar mortality and inflammation in WT and $TLR4^{-/-}$ strains within 5 days. However, increased survival in $TLR2^{-/-}$ mice was associated with reduced distant organ injury compared to WT and $TLR4^{-/-}$ mice [23]. It would be informative to repeat these studies using the low challenge dose (50 cfu) previously shown to confer chronic infection in C57BL/6 for up to 6 weeks [28]. A role for TLR4 in the pathogenesis of *B. pseudomallei* in susceptible mouse strains may become apparent only during the chronic stages of infection, such as that observed for *Mycobacterium* spp. [34,35].

The roles of TLR, particularly TLR2 and TLR4, are somewhat enigmatic in the pathogenesis of *B. pseudomallei* infections [23]. Our finding that TLR4 is not essential for protection against aerosol challenge with *B. thailandensis* is consistent with that observed for other Gram-negative intracellular pathogens, such as *F. tularensis* and *Legionella pneumophila* [33,36]. For some pathogens, differences in virulence among strains are associated with an ability to modulate host innate immune recognition, such as TLR signalling. For example, cell wall associated lipoarabinomannan (LAM) from the fast-growing, avirulent *Mycobacterium* species activates TLR2 leading to production of TNF-α and IL-12. Modification of LAM by the addition of capped mannose residues in the virulent slow-growing *Mycobacterium* species interferes with TLR2 recognition and pro-inflammatory responses [37]. In addition, *Yersinia pestis* organisms that express hexa-acylated lipid A are attenuated 350-fold compared to strains that express tetra-acylated lipid A in the murine model of pneumonic plague. The attenuated phenotype results from increased activation of TLR4 and TNF-α production [38]. Therefore, it is plausible that differences in virulence

between *B. thailandensis* and *B. pseudomallei* may be due to alterations in innate immune recognition or avoidance. Rabbit and mouse sera generated against *B. pseudomallei* or *Burkholderia mallei*, respectively, cross reacts with *B. thailandensis* LPS, suggesting that *B. thailandensis* LPS shares similar structural features with LPS molecules from highly pathogenic *Burkholderia* species [19]. Our finding that TLR4 does not contribute to protection against acute lung infection with *B. thailandensis* is similar to that observed for *B. pseudomallei* during intranasal infection of TLR4$^{-/-}$ mice [23]. Together, these *in vivo* results confirm several *in vitro* studies suggesting very similar TLR activation by the two species [14,17–19,23].

In conclusion, aerosol infection studies with *B. thailandensis* provide an excellent surrogate for modelling the acute pneumonic form of disease caused by *B. pseudomallei*. Studies to date suggest that neither TLR4 nor TLR2 provide immunity against acute lung infection with *B. pseudomallei* in the murine model [23]. Future utilization of *B. thailandensis* in knockout mice may allow the elucidation of pattern recognition receptors important for protection in inhalational melioidosis.

## 4. Materials and methods

### 4.1. Animals

Female six to eight week old pathogen-free C57BL/6, C3H/HeN, and C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME); female six to eight week old BALB/c mice were purchased from Charles River laboratories (Madison, WI). Animals were strain-segregated throughout the duration of the study and maintained five to a cage in polystyrene microisolator units. Husbandry included a 12 h light/dark cycle, sterile cages and rodent chow, and water *ad libitum*. All experimental procedures were approved by Tulane University Health Sciences Center Institutional Animal Care and Use Committee.

### 4.2. Bacterial strains and growth conditions

*B. thailandensis* strain E264 was used in this study and obtained from BEI Resources. Prior to aerosol challenge, bacteria were grown from frozen glycerol stock in Luria Bertani (LB) broth at 37 °C overnight and freshly diluted 1:100 into 100 ml of LB. The bacteria were grown to an optical density ($OD_{600}$) of 1.9 ($\approx 1 \times 10^9$ cfu/ml). The suspension was serially diluted with sterile media into 10 ml aliquots for the aerosol infections. All suspensions and dilutions were plated on LB agar for the purposes of confirming concentrations.

### 4.3. Aerosol generation

Initially, the bacterial suspension was characterized to determine the microbial efficiency when in aerosol. A 16 l plexiglass chamber was used for characterization. Serial dilutions ranging from $1 \times 10^9$ to $1 \times 10^5$ cfu/ml were used for discrete sham aerosol-generation experiments. Aerosols were generated using a Collison nebulizer (BGI Inc., Waltham, MA) operated at 18 pressure square inch gauge (PSIG) with an equivalent flow of 7.5 liters per minute (LPM). Secondary dilution flow was provided at 8.5 LPM for a total input flow of 16 LPM. In individual analyses, time-of-flight aerodynamic particle sizing was performed using the Aerodynamic Particle Sizer (Model 3321, TSI Inc., St. Paul, MN). Isokinetic continuous

aerosol sampling was performed during the sham aerosol experiments using an all glass impinger (AGI-4, Ace Glass, NJ) operated at a flow of 6 LPM. Neutral pressure was maintained in the chamber; compensatory exhaust from the aerosol system was actively maintained at 10 LPM. Sham experiments allowed determination of an efficiency factor ($C_s/C_a$; unitless ratio of the inoculum loaded into the nebulizer to the aerosol concentration both expressed in cfu/l) from which predictive target doses were calculated for the infection experiments.

### 4.4. Aerosol infection

For the experimental infections, a nose-only exposure chamber was used (CH Technologies, Westwood, NJ). This chamber was dynamically operated at 8 LPM and the collision nebulizer (BGI Inc., Waltham, MA) was used for aerosol generation. Up to 20 mice were dosed at a time; continuous aerosol sampling was performed from one of the animal ports using an AGI-4 (Ace Glass, NJ). Exposures were acute and lasted 10 min. Animals were immediately unloaded after exposure and returned to their respective caging units. Dose was determined using previously described methods with *post hoc* analysis of aerosol samples taken during each exposure event [39].

### 4.5. Post-challenge monitoring

Mice were closely monitored for survival over a 4-week course of infection and body weights were recorded. Mice that became moribund, lost 20% of their body weight, or survived to the study endpoint (4 weeks p.i.) were humanely euthanized by $CO_2$ over-dose. Groups of 10 ($1 \times 10^4$ cfu) or 6 ($1 \times 10^5$ and $1 \times 10^6$ cfu) were used for survival analysis.

### 4.6. CFU determination

After the mice were euthanized, the left lung and half of the spleen and liver were removed for determination of bacterial burden. Groups of 3–4 mice were used for each time point. Tissues were aseptically removed and individually placed in 1 ml 0.9% NaCl. Tissues were homogenized with a Power Gen 125 (Fisher Scientific) and ten-fold serial dilutions of organ homogenates were plated in triplicate on LB agar. Colonies were counted after incubation for 2–3 days at 37 °C and reported as cfu per gram of tissue. The limit of detection was 10 cfu.

### 4.7. Cytokine measurements

Cytokines in sera and lung homogenates were assayed by multiplex bead array using the Milliplex mouse 13-plex cytokine/chemokine panel (GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, MCP-1, and TNFα) according to the manufacturer's instructions (Millipore) and measured on a Bio-Plex 200 instrument (BioRad). The lower limit of detection for all analytes was 3.2 pg/ml.

### 4.8. Histological analysis

The right lung and remaining halves of the spleen and liver were used for histology. Tissues were fixed immediately by immersion in 10% neutral buffered-formalin and processed by standard paraffin-embedding methods. Sections were cut 4 μm thick, stained with

haematoxylin–eosin (H&E) and examined by light microscopy. Lesion intensity scoring on a scale of 0–4 (0 = normal; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe) was performed by a veterinary pathologist.

### 4.9. Statistical analysis

For survival analysis, Kaplan–Meier analysis followed by log rank test was performed. All other data were analyzed using Student's unpaired *t* test. Values of $P < 0.05$ were considered statistically significant. These analyses were performed using GraphPad Prism version 5.0, GraphPad Software (San Diego, CA).

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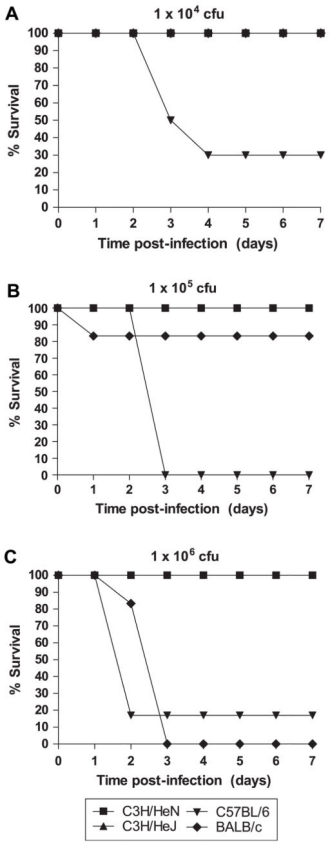


**Fig. 1.**

Survival of inbred mouse strains following aerosol infection with *B. thailandensis*. Groups of ten ($1 \times 10^4$ cfu (panel A)) or six ($1 \times 10^5$ (panel B) and $1 \times 10^6$ cfu (panel C)) mice were infected by the aerosol route and their survival monitored for up to 28 days. Mice that did not succumb within the first 4 days survived for the entire 4-week study period; therefore only the first 7 days are shown.

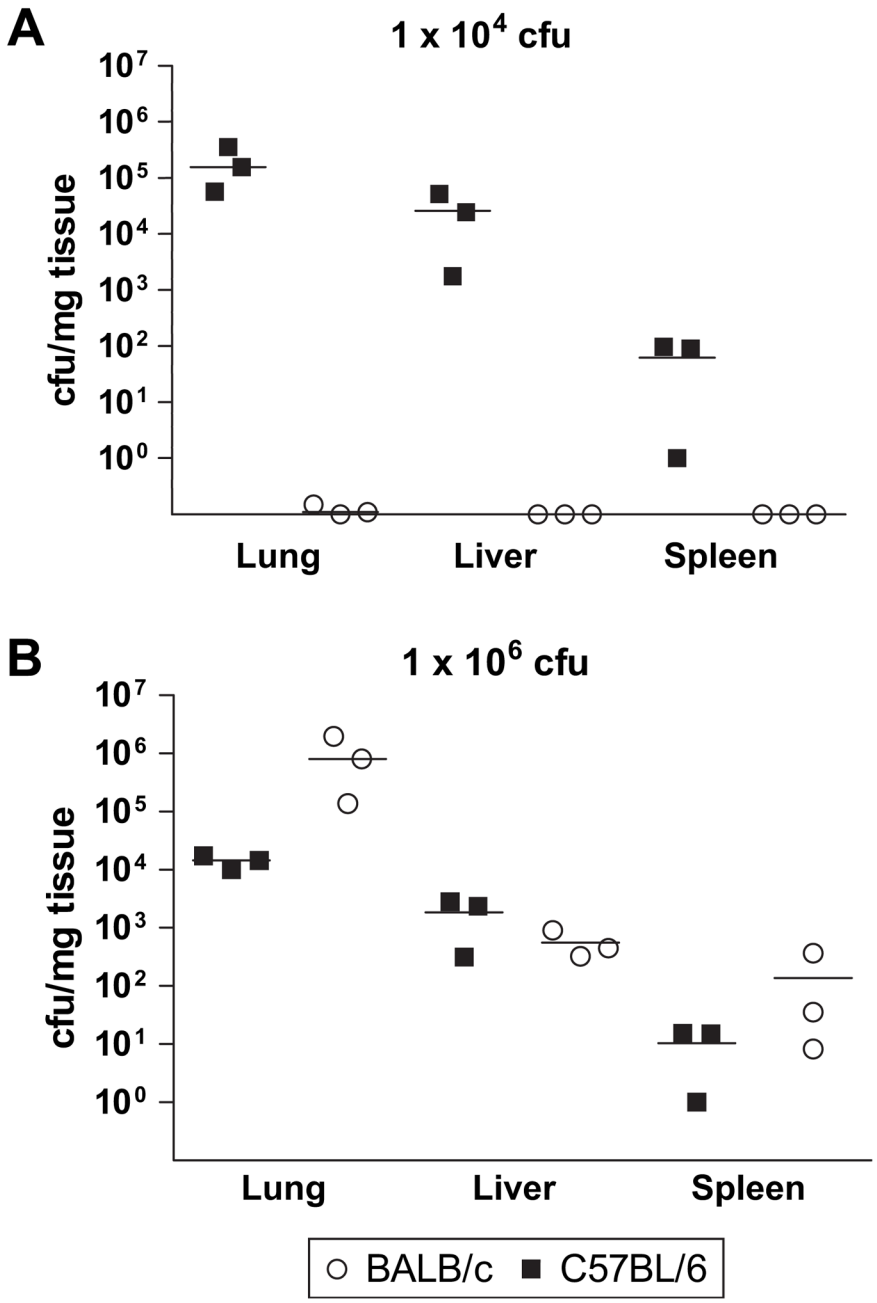


**Fig. 2.**

*B. thailandensis* recovery from lung, liver, and spleen after low ($1 \times 10^4$ cfu) and high dose ($1 \times 10^6$ cfu) aerosol challenge. (A) Bacterial burdens in the C57BL/6 mice that succumbed to aerosol challenge with $1 \times 10^4$ cfu compared to BALB/c that were asymptomatic at the time of sacrifice (72 h p.i. for both strains). (B) Bacterial burdens in the C57BL/6 and BALB/c mice that succumbed to aerosol challenge with $1 \times 10^6$ cfu at 48 h and 72 h p.i., respectively. A cohort of 3 mice per strain per time point was used. Counts of *B. thailandensis* from each mouse are presented as solid squares (C57BL/6) or open circles

(BALB/c) and are expressed as cfu/mg tissue. The median value for each strain is indicated by a bar.

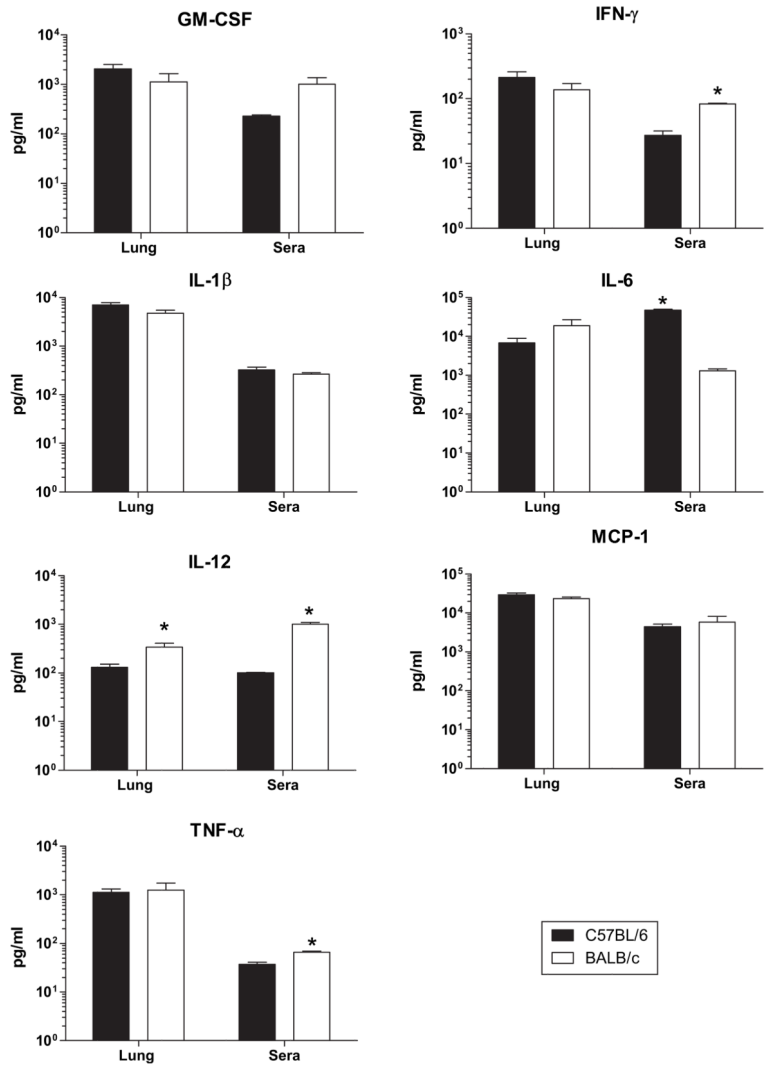


**Fig. 3.**

Pulmonary and serum cytokine levels in susceptible C57BL/6 and BALB/c strains after high dose aerosol challenge. Cytokines were measured in the lung homogenates (left columns) and the sera (right columns) of C57BL/6 (solid bars) and BALB/c (open bars) mice after succumbing to $1 \times 10^6$ cfu. Data shown are mean values with error bars showing the SEM from three mice at each time point. Samples from uninfected controls had undetectable levels of cytokines and are not shown. The assays were performed in duplicate. * indicates statistical significance between strains.

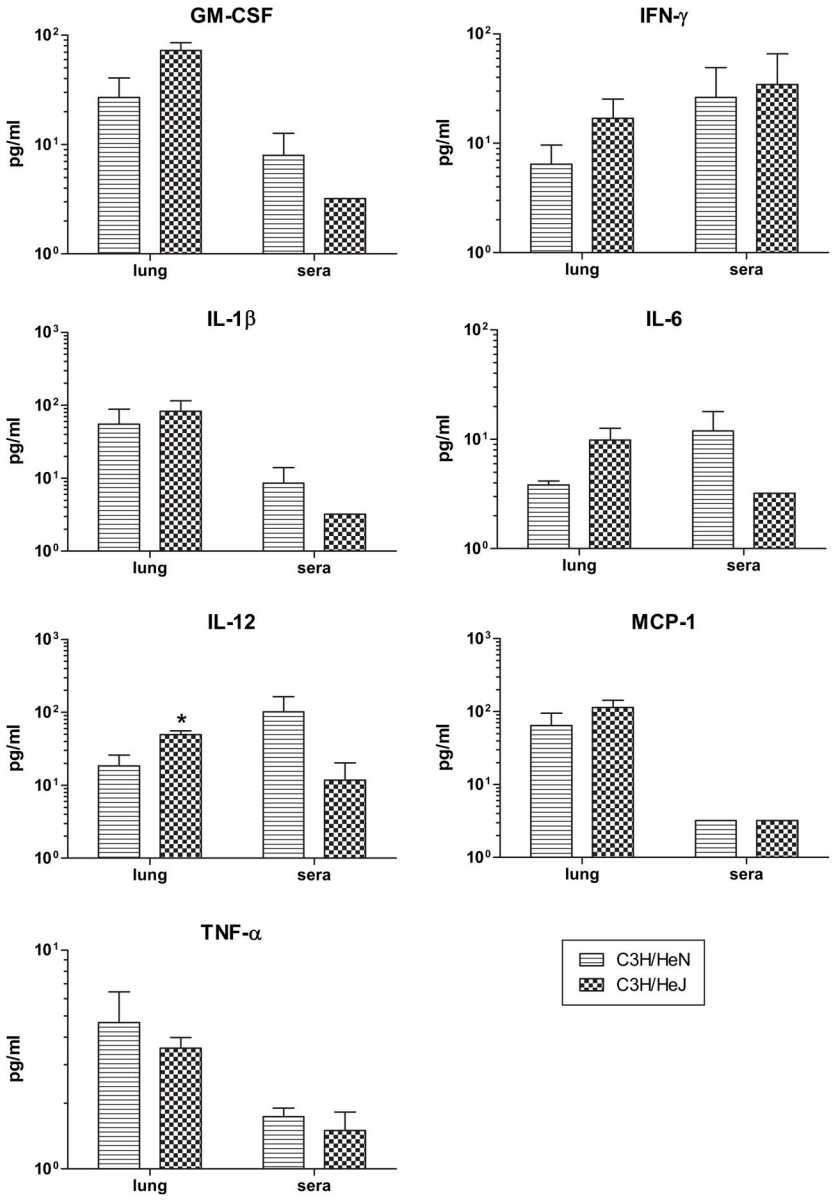


**Fig. 4.**

Pulmonary and serum cytokine levels in resistant C3H/HeN and C3H/HeJ strains after high dose aerosol challenge. Cytokines were measured in the lung homogenates (left columns) and the sera (right columns) of C3H/HeN (horizontal lines) and C3H/HeJ (cross-hatched) after 4 weeks p.i. with $1 \times 10^6$ cfu. Data shown are mean values with error bars showing the SEM from three mice at each time point. Samples from uninfected controls had undetectable levels of cytokines and are not shown. The assays were performed in duplicate. * indicates statistical significance between strains.

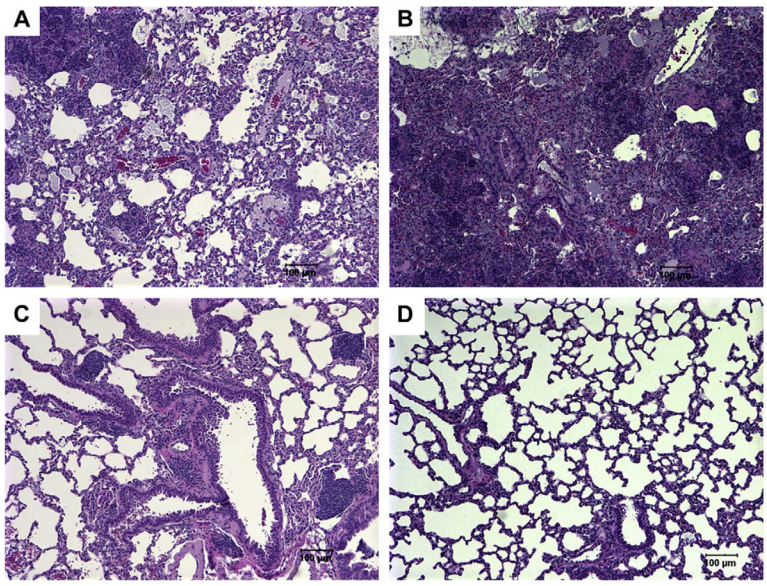


**Fig. 5.**

Lung pathology in susceptible (C57BL/6 and BALB/c) and resistant (C3H/HeN and C3H/HeJ) mice after high dose aerosol challenge. Moderate pneumonia observed in the lungs of C57BL/6 (A) compared to severe pneumonia in BALB/c (B) that succumbed to $1 \times 10^6$ cfu at 48 h and 72 h p.i., respectively. Examination of C3H/HeJ lungs revealed moderate lymphoid hyperplasia (C) at 4 weeks p.i. compared to normal lung architecture in C3H/HeN (D).