RESEARCH ARTICLE

Characterization of *Burkholderia pseudomallei* Strains Using a Murine Intraperitoneal Infection Model and *In Vitro* Macrophage Assays

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Abstract

*Burkholderia pseudomallei*, the etiologic agent of melioidosis, is a gram-negative facultative intracellular bacterium. This bacterium is endemic in Southeast Asia and Northern Australia and can infect humans and animals by several routes. It has also been estimated to present a considerable risk as a potential biothreat agent. There are currently no effective vaccines for *B. pseudomallei*, and antibiotic treatment can be hampered by nonspecific symptomology, the high incidence of naturally occurring antibiotic resistant strains, and disease chronicity. Accordingly, there is a concerted effort to better characterize *B. pseudomallei* and its associated disease. Before novel vaccines and therapeutics can be tested *in vivo*, a well characterized animal model is essential. Previous work has indicated that mice may be a useful animal model. In order to develop standardized animal models of melioidosis, different strains of bacteria must be isolated, propagated, and characterized. Using a murine intraperitoneal (IP) infection model, we tested the virulence of 11 *B. pseudomallei* strains. The IP route offers a reproducible way to rank virulence that can be readily reproduced by other laboratories. This infection route is also useful in distinguishing significant differences in strain virulence that may be masked by the exquisite susceptibility associated with other routes of infection (e.g., inhalational). Additionally, there were several pathologic lesions observed in mice following IP infection. These included varisized abscesses in the spleen, liver, and haired skin. This model indicated that commonly used laboratory strains of *B. pseudomallei* (i.e., K96243 and 1026b) were significantly less virulent as compared to more recently acquired clinical isolates. Additionally, we characterized *in vitro* strain-associated differences in virulence for macrophages and described a potential inverse relationship between virulence in the IP mouse model of some strains and in the macrophage phagocytosis assay. Strains which were more virulent for mice (e.g., HBPU10304a) were often less...
virulent in the macrophage assays, as determined by several parameters such as intracellular bacterial replication and host cell cytotoxicity.

Introduction

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative, facultative intracellular pathogen and has the ability to survive and replicate in both phagocytic and non-phagocytic cells [1–3]. Melioidosis cases are most commonly reported from countries located in Southeast Asia and the Oceania regions, with the greatest number of cases in Thailand, Vietnam, Cambodia, Laos, Malaysia, Singapore, and Northern Australia [4–9]. The disease has also been observed in the South Pacific, Africa, India, and the Middle East. In addition, sporadic cases of melioidosis have occurred in tropical locations in the western hemisphere [3,10–13]. Melioidosis cases that occur in non-endemic regions are normally associated with time spent in endemic areas, and there has been recent evidence that imported animals can harbor this bacterium [14,15]. The routes of infection include percutaneous inoculation, inhalation, or ingestion of contaminated food or water. Seasonal monsoonal rainfall is suspected to increase aerosolization of the bacteria resulting in pneumonia [7,16–18].

The incubation period varies depending upon the dose, route of infection, B. pseudomallei strain characteristics, and host predisposition. The incubation period can range from 1–21 days (mean of 9 days) [19]. However, individuals with risk factors such as diabetes, alcoholism, cirrhosis, thalassemia, lung disease, or other immunosuppressive conditions are considerably more likely to develop symptomatic infections [17,20]. Melioidosis normally presents as a febrile illness, however, this disease has an incredibly diverse list of clinical presentations including acute localized soft tissue infections, acute pulmonary infections, septicemia, and chronic localized infections [3,4]. Clinical disease with B. pseudomallei is generally caused by hematogenous spread of bacteria and seeding to various organs within the host. Although acute infections in individuals with predisposing risk factors are most common, latent infections with or without acute reactivation long after initial exposure also occur with melioidosis [19,21]. It has been postulated that after the initial phase of infection, B. pseudomallei can persist in a dormant stage in macrophages for months or years.

Intracellular survival and cell-to-cell spread may provide B. pseudomallei protection from the humoral immune response [1] and likely contributes to the need for a prolonged course of carefully selected and administered antibiotics. B. pseudomallei continues to be a public health concern in endemic areas and for military personnel serving in these regions because of its potential long latency period and ability to evade the host immune response [22–24]. Additionally, B. pseudomallei has been designated a Tier 1 agent due to its potential use as a biological agent which could represent a serious threat to public health and safety [2,25]. It is for these reasons that effective therapeutics and vaccines are needed.

A well characterized animal model must be used to generate both efficacy and safety data for such novel medical countermeasures and potentially support future pre-clinical studies. In this report, we discuss both in vitro characterization and in vivo virulence determination of a panel of 11 strains of B. pseudomallei. Our in vitro characterization included growth curves, LPS profiles, and macrophage phagocytosis/cytotoxicity assays. We described virulence as measured by median lethal dose (LD50) determinations using the intraperitoneal (IP) murine model of infection. We chose to pursue the IP infection route, in part, because it offers an easy
model that can be reproduced by many laboratories. Additionally, we highlighted several noteworthy pathologies associated with administering \textit{B. pseudomallei} to mice via the IP route.

**Materials and Methods**

**Strain characterization**

The 11 strains of \textit{B. pseudomallei} used in this study were selected by collaborating U.S. federal agencies: Defense Threat Reduction Agency (DTRA) and Biomedical Advanced Research and Development Authority (BARDA). These strains represent an index set of strains to be used in subsequent vaccine and therapeutic countermeasure development research. The strains are all human isolates for which histories and passage information are available and represent both commonly used laboratory strains and relatively new clinical isolates, as described in S1 Table and elsewhere ([26] and Critical Reagents Program/USAMRIID Unified Culture Collection, unpublished). Master seed banks (MSB) of the strains were prepared by streaking the frozen sample received onto a sheep blood agar (SBA) plate and incubating at 35°C for 24 h. A single colony from the plate was used to inoculate a starter culture of calcium-adjusted Mueller Hinton II (CaMHB II) broth. This culture was grown at 37°C with shaking to an OD\textsubscript{600} between 0.1 and 0.3 and diluted to an OD\textsubscript{600} of 0.001 in fresh CaMHB II. This culture was incubated at 37°C for approximately 12 h until it reached mid-log growth phase. The resulting culture was mixed with an equal portion of CaMHB II supplemented with 12.5% glycerol (final concentration) and frozen in aliquots. A working stock bank (WSB) was prepared using 10 \(\mu\)l of the MSB to inoculate a fresh CaMHB II culture which was grown to mid-log phase, combined with an equal volume of CaMHB II with 25% glycerol, and distributed into cryovials (500 \(\mu\)l aliquots) for storage at -70°C.

**In vitro growth kinetics analyses**

Growth curves were prepared as follows. Flasks containing 50 ml of glycerol tryptone broth (GTB, 1% tryptone, 0.5% NaCl, 4% glycerol) were each inoculated with approximately 200 \(\mu\)l of WSB material. The flasks were shaken at 200 rpm at 37°C. Aliquots were removed at selected times, and OD\textsubscript{620} readings were measured using a spectrophotometer. Samples were periodically plated on SBA plates for determination of viable colony forming units (CFU).

**Lipopolysaccharide (LPS) phenotyping of \textit{B. pseudomallei} strains**

\textit{B. pseudomallei} cells from each of the 11 strains were streaked onto SBA plates and incubated at 37°C for 48 h. From each plate, approximately 5 colonies were suspended in GTB and then incubated at 37°C with shaking at 200 rpm overnight. The cultures were then diluted with GTB to an OD\textsubscript{620} of approximately 1.0 and were heat-killed at 90°C for 90 minutes. After sterility was confirmed, LPS extractions were performed with 5 ml of killed cells for each strain using the procedure described by Yi and Hackett [27]. The purified LPS was separated on 10–20% Tricine gels (Thermo Fisher Scientific, Waltham, MA). To help analyze the LPS profiles, we used a mouse-derived monoclonal antibody which was generated against inactivated \textit{B. pseudomallei} strain 1026b (11G3-1). A western blot was performed with a 1:2000 dilution of 11G3-1 then a 1:5000 dilution of peroxidase labeled goat anti-mouse IgG secondary antibody was used (KPL, Gaithersburg, MD). The blot was developed using colorimetric detection with TMB Membrane Peroxidase Substrate (KPL). Alternatively, silver staining was conducted using the method described by Tsai and Frasch [28].
Macrophage assays

*Burkholderia* strains were examined for their ability to infect macrophage-like cells and to induce cell damage using a protocol similar to that described in other studies [29–32]. The J774. A1 murine-derived macrophage-like cell line was cultured in Dulbecco’s Modified Eagle Medium (DMEM) with glucose (Lonza, Walkersville, MD), glutamine, and 10% fetal bovine serum in 24-well plates (in wells with or without coverslips). The cells were incubated for 2 days at 37°C with 5% CO₂, at which time, growth to 90–95% confluence was achieved. On the day before the experiment, GTB was inoculated with bacterial growth from fresh SBA plate cultures of the bacteria and the flasks were incubated overnight at 37°C with shaking at 200 rpm. The densities of the overnight cultures were measured spectrophotometrically, suspensions adjusted to an OD₆₂₀ of 1.0 (approximately 1 x 1₀⁹ CFU/ml) were prepared, and the macrophages were inoculated with the *Burkholderia* strains at a multiplicity of infection (MOI) of 10–20 CFU per macrophage (MOI for each experiment is listed in figure legend). The inocula were diluted and plated on SBA plates to determine the actual MOI. The infected cultures were incubated 1 h to allow uptake; and the cells were washed and incubated with kanamycin (250–500 μg/ml) for 2 h to kill residual extracellular bacteria. Infected cells were then washed, fresh medium was added, and cultures were reincubated for a total of 7–8 h. Infected cells were then processed for bacterial survival/replication and cytotoxicity. Lysates of infected macrophages collected after 1 h, 3 h, and 8 h (final time point) were diluted and plated in triplicate on SBA plates for viable count determinations. Samples were collected from each of three separate wells per strain. Cytotoxicity activity was assessed using several parameters: the extent of cell loss (% detached cells compared to uninfected controls), the proportion of dead cells by staining with live-dead dyes (trypan blue or propidium iodide-PI), overall morphologic changes by staining with histologic dyes (Diff-Quik), and an analysis of multi-nucleated giant cell (MNGC) formation. For the latter, the relative proportions of MNGCs compared to normal cells and of nuclear morphology types (multi-nuclear vs. single nucleus) were determined. For the cytotoxicity data, cells in a minimum of six separate fields (two for each of three Diff-Quik-stained coverslips) were counted to obtain the MNGC parameters and to estimate the percentage of cell loss. For all the cytotoxicity results, the values for the infected wells were normalized by comparing them to the counts of identically treated uninfected cell wells.

Animal challenges: LD₅₀ determinations in mice

Bacteria used for challenge were harvested from a late log phase culture grown in GTB medium at 37°C with shaking at 200 rpm. The bacteria were quantified via OD₆₂₀ estimations and delivered IP in 200 μl of GTB. The delivered doses of bacteria were then verified by plate counts on SBA. Groups of 10 BALB/c mice (National Cancer Institute, Frederick, MD; female, 7–10 weeks of age at time of challenge) were challenged by the IP route with various doses of *B. pseudomallei*. Mice were monitored for clinical signs and symptoms for 60 days. Early endpoint euthanasia was employed in a uniform manner to limit pain and distress.

Histological pathology

Post-mortem tissues were collected from several euthanized mice exhibiting noteworthy pathology and treated with 10% neutral buffered formalin for at least 21 days then subjected to histological analyses. Samples were embedded in paraffin and sectioned for hematoxylin and eosin (HE) staining, as previously described [33].
Ethics statement

Animal research at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) was conducted under an animal use protocol approved by the USAMRIID Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Challenged mice were observed at least daily for 60 days for clinical signs of illness. Humane endpoints were used during all studies, and mice were humanely euthanized when moribund, according to an endpoint score sheet. Animals were scored on a scale of 0–11: 0–2 = no significant clinical signs (e.g., slightly ruffled fur); 3–7 = significant clinical symptoms such as subdued behavior, hunched appearance, absence of grooming, hind limb issues of varying severity and/or pyogranulomatous swelling of varying severity (increased monitoring was warranted); 8–11 = distress. Those animals receiving a score of 8–11 were humanely euthanized by CO2 exposure using compressed CO2 gas followed by cervical dislocation. However, even with multiple observations per day, some animals died as a direct result of the infection.

Statistical analyses

The differences in in vitro growth kinetics were evaluated statistically using GraphPad Prism, version 5.2 software (GraphPad Software, La Jolla, CA). Nonlinear regression analysis was done using the exponential model, as determined by curve fitting. The k parameter (rate constant) was used for best fit comparisons between the data sets for each strain. To estimate lethal dose response curves for each B. pseudomallei challenge strain, the following probit model was fit:

\[ p(x_j) = \Phi(x_i + \beta_i \cdot \log_{10}(\text{Dose}_j)) \]

i indexes the challenge material, j indexes the dose levels of the challenge, and \( \alpha \) and \( \beta \) are the slope and intercept, respectively. The number of non-surviving subjects was modeled as:

\[ x_j \sim \text{Binomial}(p, n_j) \]

The priors [34] for this model are:

\[ x_i \sim \text{Cauchy}(0, 10) \quad \beta_i \sim \text{Cauchy}(0, 10) \]

Samples were drawn from the posterior distributions using Hamiltonian Monte Carlo [35] as implemented in Stan [36,37] using four chains each with a warmup of 5,000 draws followed by 12,500 samples for a total of 50,000 posterior points. Inversion of the probit model formula allowed for the construction of the posterior distributions of the median lethal dose (LD50) for each challenge material. This permitted the direct comparison of the posterior LD50 distributions of each pair of challenge materials which allowed probabilistic statements to be made regarding the likelihood that the LD50 of one challenge is smaller or larger than that of another challenge. All Bayesian estimates are presented with 95% highest posterior density (HPD) credible intervals. All Bayesian analyses were performed using Stan 2.1.0. All other statistics were performed using R 3.1.1.
Results

In vitro characterization of the \textit{B. pseudomallei} strains

We performed \textit{in vitro} characterization of the 11 \textit{B. pseudomallei} strains to include growth kinetics and LPS profiling. To ensure that any differences observed during \textit{in vivo} analyses were not attributable to a defect in growth, growth curves were constructed for all 11 strains based on the OD\textsubscript{620} values recorded at intervals during incubation of the broth cultures (S1 Fig). The \textit{k} parameter (rate constant) was used for best fit comparisons between data sets for each strain, and the resulting analysis revealed that the curves of all data sets were not statistically different (\textit{P} = 0.1304).

Additionally, we were interested in identifying any apparent variations in LPS profiles. The LPS banding profiles for the 11 \textit{B. pseudomallei} strains were analyzed by SDS-PAGE and western blotting. The LPS phenotypes of all the strains appeared provisionally to be type A, and six of them (K96243, MSHR305, MSHR668, 406e, 1026b, and 1106a) have been reported previously to exhibit type A LPS [38,39]. However, a comparison of the molecular weight range of their LPS banding ladders suggested the presence of three distinct phenotypes (Fig 1). These may be type A variants or subtypes. MSHR5855 and MSHR5848 had identical LPS banding patterns with a range higher than any of the other strains (~26–60 kDa). MSHR305 displayed an intermediate range (~29–55 kDa), and the remaining strains exhibited the lowest LPS banding patterns, ranging from approximately 23–50 kDa, depending on LPS concentration. Results of the western blot in panel A were confirmed by silver stained gels, and \textit{B. pseudomallei} 576 displaying the type B LPS banding pattern is shown for comparison (Fig 1B).

Intraperitoneal mouse LD\textsubscript{50} determinations

The LD\textsubscript{50} values for each of the 11 \textit{B. pseudomallei} strains were calculated at both 21 and 60 days after IP challenge (Table 1). Direct comparisons of the posterior samples of the LD\textsubscript{50} values of each strain permit us to make probabilistic statements about how likely it is that one strain is more or less virulent than any other strain, given the observed data. Statistical analyses of day 21 data differentiated the 11 strains into three distinct virulence groups (Fig 2A), however, many of these differences were less significant when analyzing the data through day 60 (Fig 2B). The Bayesian analyses of the day 21 LD\textsubscript{50} data (posterior distributions) indicated that the 11 strains could be divided into three major virulence (“potency”) groups composed of one, five, and five strains, respectively, as follows (from most to least virulent): HBPUB10134a (group 1); MSHR668 (group 2A) and MSHR305, MSHR5855, MSHR5848, and HBPUB10303a (group 2B); and K96243, 406e, 1106a, 1026b, and MSHR5858 (group 3). Strain MSHR668 was statistically more virulent than the other four strains in the intermediate virulence group, although these five strains appear to cluster similarly as illustrated in Table 1 and Fig 2. The day 60 LD\textsubscript{50} data rankings again placed HBPUB10134a as most potent, K96243 and 1106a again as having LD\textsubscript{50} values which were the largest and distinguishable from all others, and the remaining strains fell in the intermediate range.

Noteworthy pathology associated with the IP route of infection

While the main goal of these studies was to characterize virulence using the IP route of infection and subsequently calculating LD\textsubscript{50} values for each strain, there were several interesting clinical and gross pathologic findings that were associated with the IP route of infection which warranted further investigation. The majority of these clinical signs were observed in mice regardless of the bacterial strain used. The dose of the challenge material seemed to dictate the corresponding pathology, as the higher doses resulted in acute disease and more rapid death.
compared to the mice receiving moderate to low doses of bacterial challenge material. Following early endpoint euthanasia, relevant tissues were collected from mice exhibiting these clinical abnormalities.

Abscess/pyogranuloma formation

Mice were frequently observed with protuberances or swelling on various parts of the body. In some cases, small protrusions could be observed on the face and snout of the animals. In other cases, there were putative granulomas forming on the side or limbs of the animals. Upon histologic evaluation, it was confirmed that many of these visible protrusions could be attributed to abscess or pyogranuloma formation (Figs 3 and 4). A common site for abscess/pyogranuloma formation was the spleen, and pyogranulomatous inflammation could often be observed microscopically in the liver as well (Figs 3A and 4C). Pyogranulomatous inflammation in the
Table 1. Summary of LD₅₀ statistics for *B. pseudomallei* strains in BALB/c mice challenged by IP route.

| Strain          | Dose          | Lower Interval | Upper Interval | Dose          | Lower Interval | Upper Interval |
|-----------------|---------------|----------------|----------------|---------------|----------------|----------------|
| K96243*         | 6.2x10⁴       | 2.7x10⁴        | 1.4x10⁵        | 3.5x10⁴       | 1.2x10⁴        | 1.1x10⁵        |
| 1026b           | 5.1x10⁴       | 1.7x10⁴        | 1.8x10⁵        | 1             | 0              | 1.5x10²        |
| 1106a*          | 4.2x10⁴       | 1.7x10⁴        | 9.6x10⁴        | 4.1x10⁴       | 1.7x10⁴        | 9.4x10⁴        |
| MSHR5858        | 2.7x10⁴       | 1.4x10⁴        | 6.6x10⁴        | 8.2x10²       | 1.3x10²        | 7.2x10³        |
| 406e            | 1.6x10⁴       | 4.4x10³        | 8.4x10⁴        | 6             | 1              | 32             |
| MSHR5848        | 1.5x10³       | 3.9x10²        | 6.3x10³        | 26            | 4              | 1.0x10²        |
| HPUB10303a      | 1.3x10³       | 3.8x10³        | 4.5x10³        | 11            | 2              | 32             |
| MSHR5855        | 7.5x10²       | 2.0x10²        | 2.9x10³        | 18            | 1              | 95             |
| MSHR305         | 4.0x10²       | 1.0x10²        | 1.7x10³        | 3             | 0              | 18             |
| MSHR668*        | 1.3x10²       | 37             | 4.5x10²        | 1.4x10²       | 37             | 4.5x10²        |
| HPUB10134a      | 10            | 2              | 32             | 5             | 1              | 14             |

*data included in Challacombe et al. [98]

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**Fig 2.** Statistical analyses of the LD₅₀ determinations. Kernel density plot of the posterior distributions of the LD₅₀ values of each *B. pseudomallei* isolate in BALB/c mice through **A.** 21 day survival challenge and **B.** 60 day survival challenge.

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lymphoreticular organs, particularly the spleen and liver, is typically seen in infections with *Burkholderia* species [40–45]. In some cases, the inflammation in the spleen was of sufficient severity and chronicity to cause fibrous adhesions between the spleen and adjacent organs, such as the pancreas, kidney, and ovary. Spleens were harvested from all mice that survived through day 60. The spleen weights often varied dramatically and were usually indicative of the overall bacterial burden associated with individual animals. Some spleens weighing up to approximately 3.0 g were observed (whereas normal spleens weigh between approximately 0.1 and 0.13 g), and they were somewhat difficult to remove from the peritoneum because of the fibrous adhesions. Finally, the lungs were often severely affected by this inflammatory response (Fig 3D), which may be indicative of a pneumonia that is secondary to a primary infection at a distant site. This type of secondary pneumonia has been associated with *B. pseudomallei* infections in humans [46].

Several of the mice had pathology involving one or both eyes. Eye issues were observed in mice infected with eight out of the 11 strains we examined. In some cases, the affected eye(s) appeared to be significantly swollen or protruding from the eye socket. Histological analysis revealed that the globe of the eye had only mild to moderate inflammation involving the cornea; however, the associated periorbital tissues, including extraocular muscles, optic nerve, lacrimal glands, and orbital bone were infiltrated and in some cases effaced by pyogranulomas or

![Fig 3. Representative histological lesions. A. Multifocal random areas of necrosis in the liver admixed with pyogranulomatous inflammation (BALB/c mouse challenged with 1.9 x 10^3 CFU K96243, H&E 200X). The * identifies a necrotic area. B. Pyogranulomas and abscesses closely associated with lymphatic vessels within the haired skin which were markedly distended with and occasionally obliterated by large numbers of neutrophils (BALB/c mouse challenged with 1.9 x 10^3 CFU K96243, H&E 40X). The arrow identifies the lumen of the lymphatic vessel containing inflammation. C. Pyogranulomatous inflammation (which originated from the haired skin and nasal sinuses) extending through the skull (S) into the meninges and cerebrum (C) (BALB/c mouse challenged with 1.4 x 10^4 CFU K96243, H&E 40X). The * identifies an abscess. D. Significant area of the lung parenchyma obliterated by pyogranulomatous inflammation (BALB/c mouse challenged with 1.1 x 10^2 CFU HBPUB10303a, H&E 20X). The * identifies an abscess.](https://doi.org/10.1371/journal.pone.0124667.g003)
Fig 4. Several noteworthy clinical/gross observations and their histopathological correlates. 

A. Swelling of or around the eye (white arrow). Histologic evaluation revealed abscesses and pyogranulomatous inflammation filling the retro-orbital space causing the globe of the eye (E) to protrude from the socket (BALB/c mouse challenged with $4.2 \times 10^4$ CFU 1106a, H&E 2X). The nasal cavity (N) is also marked for orientation.

B. Large swellings on the tail (often seen in conjunction with hind limb swelling and/or paralysis). Histologic evaluation revealed pyogranulomatous inflammation from the haired skin of the tail extending into adjacent bone, skeletal muscle, and adipose tissue (BALB/c mouse challenged with $1.4 \times 10^4$ CFU K96243, H&E 20X). The tail vertebra (V) is denoted for orientation.

C. Firm white nodules in the spleen. Histologic evaluation revealed marked pyogranulomatous inflammation and necrosis (identified with an *) in the spleen which effaces normal white and red pulp (BALB/c mouse challenged with $9.0 \times 10^2$ MSHR668, H&E 20X).

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abscesses. These inflammatory cells often filled the retro-orbital space and caused the globe of the eye to protrude from the socket, resulting in the described gross appearance (Fig 4A). Similar inflammatory lesions were present throughout the coronal sections of the head in several mice. There have been documented cases of ocular melioidosis with similar descriptions [47,48]. Occasionally, these infections involved the inner and middle ear canals (which may be linked to the ear infections reported grossly in some animals) or extended through the sinuses into the meninges and cerebrum (Fig 3C).

Hind limb paralysis

A more frequent observation was the significant impact to rear limb function, which was observed in mice infected with any of the 11 strains we examined. Clinically, the mice often showed an early “limp” in one rear leg, and this would progress to apparent paralysis in that leg. The first signs of single rear leg paralysis generally occurred between 6 and 30 days post-infection, depending upon the dose and strain of bacteria used. In some cases, the mice would develop double rear leg paralysis to varying degrees. There were occasional examples of mice developing double rear leg paralysis without an obvious single rear leg issue first. However, in the majority of the cases, double rear leg paralysis was observed between 1 and 11 days post onset of single rear leg issues. The observation of double rear leg paralysis was often a trigger for early endpoint euthanasia implementation. Upon pathological analyses, this hind limb involvement was associated with localized pyogranuloma formations. In several animals, significant pyogranulomatous inflammation was observed in the skin, skeletal muscle, bone, or peripheral nerves in the hind limbs which can be directly associated to the loss of motor function (paralysis) in the rear limbs. These inflammatory cells formed large pyogranulomas or abscesses often involving or associated with lymphatic vessels, which resulted in severe disruption of the aforementioned tissues (Fig 3B). Tail lesions were also observed, and although the affected region of the tail varied in severity, such lesions were a relatively common observation (Fig 4B).

Macrophage infectivity and cytotoxicity of B. pseudomallei

An in vitro assay with murine cell line J774.A1 was used to compare the abilities of the B. pseudomallei strains to infect macrophages as a potential in vitro marker for evaluating bacterial infectivity. Several parameters were used to measure both quantitative bacterial viability and the extent of bacterial cytotoxic activity for the infected cells, as described in the methods.

As shown in Fig 5A and 5B, pseudomallei 1106a survived significantly better in macrophages than did B. pseudomallei HB PUB10134a. Strain 1106a was phagocytosed to a two-fold greater extent than HBPUB10134a, as shown by the 1 h ($P = 0.0214$) and 3 h ($P < 0.0001$) viable counts; and it multiplied to a greater extent during the 5 h period between phagocytosis and the 8 h collections ($P = 0.0018$). To account for differences in strain MOIs, the counts were normalized to the inoculum (CFU added at $t_0$) or to the 3 h counts and expressed as percentages. These normalized values confirmed the differences, as shown in S2 Table. In addition to greater bacterial survival, 1106a was overall more cytotoxic for macrophages than HB PUB10134a, as shown by the greater percent cell loss from the wells, the larger proportion of MNGCs, and the higher mean number of nuclei within MNGCs (Table 2 and Fig 6). This in vitro virulence of 1106a is in contrast to the virulence in mice, as shown by the day 21 and day 60 IP LD$_{50}$ values compared to those of HBPUB10134a (Table 1 and Fig 2). Differences between these strains in their extent of adherence and phagocytosis were examined further in a cytochalasin D (CD) treatment experiment. As shown in S2A Fig, the number of HBPUB10134a adherent to the macrophages (normalized as % of inoculum) was significantly
Fig 5. Comparisons of viable counts recovered from J774.A1 macrophages infected with different strains of *B. pseudomallei*. A. The MOIs for strains 1106a and HBPU10134a were 17.2 and 13.7, respectively. Strain 1106a was phagocytosed to a greater extent than HBPU10134a, as shown by the 1 h and 3 h viable counts and multiplied to a greater extent during the 5 h period between phagocytosis and the 8 h collections. The viable counts recovered from the macrophages of 1106a for all three time points were greater than those of HBPU10134a; $P = 0.0214$ (1 h), $P < 0.0001$ (3 h), and $P = 0.0018$ (8 h). B. The MOIs for strains K96243 and HBPU10134a were 27.5 and 26.5, respectively. The viable counts recovered from the macrophages of K96243 for all three time points were greater than those of HBPU10134a; $P < 0.0001$ (1 h), 0.0006 (3 h), and 0.0052 (8 h). C. The MOIs for strains MSHR668 and 1106a were 8.9 and 5.8, respectively. The viable counts recovered from the macrophages of 1106a for all three time points were nearly or significantly greater than those of MSHR668; $P < 0.0001$ (1 h), $P = 0.070$ (3 h), and $P = 0.0003$ (8 h). D. The MOIs for strains 1106a and MSHR5855 were 12.4 and 7.7, respectively. The viable counts recovered from the macrophages of 1106a for all three time points were greater than those of MSHR5855; $P = 0.0002$ (1 h), $0.0019$ (3 h), and < 0.0001 (8 h). E. The MOIs for strains 1026b and MSHR305 were 16.2 and 16.9, respectively. The viable counts recovered from the macrophages of 1026b were greater than those of MSHR305 at the 3 h ($P = 0.0015$) and 8 h time points ($P = 0.0097$); the 1 h viable counts were not significantly different ($P = 0.077$). F. The MOI for both the 1026b and 1106a strains was 16.2. The viable counts recovered from the macrophages of 1106a for all three time points were greater than those of 1026b: $P = 0.0001$ for 1 h and 3 h time points, and $P = 0.0029$ for the 8 h time point.
less ($P < 0.0001$) than that of 1106a based on the counts from cells infected 1 h with CD-treated inocula. The extent of phagocytosis as determined by viable counts recovered after a further 2 h incubation of infected cells in kanamycin showed that HBPU10134a was phagocytosed to an extent that was 11-fold less than that of 1106a, i.e., 0.05% and 0.55% (counts normalized as a percentage of the inoculum). Furthermore, the CFU recovered from CD- and kanamycin-treated 1106a-infected cells were significantly greater than those from HBPU10134a-infected cells suggesting that fewer HBPU10134a were phagocytosed in the presence of CD than 1106a (S2A Fig). To determine whether the reduced numbers of HBPU10134a associated with the macrophages could be due to its more rapid exit or release from the cells, the CFU present in the media of cells infected and incubated in the absence of kanamycin were sampled. As shown in S2B Fig, the data indicated that there were fewer CFU of strain HBPU10134a than strain 1106a present in the medium. Thus, these data suggest that the overall differences in survival of these strains in the macrophages might be attributed to different extents of adherence and phagocytosis, as well as to different extents of replication or killing during the post-phagocytosis incubation period (Fig 5A).

*B. pseudomallei* strain HBPU10134a also demonstrated much less apparent infectivity and cytotoxicity for macrophages than *B. pseudomallei* K96243 (Fig 5B). K96243 was phagocytosed to a nearly two-fold greater extent than HBPU10134a, as shown by the 1 h and 3 h macrophage lysate counts. It also multiplied significantly during the 5 h period between uptake and the 8 h collections, whereas HBPU10134a viable counts were unchanged (Fig 5B and S2 Table). K96243 was more cytotoxic for macrophages, as shown by the more extensive cell detachment/loss, larger number of dead trypan blue-stained cells, larger proportion of MNGCs, and the higher mean number of nuclei within MNGCs (Table 2). The greater macrophage

### Table 2. Phenotypes of macrophages infected with *B. pseudomallei* strains—cytotoxicity comparisons.

| Panel | Strain | % cell loss $b$ | % cells dead (TB)$b$ | No. MNGC or necrotic cells (% of total)$c$ | No. MNGC nuclei (% of total)$c$ | Mouse IP LD$_{50}$ | day 21 | day 60 |
|-------|-------|-----------------|----------------------|----------------------------------------|----------------------------------|----------------|-------|
| A     | 1106a | 50              | 15.0                 | 8.3                                    | 45.7                             | $4.2 \times 10^4$ | 4.1$\times 10^4$ |
|       | HBPU10134a | 15–20    | $\leq 1.0$                  | 2.4                                    | 9.2                              | 10             | 5     |
| B     | K96243 | 40              | 8.3                   | 5.6                                    | 25.7                             | $6.2 \times 10^4$ | 3.5$\times 10^4$ |
|       | HBPU10134a | 25      | 4.1                    | 2.3                                    | 8.8                              | 10             | 5     |
| C     | 1106a  | 60              | 61.8                  | 12.0                                   | 73.4                             | $4.2 \times 10^4$ | 4.1$\times 10^4$ |
|       | MSHR668 | 55       | 19.0                   | 13.2                                   | 58.5                             | $1.3 \times 10^2$ | 1.4$\times 10^2$ |
| D     | 1106a  | 70–75           | 50.0                  | 15.8                                   | 60.0                             | $4.2 \times 10^4$ | 4.1$\times 10^4$ |
|       | MSHR5855 | 15      | 19.1                   | 7.7                                    | 29.4                             | $7.5 \times 10^2$ | 18    |
| E     | 1026b  | 55              | 12.5$^d$              | 5.0                                    | 35.7                             | $5.1 \times 10^4$ | 1     |
|       | MSHR305 | 15–20   | 10.0                   | 2.1                                    | 12.4                             | $4.0 \times 10^2$ | 3     |
| F     | 1106a  | $\geq 85\%$    | NA$^b$                | 12.0                                   | 70.6                             | $4.1 \times 10^4$ | 4.1$\times 10^4$ |
|       | 1026b  | 55              | 10+                   | 35.0                                   | 35.7                             | $5.1 \times 10^4$ | 1     |

$^a$Fig 6, panels A–F.

$^b$Cytotoxicity is shown as the % cells lost from the macrophage layer, by trypan blue (TB) staining; and the % dead cells among those remaining by TB and/or PI staining. However, necrotic cells and advanced stage MNGCs with degraded nuclei are unstained by TB, and the values underestimate the actual proportion of dead cells.

$^c$The values describe the approximate relative proportion of MNGCs among the total cells in six fields (%) with two from each of three replicate coverslips (600x). The major nuclear phenotypes, considering all the nuclei in the fields (typical large nuclei vs. nuclei present in MNGCs with intact stained nuclei or in necrotic fused cell masses with lightly stained nuclei), are expressed as percentages. Counts were done using Diff-Quik s

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virulence of K96243 contrasts with its significantly higher day 21 IP LD$_{50}$ for mice compared to HBPUB10134a (Table 1 and Fig 2). The macrophage infection comparison shown in Fig 5C (and S2 Table) follows the trend exhibited in the studies described in panels A and B. In this experiment, 1106a adhered to and was possibly phagocytosed by the macrophages to a greater extent than B. pseudomallei MSHR668, and multiplied more during the 5 h incubation period, as shown by the larger number of viable organisms recovered at the 8 h time point. In addition, 1106a induced even greater macrophage cytotoxicity than MSHR668 at 8 h, as evidenced by the greater percentage of trypan blue-stained cells and the presence of numerous notably large MNGCs, as well as fused, necrosing cells (Table 2). MSHR668 was significantly more virulent

Fig 6. Comparative phenotypes of macrophages infected with strain 1106a or HBPUB10134a. Morphological changes in cells and in the extent of cell death/detachment as observed in Diff-Quik stains of cultures at the 8 h time point. The typical normal appearance of uninfected semi-confluent J774.A1 cells at low power, 100x (A) and high power, 600x (D). B, E: Cells infected with 1106a, showing loss of monolayer and presence of numerous MNGCs and clusters of fused necrotic cells at 100x (B) and 600x (E). C, F: Cells infected with HBPUB10134a and viewed at 100x (C) and 600x (F) demonstrated cell loss to a lesser extent than those with 1106a and promoted MNGC formation, albeit fewer in number and smaller in size. The arrows indicate examples of MNGCs.

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than 1106a, as determined by mouse IP LD50 values at days 21 and 60 post-exposure. Similar
to the findings in Fig 5A and 5C, and as shown in Fig 5D and S2 Table, the viable counts recov-
ered from macrophages infected with 1106a were greater than those of MSHR5855 for all three
time points. MSHR5855 was much less cytotoxic than 1106a, as evidenced by the reduced cell
killing and detachment and lesser proportion and size (number of nuclei) of MNGCs associat-
ed with infection by MSHR5855 (Table 2). As illustrated in Fig 5E, a comparison between
strain 1026b and MSHR305 supported the trend observed in the other strain comparisons. The
infection with 1026b produced greater numbers of viable counts at the 3 h and 8 h times but
the 1 h recoveries of 1026b and MSHR305 were not significantly different. Also, 1026b was
more cytotoxic, inducing more necrosis (as evidenced by trypan blue-positive and unstained
necrotic MNGCs), greater cell detachment, and more extensive and larger MNGCs than
MSHR305 (Table 2). However, MSHR305 was significantly more virulent than 1106a, as deter-
mined by mouse IP LD50 values at days 21 and 60 post-exposure.

A comparison performed with strains 1106a and 1026b demonstrated an interesting varia-
tion of the relationship described in panels A–E. Strain 1106a appeared to be phagocytosed to a
greater extent than strain 1026b, and it was present in significantly higher concentrations at 8 h
compared to 1026b (Fig 5F and S2 Table). Infection with strain 1106a also appeared to be asso-
associated with a greater extent of cytotoxicity and macrophage loss (S2 Table). Thus, 1106a ap-
peared to survive, grow better, and exhibit more virulence for macrophages than 1026b. These
differences in macrophage virulence with 1106a and 1026b were observed despite the fact that
the strains had comparable day 21 IP LD50 values for BALB/c mice, although their day 60 IP
LD50 values varied greatly (Table 1).

Discussion

This work focused on characterizing a panel of 11 strains of B. pseudomallei for future use in
animal model qualification and subsequent testing for medical countermeasures. We examined
the growth kinetics of the strains and also determined their LPS profiles. We confirmed the
LPS typing for several strains [39] and also showed that previously uncharacterized strains
MSHR5858, HBPU10134a, and HBPU10303a appeared to be type A as well. Although
strain MSHR305 was previously classified as type A [39], its LPS profile was consistently distin-
guishable from the other previously established type A strains under our test conditions. In ad-
dition, we tentatively identified strains MSHR5855 and MSHR5848 as type A because their
ladder banding patterns were detected with the same monoclonal antibody (Fig 1A). However,
their consistently higher band profiles again suggest that type A variants might exist among
strains of the same known LPS phenotype. We will further investigate the strains that have po-
tentially different banding patterns using genetic and structural analyses to determine whether
they represent distinct subclasses of the known LPS types. Notably, the strains exhibiting these
altered phenotypes in our studies were Australian isolates, and strains from Australia are
known to display a higher diversity of LPS phenotypes [35]. It is also tempting to speculate
about the location of the body from which these strains were isolated. For example,
MSHR5855 and MSHR5848 were both isolated from sputum samples, and it is known that
chronic cases of melioidosis have a higher proportion of patients exhibiting pneumonia [49].
Analogously, studies with isolates of the closely related Burkholderia cepacia complex from cystic
fibrosis patients established that strains associated with chronic infection in the lungs fre-
quently exhibited an altered LPS phenotype; rough instead of smooth in this case [50,51]. This
suggests that Burkholderia LPS phenotypic changes could be associated with chronic infection.
However, chronic bacterial infections are associated with a complicated network of virulence
factors and regulatory mechanisms, as well as significant genomic alterations [52–54].
Accordingly, more work is required to fully understand the differences in *B. pseudomallei* LPS profiles and any potential effects on bacterial virulence or chronicity.

We chose the IP route of infection for these studies because this type of challenge can be readily performed. While aerosol infections are the most pertinent to the biodefense community, the IP route offered an accurate means to support initial characterization which would allow strain down-selection. Importantly, the use of BALB/c mice offered a model that could easily distinguish virulence attributes amongst these strains (Table 1). In some cases, mice are so sensitive to infections initiated by aerosolized bacteria that statistically significant strain differentiation based solely upon LD$_{50}$ calculations may be very difficult ([55] and D. Waag, unpublished data). Our data demonstrate that the IP route of infection results in a prolonged infection and much higher LD$_{50}$ values compared to those reported for inhalational routes of infection [55,56]. By using a route of infection that is less acute, it provides a means to potentially elucidate differences in bacterial strains which could be masked by using the inhalational route. The IP route also represents an important initial screening model useful for exploring pathogenesis and evaluating the efficacy of medical countermeasures prior to moving onto more difficult and costly models of inhalational disease [40,41,56–75].

This work also clearly demonstrates the importance of selecting appropriate study durations. Our data suggest that to accurately represent the disease initiated by IP inoculation, even 21 days of observation may not be sufficient. While observations made during the 14–21 day period in the BALB/c model may accurately reflect an acute stage of the disease, these observations do not necessarily reflect the overall health of the surviving animals and accordingly, the efficacy achieved when testing novel vaccines or therapeutics. As shown in this report, extended study durations will likely be required to demonstrate medical countermeasure efficacy in this (and likely any) animal model. It is important to note, however, that the LD$_{50}$ values from the day 60 lethal dose analyses are more tenuous due to the greatly diminished pool of surviving mice.

The gross and histologic lesions seen in the IP challenged mice were consistent with those expected upon exposure to *B. pseudomallei*, and the reported clinical signs can be directly attributed to the numerous abscesses and pyogranulomatous inflammation seen histologically. Further studies are required to better understand if these clinical observations are more commonly associated with specific strains of bacteria, e.g., more virulent or less virulent *B. pseudomallei* strains, as determined in this model. However, these data document some relatively common clinical signs of BALB/c mice infected intraperitoneally with different *B. pseudomallei* strains, such as the pyogranulomatous inflammatory process occurring in multiple lymphoreticular locations resembling those described in other animals (mice, hamsters, NHPs, and humans [40,41,44,76]).

There have been several previous reports describing the susceptibility of BALB/c mice to *B. pseudomallei* infection by the IP route [58,70,73,77]. However, none of these reports used all of the strains described in the present work, the strain history provided was often brief, and clinical, pathological, and bacteriological characterization was minimal or not provided, with some exceptions. Chua et al. and Atkins et al. documented *in vivo* persistence of the inoculated *B. pseudomallei* strain in host organs up through 14 or 30 days, respectively [58,70]. Nevertheless, as reported here, the strains described previously exhibited a wide range of LD$_{50}$ values, from approximately 3 to 5,650 CFU. Additionally, we determined LD$_{50}$ values at a relatively early time post-challenge (day 21), as well as after an extended period of infection (day 60).

The *in vitro* macrophage phenotypes of several of the *B. pseudomallei* strains suggested the existence of a potential inverse association between a strain’s virulence in mice and its virulence in macrophages for at least a subset of *B. pseudomallei* strains. The macrophage infection phenotypes were assessed by determining both bacterial viability and macrophage cytotoxic
activity. Fig 5 and S2 Table demonstrate the former, and Table 2 and Fig 6 illustrate the cytotoxicity parameters. As observed for other diverse in vitro B. pseudomallei phenotypes, there was some inter-experimental variability in the macrophage phenotypes, and the association was not precise for all strain comparisons. Nevertheless, the potential inverse association between mouse and macrophage virulence was observed in numerous assays and was especially clear-cut for comparisons of strains differing most in their day 21 and/or day 60 IP LD₅₀ values (Table 1 and Fig 2). The comparisons which supported this observation involved seven strains (Fig 5); comparisons involving other strains, such as MSHR5848 and HBPU10303a, also exhibited the inverse association.

In contrast, as described in the results, a comparison of the macrophage phenotypes of strains 1106a and 1026b revealed a potential variation of the possible inverse association between macrophage and mouse phenotypes (Fig 5F and Table 2 and S2 Table). Strain 1106a appeared to survive better in macrophages and induce greater cytotoxicity than 1026b, yet appeared to be comparably virulent for mice as indicated by their day 21 IP LD₅₀ values. However, strains 1106a and 1026b differed significantly (> 4 logs) in their day 60 IP LD₅₀ values (Table 1), as well as in aerosol virulence (D. Waag and C. Soffler, unpublished data). This differentiation in mouse virulence was reflected in the inverse macrophage infection parameters displayed by the two strains. This suggests that the macrophage phenotype might offer a marker of strain differences at later stages of infection. Nonetheless, the overall findings (Fig 5 and Table 2 and S2 Table) supported a potential inverse relationship between macrophage and mouse virulence for some strains of B. pseudomallei.

Interestingly, in studies with four strains of B. thailandensis, Wand et al. observed that the virulence of the strains in mice was potentially inversely associated with their macrophage cytotoxicity [78]. The two B. thailandensis strains that were most virulent in mice were much less cytotoxic for J774.A1 cells, as determined by lactate dehydrogenase (LDH) release and extent of MNGC formation, when compared to the other two strains that were highly attenuated in mice. Thus, these observations resemble those reported here for some B. pseudomallei strains. It should be noted that there are other possible explanations of our findings. For example, the reduced extent of macrophage infection by virulent strains, such as HBPUB10134a and MSHR5855, compared to lesser virulent strains, such as 1106a and K96243, might be due to faster egress of bacteria from macrophages (and killing by the antibiotic present in the medium) of the former compared to the latter strains instead of due to reduced intracellular viability. However, the consistent viable count data obtained at different time points (e.g., after removal of the antibiotic and further incubation of the infected cells) (Fig 5), the results of the CD treatment study (S2A Fig), and the quantitation of extracellular bacteria present in the medium of HBPUB10134a versus 1106a infected cells (S2B Fig) suggests that rapid release and dissemination may be a less likely explanation. However, alternate explanations for our observations, as well as the prospective studies described below are being pursued. Additionally, it is important to emphasize that we are not attempting to prove that there is a correlation or specific association between macrophage markers and the in vivo pathogenicity of, or host responses to, B. pseudomallei. Such an association requires more extensive natural history studies to validate the findings. It will also be necessary to demonstrate that an association between macrophage phenotypes and animal virulence can be reproduced in other murine cell lines and in primary cells, such as bronchial or bone marrow macrophages. Previous macrophage infection studies often used cell lines, the most common ones being the murine J774.A1 [31,32,79–83] and RAW264.7 [29,30,84–87] macrophage-like cells, as well as human monocyte-like cells, such as U937 [32,79,88]. We compared the J774.A1 and RAW264.7 cell lines in their responses to two strains of B. pseudomallei (MSHR668 and 1106a). The viable counts recovered of the strains were in similar relative proportions in both cell types, and the differences between
MSHR668 and 1106a in their infection-associated cytopathology were observed in both J774. A1 and RAW264.7 cells. However, in our hands, the J774.A1 cells tended to produce a more homogeneous monolayer in the wells compared to RAW264.7 cells and thus, were more amenable to assessment of cytotoxicity. Specifically, several values (proportion of cells detached, % dead cells, proportion and size of MNGCs) were more difficult to quantitate in RAW264.7 than in J774.A1 cells due to the more heterogeneous distribution of the former in the wells.

Only after in vitro phenotypes, such as those provided by the macrophage phagocytosis assay, is thoroughly characterized and reproduced can the mechanisms underpinning their possible association with pathogenesis be investigated. For instance, a more attenuated and less destructive course of infection in the reticuloendothelial system may promote in vivo persistence and the evolution of infection to a more chronic form. Some of the strains that were the most virulent for mice (lowest 21 and 60 day LD50 values) conversely grew less aggressively in, and exhibited reduced cytotoxicity for, macrophages compared to strains that were more attenuated in the mice (Fig 5 and Tables 1 and 2 and S2 Table). Macrophages might provide a protected niche for these intracellular pathogens against host immune responses [89–91], and it could be speculated that it might be disadvantageous for the Burkholderia to destroy its host cell during certain stages in pathogenesis. Although these hypotheses are speculative, it seems clear that further investigations could reveal additional uses for macrophage and other in vitro cell assays in probing the mechanisms of Burkholderia pathogenesis.

It is generally recognized that in vitro assays of pathogen growth and survival in macrophages are in all likelihood suboptimal models of host infection and not necessarily predictive of in vivo differences in strain virulence [32,80,92,93]. It is certainly true that no relatively simple in vitro system can model the complex processes involved in pathogenesis. However, the results of this study suggest that taking advantage of several measurable aspects of the bacterial-macrophage interaction may reveal some consistent associations between a given stage in pathogenesis and a specific bacterial or host parameter(s) of the in vitro infection. Recently, an automated image acquisition and analysis method was developed and used to characterize the ability of B. pseudomallei to induce MNGC formation [85]. It is a quantitative and promising approach although it does not measure bacterial viability, and it may be more difficult to distinguish MNGCs from cell clumps by the imaging technique than by manual staining and microscopy. However, the technique may be of value in comparing related B. pseudomallei mutants differing in virulence. The quantitative image analysis results with a wild type strain and mutant derivatives agreed with the known mutant defects in ability to induce MNGCs. The assay was also used to identify small molecules that inhibit the MNGC process and thus might provide tools for probing the molecular targets for the MNGC phenotype. This work employed one strain, K96243, and derivatives of it; although the findings do not inform those of the present study, they exemplify the application of specific macrophage phenotype as a potential in vitro biomarker for infection. Macrophage modeling has been used in studies with Burkholderia, as well as other pathogens (e.g., Yersinia pestis, Yersinia pseudotuberculosis, Francisella tularensis, and Bacillus anthracis); to probe potential mechanisms of pathogenesis and host responses, such as putative roles in infection of specific bacterial-associated mechanisms and virulence factors [29–32,79–87,89,92–96]. Macrophage models have been essential in efforts to better understand the roles of the type III and type VI secretion systems (T3SS and T6SS) in the pathogenesis of disease, the mechanisms associated with specific factors encoded by these systems (facilitating intracellular survival, replication, movement, and spread to other cells), and the cellular responses employed by the host to contain these pathogens. For example, Kespichayawattana et al., used findings from a J774.A1 cell culture model to propose that the cell-to-cell spread of B. pseudomallei is mediated by its induction of actin-associated membrane protrusion and cell fusion [31]. Tandhavanant and Chantrati noted a partial association
between colony morphotype switching of *B. pseudomallei* and survival fitness in J774.A1 and human cells, such as the U937 macrophage; however, significant strain variability was observed [32,80]. Using J774.A1 cells, Stevens and coworkers evaluated the activity of T3SS-encoded BopE and other T3SS-encoded proteins in cellular invasion and spread [82,83]. Burtnick et al. discovered a potential role of the type VI secretion system in survival of *B. mallei* and *B. pseudomallei* in animals and identified T6SS-encoded proteins which contributed to the ability of these pathogens to grow in RAW264.7 cells and induce MNGC formation [30,84]. Breitbach and coworkers and Utasincharoen et al. characterized the production of nitric oxide synthase and cytokines (interferons and tumor necrosis factor) by host cells in response to *B. pseudomallei* infection in RAW264.7 and primary bone marrow macrophages, respectively [86,87,97]. These and other reports demonstrate the importance of *in vitro* cell models in identifying potential mechanisms of bacterial pathogenesis, which could be exploited for countermeasure development. Nevertheless, it is clear that any putative inferences on the roles of bacterial virulence mechanisms or phenotypes and host cell responses that are based on cell culture data must be examined in animal models to determine their possible relevance to infection.

Supporting Information

S1 Fig. Growth curves of 11 *B. pseudomallei* isolates. The growth curves, as determined by absorbance at OD_{620}, showed no significant differences. (TIF)

S2 Fig. Infection of J774A.1 macrophages with *B. pseudomallei* strains 1106a or HBPUB10134a. A. The cells were infected in the presence or absence of cytochalasin D (CD) at MOIs of 16.3 (1106a, + CD), 31 (1106a,—CD), 32 (HBPUB10134a, + CD), or 28 (HBPUB10134a,—CD). The infected cells were incubated for 1 h, washed to remove nonphagocytosed bacteria, and incubated for 2 h in the presence of kanamycin. Shown are the mean viable counts recovered after the 1 h uptake period and after incubation with kanamycin (3 h). The number of HBPUB10134a (white bars) adherent to the macrophages (normalized as % of inoculum) was two-fold less than that of 1106a (black bars), as determined by the viable counts from cells infected 1 h with CD-treated inocula (*P* < 0.0001). The direct mean numbers of phagocytosed bacteria shown after washing and incubation (3 h,—CD) were not significant (*P* = 0.161). However, the extent of phagocytosis of HBPUB10134a was 11-fold less than that of 1106a when viable counts were normalized as a percentage of the inoculum; these values were 0.05% and 0.55%, respectively. The direct mean number of CFU recovered from—CD and kanamycin-treated 1106a-infected wells was significantly greater than that from HBPUB10134a-infected wells, *P* = 0.0353 (GraphPad t-test). These were the number of bacteria phagocytosed in the presence of CD (inset graph). B. The cells were infected at MOIs of 16.5 (1106a) or 19.2 (HBPUB10134a). The infected cells were incubated for 1 h, washed to remove unphagocytosed bacteria, and incubated for 2 h in medium with no antibiotic (3 h). Shown are the mean viable counts recovered after the 1 h uptake period and in either the medium recovered from the wells or from cell lysate after the 2 h incubation (3 h). At the 3 h time point, the medium was first removed, and the cells were washed twice before being lysed to recover intracellular bacteria. Strain 1106a counts were greater than those of HBPUB10134a for the 1 h, 3 h lysate, and 3 h medium samples: *P* = 0.0047, *P* < 0.0001, and *P* = 0.0056, respectively. In addition, the viable counts recovered from the macrophage lysates at the 1 h and 3 h time points for 1106a were twice those of HBPUB10134a when normalized as a percentage of each strain’s cell inoculum. (TIF)
S1 Table. Brief summary of human clinical history of *B. pseudomallei* isolates.

(DOCX)

S2 Table. Phenotypes of macrophages infected with *B. pseudomallei* strains: Bacterial survival

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: CKC SLW DMW PLW. Performed the experiments: CKC SLW KA CPK JAB JJB RCB. Analyzed the data: CKC SLW SJK JJB. Contributed reagents/materials/analysis tools: SRT. Wrote the paper: CKC SLW RCB JJB.

**References**

1. Allwood EM, Devenish RJ, Prescott M, Adler B, Boyce JD (2011) Strategies for Intracellular Survival of *Burkholderia pseudomallei*. Front Microbiol 2: 170. doi:10.3389/fmicb.2011.00170 PMID: 22007185

2. Vietri NJ, DeShazer D (2007) Melioidosis. In: Dembek Z, editor. Textbook of Military Medicine: Medical Aspect of Biological Warfare. Washington, D.C.: Borden Institute Walter Reed Army Medical Center. pp. 147–166.

3. Wiersinga WJ, Currie BJ, Peacock SJ (2012) Melioidosis. N Engl J Med 367: 1035–1044. doi: 10.1056/NEJma1204699 PMID: 22970946

4. Cheng AC, Currie BJ (2005) Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 18: 383–416. PMID:15831829

5. Dance DA (1991) Melioidosis: the tip of the iceberg? Clin Microbiol Rev 4: 52–60. PMID: 19861063

6. Dance DA (2000) Melioidosis as an emerging global problem. Acta Trop 74: 115–119. PMID: 10674638

7. Lo TJ, Ang LW, James L, Goh KT (2009) Melioidosis in a tropical city state, Singapore. Emerg Infect Dis 15: 1645–1647. doi: 10.3201/eid1510.090246 PMID: 19861063

8. Rammaert B, Beaute J, Borand L, Hem S, Buchy P, Goyet S, et al. (2011) Pulmonary melioidosis in Cambodia: a prospective study. BMC Infect Dis 11: 126. doi: 10.1186/1471-2334-11-126 PMID: 21569563

9. Yabuuchi E, Arakawa M (1993) *Burkholderia pseudomallei* and melioidosis: be aware in temperate area. Microbiol Immunol 37: 823–836. PMID: 7507550

10. O’Sullivan BP, Torres B, Conidi G, Smole S, Gauthier C, Stauffer KE, et al. (2011) *Burkholderia pseudomallei* infection in a child with cystic fibrosis: acquisition in the Western Hemisphere. Epidemiol Infect 139: 871–875. PMID: 16181507

11. Aardema H, Luijnenburg EM, Salm EF, Blijmer HA, Visser CE, Van't Wout JW. (2005) Changing epidemiology of melioidosis? A case of acute pulmonary melioidosis with fatal outcome imported from Brazil. Clin Infect Dis 33: 1–5. doi: 10.1086/429857 PMID: 15903987

12. Doker TJ, Sharp TM, Rivera-Garcia B, Perez-Padilla J, Benoit TJ, Ellis EM, et al. (2014) Contact Investigation of Melioidosis Cases Reveals Regional Endemicity in Puerto Rico. Clin Infect Dis 60(2): 239–242. doi: 10.1093/cid/ciu764 PMID: 25270646

13. Gee JE, Allender CJ, Tuanyok A, Elrod MG, Hoffmaster AR (2014) *Burkholderia pseudomallei* type G in Western Hemisphere. Emerg Infect Dis 20: 682–684. doi: 10.3201/eid2004.130960 PMID: 24655932

14. Sprague LD, Neubauer H (2004) Melioidosis in animals: a review on epizootiology, diagnosis and clinical presentation. J Vet Med B Infect Dis Vet Public Health 51: 305–320. PMID: 15525357

15. Zehnder AM, Hawkins MG, Koski MA, Littal B, Byrne BA, Swanson AA, et al. (2014) *Burkholderia pseudomallei* isolates in 2 pet iguanas, California, USA. Emerg Infect Dis 20: 304–306. doi: 10.3201/eid2002.131314 PMID: 24447394
16. Currie BJ, Jacups SP (2003) Intensity of rainfall and severity of melioidosis, Australia. Emerg Infect Dis 9: 1538–1542. PMID: 14720392

17. Hassan MR, Pani SP, Peng NP, Voralu K, Vijayalakshmi N, Mehanderkar R, et al. (2010) Incidence, risk factors and clinical epidemiology of melioidosis: a complex socio-ecological emerging infectious disease in the Alor Setar region of Kedah, Malaysia. BMC Infect Dis 10: 302. doi: 10.1186/1471-2334-10-302 PMID: 20964837

18. Liu X, Pang L, Sim SH, Goh KT, Ravikumar S, Win MS, et al. (2015) Association of melioidosis incidence with rainfall and humidity, Singapore, 2003–2012. Emerg Infect Dis 21: 159–162. doi: 10.3201/eid2101.140042 PMID: 25531547

19. Currie BJ, Fisher DA, Anstey NM, Jacups SP (2000) Melioidosis: acute and chronic disease, relapse and re-activation. Trans R Soc Trop Med Hyg 94: 301–304. PMID: 10975006

20. Suputtamongkol Y, Chaowagul W, Chuchitchasakd P, Lertpatanasuwun N, Intaranongpai S, Ruchutra-kool T, et al. (1999) Risk factors for melioidosis and bacteremic melioidosis. Clin Infect Dis 29: 408–413. PMID: 10476750

21. Gan YH (2005) Interaction between Burkholderia pseudomallei and the host immune response: sleeping with the enemy? J Infect Dis 192: 1845–1850. PMID: 16235187

22. Kronmann KC, Truett AA, Hale BR, Crum-Cianflone NF (2009) Melioidosis after brief exposure: a sero-logic survey in US Marines. Am J Trop Med Hyg 80: 182–184. PMID: 19190209

23. Ngauy V, Lemeshev Y, Sadkowski L, Crawford G (2005) Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. J Clin Microbiol 43: 970–972. PMID: 15695721

24. Zhang B, Wear DJ, Kim HS, Weina P, Stojadinovic A, Izadjoon M. (2012) Development of hydrolysis probe-based real-time PCR for identification of virulent gene targets of Burkholderia pseudomallei and B. mallei—a retrospective study on archival cases of service members with melioidosis and glanders. Mil Med 177: 216–221. PMID: 22360070

25. Dance D (2014) Treatment and prophylaxis of melioidosis. Int J Antimicrob Agents 43: 310–318. doi: 10.1016/j.ijantimicag.2014.01.005 PMID: 24613038

26. Van Zandt KE, Tuanyok A, Keim PS, Warren RL, Gelhaus HC (2012) An objective approach for Burkholderia pseudomallei strain selection as challenge material for medical countermeasures efficacy testing. Front Cell Infect Microbiol 2: 120. doi: 10.3389/fcimb.2012.00120 PMID: 23057010

27. Yi EC, Hackett M (2000) Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst 125: 651–656. PMID: 10892021

28. Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119: 115–119. PMID: 6176137

29. Arjcharoen S, Wilraiphat C, Pudla M, Limposuwan K, Woods DE, Sirisinha S, et al. (2007) Fate of a Burkholderia pseudomallei lipopolysaccharide mutant in the mouse macrophage cell line RAW 264.7: possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. Infect Immun 75: 4298–4304. PMID: 17576760

30. Burtnick MN, Brett PJ, Harding SV, Ngugi SA, Ribot WJ, Chantratita N, et al. (2011) The cluster 1 type VI secretion system is a major virulence determinant in Burkholderia pseudomallei. Infect Immun 79: 1512–1525. doi: 10.1128/IAI.01218-10 PMID: 21300775

31. Kespichayawattana W, Rattanachetkul S, Wanun T, Utasinchoncharoen P, Sirisinha S (2000) Burkholderia pseudomallei induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun 68: 5377–5384. PMID: 10948167

32. Tandhavanant S, Thanwisai A, Limmathurotsakul D, Korbsrisate S, Day NP, Peacock SJ, et al. (2010) Effect of colony morphology variation of Burkholderia pseudomallei on intracellular survival and resistance to antimicrobial environments in human macrophages in vitro. BMC Microbiol 10: 303. doi: 10.1186/1471-2180-10-303 PMID: 21114871

33. Davis KJ, Vogel P, Fritz DL, Steele KE, Pitt ML, Welkos SL, et al. (1997) Bacterial filamentation of Yersinia pestis by beta-lactam antibiotics in experimentally infected mice. Arch Pathol Lab Med 121: 865–868. PMID: 9278616

34. Gelman A, Jakulin A, Pittau MG, Su YS (2008) A weakly informative default prior distribution for logistic and other regression models. The Annals of Applied Statistics: 1360

35. Hoffman MD, Gelman A (2011) The No-U-Turn Sampler: Adaptively Setting Path Lengths in Hamiltonian Monte Carlo. Version 1 ed.

36. Team SD (2013) Atna: A C++Library for probability and sampling. 2.1 ed.

37. Team SD (2014) RStan: the R interface to Stan. 2.2 ed.
38. Anuntagool N, Wuthiekanun V, White NJ, Currie BJ, Sermswan RW, Wongratanacheewin S, et al. (2006) Lipopolysaccharide heterogeneity among Burkholderia pseudomallei from different geographic and clinical origins. Am J Trop Med Hyg 74: 348–352. PMID: 16525990

39. Tuanyok A, Stone JK, Mayo M, Kaestli M, Gruendike J, Georgia S, et al. (2012) The genetic and molecular basis of O-antigenic diversity in Burkholderia pseudomallei lipopolysaccharide. PLoS Negl Trop Dis 6: e1453. doi: 10.1371/journal.pntd.0001453 PMID: 22235357

40. Dannenberg AM Jr., Scott EM (1958) Melioidosis: pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of Malleomyces pseudomallei. J Exp Med 107: 153–166. PMID: 13481262

41. Fritz DL, Vogel P, Brown DR, Deshazer D, Waag DM (2000) Mouse model of sublethal and lethal intra-peritoneal glanders (Burkholderia mallei). Vet Pathol 37: 626–636. PMID: 11105952

42. Hoppe I, Brenneke B, Rohde M, Kreft A, Haussler S, Reganzerowski A, et al. (1999) Characterization of a murine model of melioidosis: comparison of different strains of mice. Infect Immun 67: 2891–2900. PMID: 10338496

43. Tarlow MJ, Lloyd J (1971) Melioidosis and chronic granulomatous disease. Proc R Soc Med 64: 19–20. PMID: 4101498

44. Wong KT, Puthucheary SD, Vadivelu J (1995) The histopathology of human melioidosis. Histopathology 26: 51–55. PMID: 7713483

45. Santanirand P, Harley VS, Dance DA, Drasar BS, Bancroft GJ (1999) Obligatory role of gamma interferon for host survival in a murine model of infection with Burkholderia pseudomallei. Infect Immun 67: 3593–3600. PMID: 10377114

46. Meumann EM, Cheng AC, Ward L, Currie BJ (2012) Clinical features and epidemiology of melioidosis pneumonia: results from a 21-year study and review of the literature. Clin Infect Dis 54: 362–369. doi: 10.1093/cid/cir808 PMID: 22057702

47. Saonanon P, Tirakunwichcha S, Chierakul W (2013) Case report of orbital cellulitis and necrotizing fasciitis from melioidosis. Ophthal Plast Reconstr Surg 29: e81–84. doi: 10.1097/IOP.0b013e318275b601 PMID: 23303132

48. Parkes JD, Kline KL, Riedesel EA, Haynes JS (2009) A vascular hamartoma arising from the cervical spine of a cat. J Feline Med Surg 11: 724–727. doi: 10.1016/j.jfms.2008.09.009 PMID: 19564125

49. Currie BJ (2003) Melioidosis: an important cause of pneumonia in residents of and travellers returned from endemic regions. Eur Respir J 22: 542–550. PMID: 14516149

50. Evans E, Poxton IR, Govan JR (1999) Lipopolysaccharide chemotypes of Burkholderia cepacia. J Med Microbiol 48: 825–832. PMID: 10482293

51. Shaw D, Poxton IR, Govan JR (1995) Biological activity of Burkholderia (Pseudomonas) cepacia lipopolysaccharide. FEMS Immunol Med Microbiol 11: 99–106. PMID: 7640678

52. Nandi T, Tan P (2013) Less is more: Burkholderia pseudomallei and chronic melioidosis. MBio 4: e00709–00713. doi: 10.1128/mBio.00709-13 PMID: 24065633

53. Price EP, Hornstra HM, Limmathurotsakul D, Max TL, Sarovich DS, Vogler AJ, et al. (2010) Within-host evolution of Burkholderia pseudomallei in four cases of acute melioidosis. PLoS Pathog 6: e1000725. doi: 10.1371/journal.ppat.1000725 PMID: 20090837

54. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D’Argenio DA, et al. (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103: 8487–8492. PMID: 16687478

55. Massey S, Yeager LA, Blumentritt CA, Vijayakumar S, Sbrana E, Peterson JW, et al. (2014) Comparative Burkholderia pseudomallei natural history virulence studies using an aerosol murine model of infection. Sci Rep 4: 4305. doi: 10.1038/srep04305 PMID: 24603493

56. Titball RW, Russell P, Cuccui J, Easton A, Haque A, Atkins T, et al. (2008) Burkholderia pseudomallei: animal models of infection. Trans R Soc Trop Med Hyg 102 Suppl 1: S111–116. doi: 10.1016/S0035-9203(08)70026-9 PMID: 19121670

57. Nieves W, Petersen H, Judy BM, Blumentritt CA, Russell-Lodrigue K, Roy CJ, et al. (2014) A Burkholderia pseudomallei outer membrane vesicle vaccine provides protection against lethal sepsis. Clin Vaccine Immunol 21: 747–754. doi: 10.1128/CVI.00119-14 PMID: 24671550

58. Chua KL, Chan YY, Gan YH (2003) Flagella are virulence determinants of Burkholderia pseudomallei. Infect Immun 71: 1622–1629. PMID: 12654773

59. Tan GY, Liu Y, Sivalingam SP, Sim SH, Wang D, Paucod JC, et al. (2008) Burkholderia pseudomallei aerosol infection results in differential inflammatory responses in BALB/c and C57Bl/6 mice. J Med Microbiol 57: 508–515. doi: 10.1099/jmm.0.07596-0 PMID: 18349373

60. Barnes JL, Ketheesan N (2005) Route of infection in melioidosis. Emerg Infect Dis 11: 638–639. PMID: 15834987
61. Harland DN, Chu K, Haque A, Nelson M, Walker NJ, Sarkar-Tyson M, et al. (2007) Identification of a LoIC homologue in Burkholderia pseudomallei, a novel protective antigen for melioidosis. Infect Immun 75: 4173–4180. PMID: 17517877

62. Manzeniuk IN, Galina EA, Dorokhin VV, Kalachev I, Borzenkov VN, Svetoch EA. (1999) [Burkholderia mallei and Burkholderia pseudomallei. Study of immuno- and pathogenesis of glanders and melioidosis. Heterologous vaccines]. Antibiot Kimioter 44: 21–26. PMID: 10494379

63. Nelson M, Prior JL, Lever MS, Jones HE, Atkins TP, Titball RW. (2004) Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. J Med Microbiol 53: 1177–1182. PMID: 15585494

64. Sarkar-Tyson M, Smither SJ, Harding SV, Atkins T, Titball RW (2009) Protective efficacy of heat-inactivated B. thailandensis, B. mallei and B. pseudomallei against experimental melioidosis and glanders. Vaccine 27: 4447–4451. doi:10.1016/j.vaccine.2009.05.040 PMID: 19490962

65. Smith MD, Angus BJ, Wuthiekanun V, White NJ (1997) Arabinose assimilation defines a nonvirulent strain of Burkholderia pseudomallei. Infect Immun 65: 4319–4321. PMID: 9317042

66. Valade E, Thibault FM, Gauthier YP, Palencia M, Popoff MY, Vidal DR. (2004) The PmlI-PmlR quorum-sensing system in Burkholderia pseudomallei plays a key role in virulence and modulates production of the MprA protease. J Bacteriol 186: 2288–2294. PMID: 15060030

67. Wikraiphat C, Charoensap J, Utaisincharoen P, Wongratanacheewin S, Taweechaisupapong S, Woods DE, et al. (2009) Comparative in vivo and in vitro analyses of putative virulence factors of Burkholderia pseudomallei using lipopolysaccharide, capsule and flagellin mutants. FEMS Immunol Med Microbiol 56: 253–259. doi:10.1111/j.1574-695X.2009.00574.x PMID: 19549172

68. Warawa JM (2010) Evaluation of surrogate animal models of melioidosis. Front Microbiol 1: 141. doi:10.3389/fmicb.2010.00141 PMID: 21772830

69. Atkins T, Prior R, Mack K, Russell P, Nelson M, Prior J, et al. (2002) Characterisation of an acapsular mutant of Burkholderia pseudomallei identified by signature tagged mutagenesis. J Med Microbiol 51: 539–547. PMID: 12132769

70. Atkins T, Prior RG, Mack K, Russell P, Nelson M, Oyston PC, et al. (2002) A mutant of Burkholderia pseudomallei, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. Infect Immun 70: 5290–5294. PMID: 12183585

71. Chin CY, Tan SC, Nathan S (2012) Immunogenic recombinant Burkholderia pseudomallei MprA serine protease elicits protective immunity in mice. Front Cell Infect Microbiol 2: 85. doi: 10.3389/fcimb.2012.00085 PMID: 22919676

72. Hara Y, Mohamed R, Nathan S (2009) Immunogenic Burkholderia pseudomallei outer membrane proteins as potential candidate vaccine targets. PLoS One 4: e6496. doi: 10.1371/journal.pone.0006496 PMID: 19654871

73. Jones SM, Ellis JF, Russell P, Griffin KF, Oyston PC (2002) Passive protection against Burkholderia pseudomallei infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins. J Med Microbiol 51: 1055–1062. PMID: 12466403

74. Scott AE, Burtnick MN, Stokes MG, Whelan AO, Williamson ED, Atkins TP, et al. (2014) Burkholderia pseudomallei capsular polysaccharide conjugates provide protection against acute melioidosis. Infect Immun 82: 3206–3213. doi: 10.1128/IAI.00113-14 PMID: 24866807

75. Scott AE, Laws TR, D’Elia RV, Stokes MG, Nandi T, Williamson ED, et al. (2013) Protection against experimental melioidosis following immunization with live Burkholderia thailandensis expressing a manno-heptose capsule. Clin Vaccine Immunol 20: 1041–1047. doi: 10.1128/CVI.00113-13 PMID: 23677322

76. Yingst SL, Facemire P, Chuvala L, Norwood D, Wolcott M, Alves DA. (2014) Pathological findings and diagnostic implications of a rhesus macaque (Macaca mulatta) model of aerosol-exposure melioidosis (Burkholderia pseudomallei). J Med Microbiol 63: 118–128. doi: 10.1099/jmm.0.059063-0 PMID: 24105842

77. Srilunanch T, Proungvitaya T, Wongratanacheewin S, Strugnell R, Homchampa P (2009) Construction and characterization of an unmarked aroC deletion mutant of Burkholderia pseudomallei strain A2. Southeast Asian J Trop Med Public Health 40: 123–130. PMID: 19323044

78. Wand ME, Muller CM, Titball RW, Michell SL (2011) Macrophage and Galleria mellonella infection models reflect the virulence of naturally occurring isolates of B. pseudomallei, B. thailandensis and B. oklahomensis. BMC Microbiol 11: 11. doi: 10.1186/1471-2180-11-11 PMID: 21241461

79. Chantratita N, Tandhavanant S, Wikraiphat C, Trunck LA, Rholl DA, Thanwisai A, et al. (2012) Proteomic analysis of colony morphology variants of Burkholderia pseudomallei defines a role for the arginine deiminase system in bacterial survival. J Proteomics 75: 1031–1042. doi: 10.1016/j.jprot.2011.10.015 PMID: 22062159
80. Chantratita N, Wuthiekanun V, Boonburrunk K, Tiyawisutri R, Vesaratichavest M, Limmathurotsakul D, et al. (2007) Biological relevance of colony morphology and phenotypic switching by Burkholderia pseudomallei. J Bacteriol 189: 807–817. PMID: 17114252

81. Haussler S, Rohde M, von Neuhoff N, Nimtz M, Steinmetz I (2003) Structural and functional cellular changes induced by Burkholderia pseudomallei rhamnolipid. Infect Immun 71: 2970–2975. PMID: 12704181

82. Stevens MP, Friebel A, Taylor LA, Wood MW, Brown PJ, Hardt WD, et al. (2003) A Burkholderia pseudomallei type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. J Bacteriol 185: 4992–4996. PMID: 12897019

83. Stevens MP, Wood MW, Taylor LA, Monaghan P, Hawes P, Jones PW., (2002) An Inv/Mxi-Spa-like type III protein secretion system in Burkholderia pseudomallei modulates intracellular behaviour of the pathogen. Mol Microbiol 46: 649–659. PMID: 12410823

84. Burtnick MN, DeShazer D, Nair V, Gherardini FC, Brett PJ (2010) Burkholderia mallei cluster 1 type VI secretion mutants exhibit growth and actin polymerization defects in RAW 264.7 murine macrophages. Infect Immun 78: 88–99. doi: 10.1128/IAI.00985-09 PMID: 19884331

85. Pegoraro G, Eaton BP, Ulrich RL, Lane DJ, Ojeda S, et al. (2014) High-content imaging assay for the quantification of the Burkholderia pseudomallei induced multicellular giant cell (MNGC) phenotype in murine macrophages. BMC Microbiol 14: 98. doi: 10.1186/1471-2180-14-98 PMID: 24750902

86. Utasinscharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, Sirisinha S. (2004) Induction of iNOS expression and antimicrobial activity by interferon (IFN)-beta is distinct from IFN-gamma in Burkholderia pseudomallei-infected mouse macrophages. Clin Exp Immunol 133: 277–283. PMID: 15086391

87. Utasinscharoen P, Tangthawornchaikul N, Kespichayawattana W, Anuntagool N, Chaisuriya P, Sirisinha S. (2000) Kinetic studies of the production of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-alpha) in macrophages stimulated with Burkholderia pseudomallei endotoxin. Clin Exp Immunol 122: 324–329. PMID: 11122236

88. Chieng S, Carreto L, Nathan S (2012) Burkholderia pseudomallei transcriptional adaptation in macrophages. BMC Genomics 13: 328. doi: 10.1186/1471-2164-13-328 PMID: 22823543

89. Galyov EE, Brett PJ, DeShazer D (2010) Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. Annu Rev Microbiol 64: 495–517. doi: 10.1146/annurev.micro.112408.134030 PMID: 20528691

90. Mulye M, Bechill MP, Grose W, Ferreira VP, Lafontaine ER, Wooten RM. (2014) Delineating the importance of serum opsonins and the bacterial capsule in affecting the uptake and killing of Burkholderia pseudomallei by murine neutrophils and macrophages. PLoS Negl Trop Dis 8: e2988. doi:10.1371/journal.pntd.0002988 PMID: 25144195

91. Lauriano CM, Barker JR, Yoon SS, Nano FE, Arulananandam BP, Hassett DJ, et al. (2004) MglA regulates transcription of virulence factors necessary for Franciselina tularensis intraamoebae and intramacrophage survival. Proc Natl Acad Sci U S A 101: 4246–4249. PMID: 15010524

92. Pujol C, Grabenstein JP, Perry RD, Bliska JB (2005) Replication of Yersinia pestis in interferon gamma-activated macrophages requires ripA, a gene encoded in the pigmentation locus. Proc Natl Acad Sci U S A 102: 12909–12914. PMID: 16120661

93. Pujol C, Klein KA, Romanov GA, Palmer LE, Cirotta C, Zhao Z, et al. (2009) Yersinia pestis can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. Infect Immun 77: 2251–2261. doi: 10.1128/IAI.00668-09 PMID: 19289509

94. Breitbach K, Rottner K, Kloccke S, Rohde M, Jenzora A, Weiland J, et al. (2003) Actin-based motility of Burkholderia pseudomallei involves the Arp 2/3 complex, but not N-WASP and Ena/VASP proteins. Cell Microbiol 5: 385–393. PMID: 12780776

95. Challacombe JF, Stubben CJ, Klimko CP, Welkos SL, Kern SJ, Bozue JA, et al. (2014) Interrogation of the Burkholderia pseudomallei Genome to Address Differential Virulence among Isolates. PLoS One 9: e115951. doi: 10.1371/journal.pone.0115951 PMID: 25536074