DISSERTATION

INVESTIGATION OF INNATE IMMUNITY, MUCOSAL THERAPEUTICS AND PATHOGENESIS OF SELECT AGENT *BURKHOLDERIA* SPECIES.

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ABSTRACT

INVESTIGATION OF INNATE IMMUNITY, MUCOSAL THERAPEUTICS AND PATHOGENESIS OF SELECT AGENT *BURKHOLDERIA* SPECIES.

Burkholderia mallei and *B. pseudomallei* are important human pathogens and cause the diseases glanders and melioidosis, respectively. Both organisms are gram-negative bacteria and due to their potential use as bioweapons both have been classified as category B select agents by the Centers for Disease Control and Prevention (CDC). Both bacteria are highly infectious when inhaled and are inherently resistant to many antimicrobials. The protective innate immune responses to *Burkholderia* infection, specifically *B. mallei* infection, are poorly characterized. The goal of these studies was to gain a better understanding of innate immunity and pathogenesis to improve development of therapeutics for treatment of both diseases. A mouse model of acute respiratory glanders was developed to investigate the role of monocytes following *B. mallei* infection. Mice lacking monocyte chemoattractant protein-1 (MCP-1), or chemokine receptor 2 (CCR2), and wild type (WT) mice treated with liposomal clodronate were all highly susceptible to *B. mallei* infection. Following *B. mallei* infection neutrophil recruitment and TNF-α production remained intact in $CCR2^{-/-}$ mice. However, $CCR2^{-/-}$ mice were unable to recruit monocytes or dendritic cells, and produced less IL-12 and IFN-γ than WT mice. Treatment of $CCR2^{-/-}$ mice with recombinant IFN-γ (rIFN-γ) was sufficient to protect mice against disease, highlighting the importance of this cytokine for protection.

To expand on these studies, the necessity of myeloid differentiation factor 88 (MyD88) dependent Toll-like receptor (TLR) signaling was investigated. TLRs are pattern recognition receptors which recognized conserved pathogen associated molecular patterns (PAMP)

expressed by invading organisms. The majority of bacterial associated PAMPs recognized by TLRs signal through the MyD88 dependent pathway. MyD88$^{-/-}$ mice were highly susceptible to *B. mallei* infection, and recruitment of multiple cell types including neutrophils, monocytes and dendritic cells was impaired. Intracellular cytokine staining revealed that dendritic cells and monocytes were the major source IL-12, and natural killer (NK) cells were the major source of IFN-γ. While early production of IL-6 and TNF-α was reduced, MyD88$^{-/-}$ mice were completely unable to produce IFN-γ. Similar to monocyte deficient mice, treatment of MyD88$^{-/-}$ mice with rIFN-γ provided partial protection against infection. Therefore monocyte derived dendritic cell production of IL-12, and subsequent production of IFN-γ by NK cells is critical for protection against acute glanders infection.

Treatment of glanders and melioidosis with antibiotics requires prolonged treatment, and even optimal antibiotic regimens can fail. Therefore the ability of an immune based therapeutic to protect mice following a respiratory challenge was investigated. Cationic liposome DNA complexes (CLDC) are potent activators of innate immunity and induce high levels of IFN-γ production. CLDC administration 24 hours prior to infection with either *B. mallei* or *B. pseudomallei* provided complete protection. Administration at the time of infection provided partial protection, although therapeutic treatment was not effective. The protective effect of CLDC was found to be dependent on MyD88 signaling and IFN-γ production. In contrast, neither monocyte recruitment nor nitric oxide production was necessary for CLDC protection. These studies demonstrated that prophylactic administration of CLDC could provide protection against an intentional release of either pathogen.

While acute disease following *B. pseudomallei* has been well studied, little is known about chronic disease. Chronic disease is recognized as a major complication of melioidosis, and

while disease has been reported to develop in patients up to 62 years after exposure, the site of bacterial persistence is not known. *B. pseudomallei* is an environmental saprophyte and therefore the potential for infection following inoculation, inhalation or ingestion exists. Despite the potential of oral infection, ingestion has not been well studied. In this study an improved selective medium was developed allowing for detection of low level enteric colonization by *B. pseudomallei*. A mouse model of persistent enteric *B. pseudomallei* infection was developed to investigate the ability of chronic melioidosis to develop following oral infection. Oral infection with *B. pseudomallei* resulted in bacterial persistence for up to 60 days in all gastrointestinal (GI) organs, fecal shedding, and a focus of infection localized to the mucosa of the stomach. Despite colonization of the stomach and dissemination into the distal GI tract, no tissue pathology was observed in any GI organ. Multiple *B. pseudomallei* strains were shown to colonize the stomach, and all strains disseminated to the spleen and liver. All mice were shedding bacteria in their feces although bacterial burdens in both GI organs and feces were low, which may make detection of GI colonization in humans difficult. These studies suggest the GI tract may be a reservoir for asymptomatic carriage of *B. pseudomallei*, and if confirmed in humans, could have implications for screening and treatment of melioidosis.

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CHAPTER 1.

LITERATURE REVIEW

BURKHOLDERIA MALLEI AND *B. PSEUDOMALLEI*

1.1 Background and history.

The genus *Burkholderia* consists of gram-negative environmental bacteria which are typically plant pathogens or plant symbionts, but can also be pathogenic to animals and humans (1). Two of these pathogenic species include *B. mallei* and *B. pseudomallei*. Both bacteria are non-spore forming aerobic rods 2-5 μm long and ~0.5 μm wide, and while *B. mallei* is non-motile, *B. pseudomallei* is motile (2-3).

B. mallei is an obligate mammalian pathogen which causes the disease glanders in equines and humans, and contact with infected horses is reported in nearly all human cases (4-7). Glanders is an ancient disease originally described in Greece by Vegetius in 400 BC, and was named "malleus" by Aristotle, which translates to, "bad disease or endemic" (8). Glanders is thought to be the cause of the sixth plague of Egypt described in the bible, and was the inspiration for the establishment of the first veterinary school in Lyons France by Louis XV in 1762 (9-11). The term "snotty nose" appears to have derived from glanders as Dutch physicians used the term snot to describe glanders in horses. In addition, the direct translation of both the French (la morve) and German (rotz) names for glanders is snot (9, 12-13).

In 1886 Friedrich Loffler identified *B. mallei* as the causative agent of glanders by infecting animals with pure cultures (8, 14-15). The frequent use of horses by man in the 18th -

20th centuries resulted in a large number of glanders cases in horses. For example, in Russia 21,305 cases were diagnosed in 1908, and 10,000 horses a year were destroyed at the turn of the 20th century (8). Estimates from the First World War vary greatly, with the number of horses euthanized ranging from 5,776 to 20,879 horses, with 35,928 to 58,843 cases being identified at field stations (8, 15). Legislation and diagnostic testing were critical in controlling glanders. For instance, the Glanders and Farcy order of 1894 in England allowed for a 2 pound compensation to horse owners following euthanasia of a diseased horse (16). In addition, screening and euthanasia of infected horses rapidly reduced the number of glanders cases. For example, in 1898 there were 2443 cases in England, but by 1920 only 15 cases were reported (8).

B. pseudomallei is a saprophytic bacterium found in soil and surface water, and is considered to be an opportunistic pathogen of humans and animals (17). *B. pseudomallei* was originally identified by Alfred Whitmore in 1911 as a disease similar to glanders observed during post mortem autopsies of morphia users in Rangoon (18-20). This new bacterium was found to be motile, while *B. mallei* was known to be non-motile. Furthermore, melioidosis was identified in subjects recently released from prison with no contact with horses. The disease was given the name melioidosis by Stanton and Fletcher in 1921 after "melis", the Greek term used to describe conditions which resembled glanders (3, 21). *B. pseudomallei* was identified in sheep in Australia in 1949, and southeast Asia (S.E. Asia) and northern Australia (N. Australia) remain the major endemic areas of melioidosis (22-23).

Additional *Burkholderia* species which are relevant to experimental investigation, or which cause disease in immunosuppressed patients include *B. thailandensis*, and a group of bacteria known as the *B. cepacia* complex. *B. thailandensis* is a soil bacteria closely related to *B. pseudomallei*, but the ability of *B. thailandensis* to utilize L-arabinose results in reduced

virulence in both animals and humans (24-27). Although, at high infectious doses *B. thailandensis* still retains some virulence in animals, and has been used as a model of *B. pseudomallei* infection (28).

The *B. cepacia* complex consists of 10 genomvars which can cause disease in immunosuppressed patients, mainly cystic fibrosis patients (29). *B. cepacia* was originally identified as the causative agent of onion rot in 1950 by William Burkholder, for whom the genus was later named (30-31).

1.2 Biological weapon potential.

B. mallei is one of the few bacteria to have been used as a biological weapon. During the American Civil War, Union States soldiers observed that upon withdrawal, Confederate States soldiers left glandered horses behind (9-10). During World War I (WWI) both Germany and France undertook a series of sabotage missions aimed at infecting horses bound for Europe with glanders. German agents infected horses with *B. mallei* orally or cutaneously in the United States, Romania, and Spain, while an attempted glanders attack on reindeer in Norway was detected before it could be initiated. France infected horses with glanders in Switzerland which were headed for the German front. In addition, captured French soldiers were found to contain *B. mallei* cultures, supposedly to be used against German soldiers (32-34). Glanders was also used in WWII by Ishii Shiro of Japan against prisoners at the Unit 731 camp in Manchuria, although *B. mallei* was never incorporated into weapons (32). Finally, it is considered likely that *B. mallei* was used by the Soviet Union against the mujahideen in Afghanistan in the 1980s (9, 35).

Although no documented evidence exists regarding the use of *B. pseudomallei* in bio-warfare, the capacity of *B. pseudomallei* as a bioweapon has been recognized for some time. In the Sherlock Holmes novel *The Dying Detective*, written by Sir Arthur Conan, the infectious disease called "Tapanuli fever" was used as a biological weapon. Tapanuli fever is thought to be melioidosis as symptoms are similar to melioidosis and the novel was written in 1913; at the same time melioidosis was identified by Whitmore (36-37). Outside of fiction novels, both the United States and Egypt are thought to have investigated the bioweapon potential of *B. pseudomallei* (32).

Following the anthrax attacks of 2001, both *B. mallei* and *B. pseudomallei* were classified as category B select agents by the United States Centers for Disease Control and Prevention (CDC) due to their potential use as biological weapons (38). Both bacteria can be aerosolized, and are highly infectious by the aerosol route. *B. mallei* has been used as a bioweapon in the past, and both species are highly antibiotic resistant. Due to their classification as potential bio-weapons, there has been an increase in research focusing on development of novel therapeutics for both bacteria.

1.3 Epidemiology.

1.3(1) Natural bacterial reservoirs.

B. mallei is an obligate mammalian pathogen and the only known reservoir is Equidae, including horses, mules and donkeys (6-9, 39). Although naturally occurring infection can occur in a number of animals such as house cats, dogs, camels, goats, sheep, wolves, and lions fed glandered horse meat (9, 39-42). Among Equidae the horse is thought to be the main reservoir as horses typically develop chronic disease, mules are more likely to develop acute disease, and

donkeys are highly susceptible succumbing to infection within 2 weeks of exposure (4, 8). The requirement of horses for disease transmission is highlighted by the absence of glanders in Cuba and Mexico prior to importation of glandered horses from the United States in 1872 and 1890, respectively. Subsequently, 89 cases of human glanders developed in Cuba between 1888 and 1893 (4, 10). Conversely, glanders does not occur in the absence of diseased horses. For instance, glanders was highly prevalent in the United States with 17,147 horses succumbing to glanders at a large stable used by the Union army during the Civil War (10). Following identification and removal of infected horses, no cases of equine glanders have occurred in the United States since 1938, and screening by the complement fixation assay prevents re-introduction (9, 32, 43-45).

Although *B. mallei* is not highly stable in the environment, studies have shown that *B. mallei* can survive for 6-11 days in a moist stall, 14-24 days in rotting material, though drying kills the bacteria in 1-2 weeks (8, 15, 46). Pus from glanders lesions persisted in water for 18 days, and pure cultures have been shown to survive in water for 28 to 31 days (8, 15, 46-47, 48). Furthermore, *B. mallei* is known to survive in stomach secretions and urine for up to 40 hours (8). Therefore, the moderate environmental survival of *B. mallei* allows for disease transmission between horses, and possibly transmission to humans.

In contrast to *B. mallei*, *B. pseudomallei* is an environmental saprophyte. Early researchers considered rodents to be the natural reservoir of *B. pseudomallei*, with fecal shedding resulting in disease transmission (18, 21, 49), although subsequent surveys of rodents from endemic regions failed to show frequent rodent colonization with *B. pseudomallei* (50-57). French researchers in Vietnam demonstrated that *B. pseudomallei* could be isolated from water

and soil (51, 58-61). Subsequently, a number of studies have confirmed that *B. pseudomallei* is prevalent in soil and surface water and can contaminate community water supplies **(Table 1.1).**

Table 1.1. Previous reports of *B. pseudomallei* prevalence in soil, surface water, and contamination of water supplies.

Soil		
Country	**Prevalence (Reference)**	
Thailand	68% (62), 65% (63), 54% (64), 30.1%[a] (65), 28% (66), 20%[a] (56), 4.4-50.1% (67)	
Australia	65% (68), 19.2% (69), 13% (70), 4% (71), < 2% (72), 1.7% (73)	
China	31.6% (74), 8.4% (75), 5% (76), 4.2% (77)	
Malaysia	11% (78), 3% (79), 1.9% (80)	
Laos	44% (81), 36% (82)	
Taiwan	26.4% (83), 19.5% (84)	
Vietnam	6% (85)	
Singapore	1.8% (86)	
Cambodia	30% (87)	
Papua New Guinea	2.8% (88)	
Iran	12% (89-90)	
Surface water		
Country	**Prevalence (Reference)**	
Thailand	50%[a] (91), 26%[a] (56)	
Australia	93% (68), 9% (71), 3% (73), < 2% (72)	
Malaysia	8% (78), 2.9% (80)	
Singapore	5.9%[a] (92)	
Italy	7% (93)	
Contaminated water supplies		
Country	**Source**	**Reference**
Australia	Human outbreak (1994-1996, N. Australia)	(94)
Australia	Human outbreak (1997, W. Australia)	(95-97)
Australia	Pig outbreak	(98)
Australia	Pig outbreak	(99)
Australia	Water bores	(100-101)
Thailand	Roadside and community wells[a]	(56, 62)

[a] Determined by culture prior to discovery of *B. thailandensis*.

Abbreviations: **N. Australia** northern Australia; **W. Australia** western Australia.

Early environmental survey studies identified *B. pseudomallei* more frequently in southern Thailand, while disease occurred more frequently in northeast Thailand (N.E. Thailand) (65, 102-103). The identification of the avirulent soil bacterium *B. thailandensis* in 1996 provided an explanation for these results (24, 26-27). Subsequent studies have confirmed that *B.*

pseudomallei is not evenly distributed in the soil. For example, studies in Thailand revealed that *B. pseudomallei* was isolated 50.1% of samples from N.E. Thailand, but from only 13.8% of samples from northern Thailand (67). These distributions also correlated with disease incidence, suggesting that *B. pseudomallei* exists at high levels in certain areas (104). Studies in N.E. Thailand investigating factors associated with increased soil prevalence have shown that the highest rates of isolation in soil with a pH of 5-6, and in sandy soils which are thought to allow the bacteria to move with changing water conditions (105). In addition, high levels of localized genetic variability amongst *B. pseudomallei* strains has also been observed (within a 2 meter^2 area), suggesting there are high levels of microevolution occurring in the soil (106).

Both optimal and extreme conditions associated with presence and survival of *B. pseudomallei* in the environment have been investigated (107). *B. pseudomallei* has been isolated from soil at a depth of 25-90 centimeters (62, 108-109). *B. pseudomallei* can survive in distilled water without the addition of nutrients for up to 2 decades (47-48, 110-111), and is also resistant to acidic conditions. For instance, *B. pseudomallei* and can survive in soil slurries with a pH of 4, but not 3.5, and in saline at a pH of 2 or 10 for 1 day (112-113). Although the optimal soil pH is 5-6, environmental surveys have shown that *B. pseudomallei* can be isolated from soil over a wide pH range (from 2.8 to 7.4), and from water with a pH range of 2 to 9 (78, 102, 105). These results suggest that *B. pseudomallei* may be more acid tolerant in certain environmental niches as compared to *in vitro* culture. Finally, water appears to be important for the persistence of *B. pseudomallei* in the environment. Studies show a soil water content of 10%-15% is required for survival (69, 112-114).

Contamination of community water supplies has also been documented. For instance, *B. pseudomallei* has been isolated from 26% of drinking water sources in Thailand, and in Australia

26% of water bores were positive (56, 62, 100-101). Furthermore two separate outbreaks of human melioidosis have been linked to contaminated water supplies in Australia (94, 96).

Although a number of studies have shown that chlorine treatment kills *B. pseudomallei* (115-117), other studies have shown that chlorine induces *B. pseudomallei* to enter a viable but non-culturable (VBNC) state (118-119). The VBNC state of bacteria occurs following exposure to some form of stress such as changes in temperate, osmotic conditions, oxygen concentrations, heavy metals and food preservatives. Bacteria in the VBNC state are unable to grow on common agar media, and therefore viability is typically confirmed using fluorescent metabolic dyes. The VBNC state has been described in ~50 bacteria thus far including *B. pseudomallei*, *B. cepacia*, *Helicobacter pylori*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. Although VBNC bacteria are not thought to be virulent, vegetative bacteria which have recovered from a VBNC state are known to be virulent. Factors inducing bacteria to recover from a VBNC state are highly variable and range from removal of the stress or differential culture methods, to passage through amoeba species or infection of egg yolk (120). *B. pseudomallei* can also infect amoeba such as *Acanthamoeba astronyxis*, potentially protecting the bacterium from the harmful effects of chlorine (121).

Finally, the epidemiological link between infected horses and humans in glanders; as well as the connection between environmental and human isolates in melioidosis has been demonstrated. For instance, Pulsed-field gel electrophoresis (PFGE) analysis of 21 *B. mallei* strains resulted in identical PFGE banding patterns in two sets of human and horse isolates (122). Human melioidosis isolates from Taiwan have been matched to soil strains (84), and in Australia clinical isolates have been matched to *B. pseudomallei* from soil (123), contaminated detergent (124), and community water supplies (94-97).

1.3(2) Endemic regions.

Until horses were replaced by other modes of transportation glanders was present throughout the world, regardless of environmental conditions (4, 8-9). Glanders is still endemic in parts of the Middle East, Asia, Africa, South and Central America, and regions of the former Union of Soviet Socialist Republics (USSR) (9, 32, 39). Recently outbreaks in horses have occurred in Pakistan as well as Bahrain, Iran, and Lebanon in 2011, and a possible outbreak in Brazil occurred in 2010 (125-129). Therefore, although rare, natural outbreaks of glanders do still occur.

The prevalence of *B. pseudomallei* appears to be restricted mainly by the requirement of a tropical environment, and new areas of isolation are growing rapidly. Originally *B. pseudomallei* was thought to exist between 20° longitude and 20° latitude (57, 130). More recent studies have demonstrated that the current distribution likely represents the "tip of the iceberg". While S.E. Asia and N. Australia remain the major endemic regions for disease, more recently melioidosis has been identified in the Indian subcontinent, China, Africa, both Central and South America, and numerous countries in tropical latitudes (17, 23, 90). In most cases, identification in new regions of the world is likely due to improved diagnostic capability in these regions, rather than spread from endemic areas.

Although rare, outbreaks of melioidosis have occurred in non-tropical areas. Multiple non-tropical outbreaks have occurred in Australia. In western Australia outbreaks have occurred in sheep at 32°S and 25.5°S, and a human infection occurred at 31°S (99, 131-132). In addition, two human outbreaks have occurred in non-tropical areas of eastern Australia (133-134).

An outbreak of melioidosis also occurred in a temperate region of France at a Paris zoo. The source of the infection is thought to be a Panda delivered to the zoo, or horses imported from

Iran. *B. pseudomallei* subsequently spread to neighboring zoos and equestrian centers, and was able to persistently colonize the soil. Spread of the bacteria is thought to have occurred through transportation of animals, and excretion of bacteria in the manure. Two human deaths were attributed to this outbreak and extensive environmental cleanup and disinfection was required. Although the outbreak was ultimately contained, *B. pseudomallei* persisted in the environment for years (17, 90, 135-137).

In the United States cases of infection with *B. pseudomallei* were reported in Oklahoma and Georgia, and in neither case had the patient traveled outside of the United States (138-139). Subsequent studies have shown these strains to be a unique species named *B. oklahomensis* (140). Studies of these two *B. oklahomensis* isolates, and two *B. thailandensis* isolates from the United States, have demonstrated that these strains are not highly virulent in animal models (141-142). More recently a case of melioidosis occurred in southern Arizona in a patient who had not traveled outside of the United States. The source of *B. pseudomallei* was not conclusively identified, although contaminated medication was considered to be the most likely source (143).

In conclusion, *B. pseudomallei* exists mainly in tropical environments of S.E. Asia and N. Australia, although the number of countries where *B. pseudomallei* has been identified is increasing. Animal transportation may be responsible for outbreaks in non-tropical regions, and dissemination from S.E. Asia and N. Australia. Cases from Australia and France demonstrate that non tropical foci of soil persistence can occur.

1.3(3) Incidence and mortality.

The incidence of *B. mallei* is largely unknown, human infection is rare, and incidence likely varies depending on the frequency and duration of exposure to infected animals.

In contrast to *B. mallei*, incidence and mortality rates are known for *B. pseudomallei*, and data from S.E. Asia and Oceana (Australia, Papua New Guinea (PNG) and the Torres Strait Islands) are summarized in **Table 1.2**.

Table 1.2. Previously reported mortality and incidence rates for melioidosis.
Adapted From (144)

Country	Years	Mortality[a]	Incidence[b]	Reference
Thailand	1989-1994	NA	4.4	(145)
	1997-2006	42.6	12.7	(146)
Malaysia	2000-2003[c]	64	6.07	(147)
	2005-2006	44	4.3	(148)
	2005-2008	33.8	16.4	(149)
Singapore	1987-1994	NA	1.6	(150)
	1989-1996	39.5	1.7	(86)
Taiwan	2000-2006	NA	0.7	(84)
Australia	1990-2002	18.4	18.7	(151)
Papua New Guinea	1994-1998	46	20	(152)
Australia + PNG	1989-1998	21	16.5	(153)
Torres Strait	1995-2000	22	42.7	(154)

[a] Mortality rates reported as the percent of patients that died from melioidosis.

[b] Incidence rates reported as cases per 100,000 population.

[c] Pediatric melioidosis cases.

Abbreviations: **PNG** Papua New Guinea; **NA** Not Available

Data from Thailand, Australia and Malaysia indicate that the incidence of melioidosis appears to be increasing, which may be due to an increase in diagnosis and reporting of melioidosis cases (146-149, 151). In addition to day to day environmental exposure severe weather events or high levels of rainfall can also increase melioidosis incidence rates. For

example following the Tsunami of 2004, or during monsoonal rains, increased rates of melioidosis have been observed (151, 155-156). Furthermore, in Australia, the Torres Strait Islands, Papua New Guinea, and Malaysia, but not in Thailand, the number of melioidosis is associated with rainfall (146, 149, 151-152, 154).

Although glanders and melioidosis are relatively rare, once acquired both diseases are highly lethal. Reports from before the antibiotic era indicate that both diseases are almost universally fatal if untreated. For instance, in Robin's description of 156 chronic glanders cases in 1906, only 9 patients were considered cured (mortality rate of 94%) (4). Similar mortality rates were described in Stanton and Fletcher's report of 83 human melioidosis cases in 1932, where only 2 patients were known to have recovered (mortality rate of 98%) (49).

The use of antibiotics has improved the survival rate in melioidosis patients, although mortality rates in Thailand are still ~40% (146). In Australia the mortality rate has dropped from 30% in 1989 to 9% from 2005-2010 (157). Overall the mortality rate appears to be decreasing in Thailand, Australia and Malaysia (Table 1.2 (146-149, 151)). These decreases in mortality rates, as well as differences in mortality rates between Thailand and Australia, are likely due to improved supportive care (144). For instance, patients in Australia are treated in intensive care units, and the current mortality rate is 19% (157). In a 2010 report from Thailand, due to limited resources, most cases in Ubon Ratchathani province were not treated in intensive care units and the mortality rate was 42.6%. In contrast, patients from the neighboring Khon Kaen province in Thailand were treated in intensive care units, and the mortality rate was 20% (146). Currently all melioidosis patients are treated in intensive care units, although these studies highlight the fact that in addition to appropriate antimicrobial treatment, supportive care is also critical.

While natural human infection with glanders is rare, antibiotic treatment of glanders appears to be an effective means of treatment, although only a small number of human cases are available for analysis (9, 43, 158-160).

1.4 Diagnostic methods.

1.4(1) Selective media.

Early studies with *B. mallei* showed that culture on a boiled potato resulted in characteristic growth. *B. mallei* cultured on potato results in a honey yellow growth in 2 days, and colonies then coalesce into a lawn which turns a chocolate color, and in some cases the potato around the lawn can turn a blue/green color. Although *B. mallei* did not always grow on potato, so glanders could not be excluded due to a lack of growth (8, 15, 161).

B. pseudomallei is known to grow on most commonly used media, and can grow on MacConkey's medium (MAC) (49, 73, 91, 108, 139, 162-163), but not Salmonella-Shigella medium (SS) (92, 164-165). Isolation of *B. pseudomallei* from sterile sights is straight forward, although isolation from complex microbial sites such as skin, soil, or surface water is more challenging. The unique biochemistry and high levels of antibiotic resistance of *B. pseudomallei* have allowed for multiple selective mediums to be developed.

Previous studies identified that growth occurred in media containing 3 to 5 % glycerol and neutral red resulted in ruffled colonies and uptake of neutral red (18, 166). In addition, both *B. mallei* and *B. pseudomallei* were found to be resistant to crystal violet at concentrations which were bactericidal to gram-positive organisms (115). Leslie Ashdown combined all of these observations into one medium and included gentamicin to increase the selectivity (167). This medium is typically referred to as Ashdown's medium (ASH) and has been shown to provide the

best combination of specificity and sensitivity for isolation of *B. pseudomallei* from complex microbial samples (168-169).

Additional selective mediums have also been developed including *Burkholderia pseudomallei* selective agar (BPSA), a media designed provide enhanced isolation of mucoid forms of *B. pseudomallei* (170); and Francis medium which prevents growth of *B. cepacia*, which can occasionally occur on ASH medium (171). Although ASH has greatly improved isolation of *B. pseudomallei*, studies have shown that enteric bacteria can overgrow *B. pseudomallei* (172). Various selective mediums, including modifications of ASH, have been used in the past employing antibiotics such as colistin S (polymyxin E), polymyxin B, vancomycin, streptomycin, amikacin, penicillin G or ampicillin to increase specificity (62, 64, 73, 85, 139, 163, 173-174).

Studies investigating the presence of *B. pseudomallei* in soil have demonstrated that culturing the sample in a selective broth prior to culture on agar medium can increase isolation rates of *B. pseudomallei* (62, 64, 172, 175-177). Studies have demonstrated that a threonine-basal salt solution (TBSS) with colistin is preferable over ASH broth with colistin, although both increased sensitivity over direct agar plating (62, 175, 177-178). Still other studies have demonstrated that growth on TBSS was better during the wet season, while ASH improved isolation during the dry season (176).

B. mallei is more fastidious, and the addition of glycerol is necessary for rapid growth (9, 161). In contrast to *B. pseudomallei*, *B. mallei* does not grow well on MAC, and is not able to grow on ASH (49, 168). Selective mediums have not been developed specifically for *B. mallei*, although *Pseudomonas cepacia* medium (PC) which contains crystal violet, ticarcillin and polymyxin B is known to sustain growth of *B. mallei* (168-169, 179).

1.4(3) Molecular techniques for identification of *B. mallei* and *B. pseudomallei*.

A number of polymerase chain reaction (PCR) assays have been developed to detect both *B. mallei* and *B. pseudomallei* (180-194), including assays that can differentiate *B. mallei*, *B. pseudomallei* and *B. thailandensis* (185, 192). The use of PCR in environmental survey studies has demonstrated that PCR is a sensitive and specific method of *B. pseudomallei* isolation (63, 69). In contrast, attempts to detect *B. pseudomallei* in patients using PCR have been less successful. For instance, compared to culture, sensitivity and specificity (Sens. / Spec.) rates of PCR from different studies include 92.5% / 91.7% (193), 100% / 67% (181), 65% / 100% (186) and 71% / 82% (194). Newer primer sets based on genomic screens which have indentified single nucleotide polymorphisms (SNP), multiplex PCR, or improved DNA isolation procedures may provide increased clinical effectiveness (74, 195-197).

In addition to PCR assays a fluorescent *in situ* hybridization (FISH) assay has recently been developed for the identification of *B. pseudomallei* or *B. mallei* (198). The assay is rapid, requiring only 1 hour for hybridization, and allows for differentiation of *B. pseudomallei* and *B. thailandensis*; although the clinical usefulness of this assay still needs to be determined.

1.4(4) Antibody mediated detection.

Perhaps the most rapid and specific assay is the latex agglutination assay (199-200). This assay uses antibody conjugated latex beads to detect *B. pseudomallei*. The specificity of the assay is determined by the specificity of the antibody conjugated to the latex bead (201). Therefore, the use of a monoclonal antibody allows for differentiation of *B. pseudomallei* and *B. thailandensis*, although some cross reactivity with *B. mallei* has been observed (202).

Immunofluorescent (IFA) assays for detection of *B. pseudomallei* have been developed although clinical studies show that these assays have poor sensitivity (203-205).

1.4(5) Serological assays.

Serological assays for *B. mallei* have been useful for detection of antibody production in both humans and horses. Serological assays including the indirect hemagglutination (IHA) assay, complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA) have been developed (8-9). Both the IHA and CF assays have been used successfully to identify human glanders (6-7, 43, 206).

Similar to *B. mallei* both the CF and IHA assays have been shown to be useful in the diagnosis of melioidosis. The IHA assay has been shown to be sensitive and specific, is inexpensive and easy to perform, and is the most common assay used to screen people in endemic areas (207-210). Due to the high number of seropositive individuals without symptoms, IHA titers are thought to be a good indicator of exposure, while IFA assays for IgM are thought to be better indicators of active disease (211-212). Background IHA titers in patients from non endemic areas have been shown to be < 1:40, and therefore titers ≥ 1:40 are considered to indicate subclinical exposure, while some studies consider titers ≥ 1:160 to indicate active infection (208-209, 213-214). Different cutoff titers used to determine the number of "positive individuals" makes comparisons between studies difficult.

Seroprevalence and cutoff titers reported in previous studies are presented in **Table 1.2**. Prevalence rates vary greatly from 0.2% in Singapore to 41.7% in Thailand. In addition, studies from Thailand suggest that exposure occurs at a young age with approximately 80% of children in exposed by 4 years of age. Exposure rates of 12% were observed in 0-6 month old children

Table 1.3. Previously reported seroprevalence of melioidosis in endemic areas.

Country	Percent positive	Cutoff titer	Reference
Thailand	41.7%, 29.5%[a]	1:40	(212)
	29.1%	1:40	(208)
	21.0%	1:40	(214)
	10.3%	1:160	(219)
China	34.3%	1:40	(220)
	14%	1:80	(221)
	3.8-15.2%	NA	(222)
	1.3-15%	1:40	(76)
Vietnam	21%	1:40	(223)
	11.5%, 3%[b]	1:80	(224)
	1-2%[c]	1:40	(225)
	6%, 2%[d]	1:40	(225)
Taiwan	10.9-36.6%[e]	NA[e]	(83)
Malaysia	7.3%	1:40	(213)
	2%[f]	1:40	(226)
India	8.3%	1:40	(227)
Myanmar	7%	1:160	(228)
Papua New Guinea	8.2%	1:40	(152)
Torres Strait	7.8%	1:40	(229)
Australia	13%	1:40	(230)
	5-13%	1:40	(153)
	5.7%	1:40	(229)
	2.5%	1:40	(231)
Singapore	0.2%	1:16	(86)
Brazil	58.5%[e]	1:200[e]	(232)

[a] 41.7% of blood donors, 29.5% of sera submitted for other tests.

[b] 11.5% of wounded soldiers, 3% of non-wounded soldiers.

[c] Uninjured US soldiers from Vietnam.

[d] 6% of native population, 2% of Europeans living in Vietnam.

[e] Seroprevalence determined by ELISA.

[f] British, New Zealand or Australian soldiers serving in West Malaysia.

Abbreviations: **NA** Not available.

and increased at a rate of about 24% per year to a peak at ~80% at 4 years of age, with titers remaining stable from 4-15 years of age (215-217). Despite frequently positive IHA titers in children, estimates from Thailand suggest that only 1/4600 children develop melioidosis (~ 0.02%) (218).

The disconnect between serology surveys and incidence rates of melioidosis (Table 1.2) is quite striking. For instance in Thailand serology reports put exposure rates at ~10-40%, and in a recent study from Thailand the highest incidence rate of melioidosis from 1997-2006 was 21.3 per 100,000 people (~0.02%) (146). Similar trends are observed in Australia where reports put exposure rate at 2.5-13%, while the highest melioidosis incidence rate from 1990-2002 was 41.7 per 100,000 people (~0.04%) (151). Exposure to avirulent environmental *Burkholderia* spp., such as *B. thailandensis*, may be responsible for a large portion of these antibody responses. Although recent studies suggest subclinical exposure to *B. pseudomallei* may be a more likely explanation. For example, *B. pseudomallei* antibodies from people in endemic areas cross reacted with *B. mallei* but not *B. thailandensis*, or showed weak cross reactivity to *B. thailandensis* (218, 233-235).

1.5 Glanders and melioidosis.

1.5(1) Risk factors.

The major risk factor for development of glanders in both horses and humans is exposure to infected animals. Glanders develops in otherwise healthy people, and disease does not appear to occur more frequently in patients with underlying conditions (32). There does seem to be an association with male sex, and age as 66% of patients were 20-40 years old, although this likely represents an increased exposure to horses (4).

In contrast to glanders, a number of underlying conditions are associated with development of melioidosis. Major risk factors associated with melioidosis include diabetes, chronic kidney disease and alcoholism (146, 151, 157). Diabetes is the most common risk factor occurring in 18%, 39%, 38-89%, 46%, 50%, 57.5% and 78% of patients in Cambodia, Australia, Malaysia, Thailand, Taiwan, Singapore and the Torres Strait Islands, respectively (146, 148-149, 151, 154, 157, 236-240). Chronic kidney disease occurs in 10%, 10%, 12%, 13-27% and 20% of patients in Malaysia, Cambodia, Australia, Thailand and Taiwan, respectively (145, 148-149, 151, 157, 237-242). Finally, excessive alcohol consumption occurs in 20%, 31%, and 39% of patients in Taiwan, Cambodia and Australia, respectively (151, 157, 237-238). Additional risk factors associated with disease are male sex, age ≥ 45, and in Australia chronic lung disease and aboriginal descent (86, 146, 151, 157). There is also a large list of risk factors which occur less frequently, or in individual patients (146, 148-149, 151, 154, 157, 236-240). Melioidosis also develops in patients without any risk factors in 10-29%, 14%, 22.6%, and 30-47% of patients in Malaysia, Australia, Cambodia and Thailand, respectively (145, 151, 157, 238-242). Therefore while most patients have an underlying condition, disease can develop in otherwise healthy individuals.

In contrast to studies from S.E. Asia and N. Australia, patients from Papua New Guinea did not have diabetes, alcoholism or chronic kidney disease, as all of these diseases are rare in Papua New Guinea. In a recent report of 11 melioidosis cases the median age was 9.5 years of age, which is much lower than other areas where old age (≥ 45 years) is a common risk factor for disease (86, 146, 149, 151-152, 154). The young median age in this study is in agreement with serology data from Thailand indicating exposure occurs at a young age (215-217).

Attempts to identify a commonality between the major risk factors have identified reduced neutrophil function (17). Defects in neutrophil function observed in patients with diabetes, chronic kidney disease, or alcoholism include reduction in chemotaxis (243-252), adherence (249, 253), phagocytosis (245, 247, 250-252, 254-257), oxidative burst (252, 256, 258), and bacterial killing (243, 245, 247, 250, 254-255, 259-261). In response to *B. pseudomallei* infection neutrophils are able to phagocytose the bacterium; although while neutrophils appear to kill opsonized *B. pseudomallei* (262), most studies indicate that *B. pseudomallei* is not efficiently killed by neutrophils (263-265). Studies in diabetic melioidosis patients have shown that neutrophils have reduced chemotaxis towards IL-8, reduced phagocytosis and trends towards reduced oxidative burst were observed (266). Therefore patients with underlying conditions associated with melioidosis have multiple functional defects in neutrophil function.

Clinical trials testing the ability of granulocyte-colony stimulating factor (G-CSF) to improve melioidosis treatment have shown reductions in morbidity, but no differences in mortality (267). G-CSF is known to increase neutrophil number as well as neutrophil function, and G-CSF receptors are highly expressed on mature neutrophils (268-269). It is possible that if the underlying conditions are not properly treated then neutrophils could remain suppressed as control of diabetes (248, 250, 253-254, 259), and kidney disease (258) has been shown to improve neutrophil function. Therefore, G-CSF may not be sufficient to activate neutrophils due to the basal suppression of neutrophil function in patients with these underlying diseases. Neutrophils are known to express a number of TLRs and treatment with TLR agonists may induce cytokines such as TNF-α and IL-1β which may further enhance neutrophil activation (270-271). Another possibility is that because *B. pseudomallei* is not highly susceptible to

neutrophil mediated killing, even fully activated neutrophils may not be sufficient to control *B. pseudomallei* (263-265). Finally, there may be additional co-morbidities associated with these conditions involved with the increased rate of melioidosis in these patients.

Further analysis of risk factors commonly observed in melioidosis patients demonstrates that these are all complex diseases associated with a number of compilations, increased risk for infection, and immunosuppression. Indeed, entire journals have been dedicated to complications associated with diabetes (i.e., Journal of Diabetes and its Complications; ISSN 1056-8727; Publication start year: 1987). Typical complications associated with diabetes include kidney disease, hypertension, peripheral and autonomic neuropathy, myocardial infarct, atherosclerosis, cerebral hemorrhage, retinopathy, peripheral vascular atherosclerosis, gangrene and infections (272). Compared to the general population diabetes patients are known to be at an increased risk for lower respiratory, urinary tract, skin and mucous membrane infections; as well as fungal infections of the skin and mucous membranes (273). Specifically, increased incidence of *M. tuberculosis*, *Escherichia coli* O157H7 and hepatitis C infection have been observed in diabetics (274-276).

Studies on immune function in diabetics have shown that, in addition to defects in neutrophil function, reduced macrophage and monocyte function as well as decreased inflammasome function have been observed (277-278). Recent studies investigating melioidosis in diabetic mouse models have shown that while neutrophil and dendritic cell function remained intact, reduced macrophage function was observed (279). Other studies have demonstrated the complexity of diabetes as microarray analysis showed that following *B. pseudomallei* infection over 1000 genes were differentially expressed in diabetic mice as compared to wild type (WT) mice (280).

In chronic kidney disease complications include gastrointestinal bleeding, gastritis, esophagitis, colitis, nausea and vomiting, hypertension, congestive heart failure, anemia, peripheral neuropathy, and many other conditions (281). Uremia interferes with T cells, B cells and macrophages, and is likely responsible for many infections observed in hemodialysis patients. Infections in these patients include gram-negative bacterial infections, *Candida albicans*, *Staphylococcus aureus* and *M. tuberculosis* (282-284). While recruitment of macrophages, monocytes and T cells appears to be intact in chronic kidney disease patients, the function of all these cell types, as well as the inflammasome response are impaired (277, 285-286).

Chronic alcohol abuse can also cause a wide range of complications. For instance, alcohol abuse can result in gastritis and pancreatitis, hypertension, peripheral neuropathy, liver failure, acute hepatitis, alcoholic cirrhosis, cerebella degeneration, and other conditions (287). Frequent infections associated with excessive alcohol consumption include *Legionella* spp., *M. tuberculosis* and *Klebsiella pneumoniae*. Similar to the other risk factors reduced macrophage, monocyte, and T cell function have all been observed following excessive alcohol consumption (288).

Therefore there are multiple common complications amongst the major risk factors for melioidosis which are both immune and non-immune based. Impaired monocyte function is also common to all three, including reduced chemotaxis (245, 289), phagocytosis (245), as well as altered cytokine and chemokine production (245, 290-291). Therefore treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF), or monocyte chemoattractant protein-1 (MCP-1), to enhance monocyte and dendritic cell function in addition to neutrophil

function may improve current treatments (292). Furthermore, co-morbidities independent of the immune system may also be responsible for increased rates of melioidosis in these patients.

1.5(2) Infection routes.

Both glanders and melioidosis are thought to be acquired through inoculation, inhalation or ingestion, although the relative contribution of each route to infection is not well known. Most glanders cases were thought to have been acquired from cutaneous inoculation following contact with glandered horses. For example, 95 cases were thought to be acquired through cutaneous inoculation, and of 105 cases confirmed to be of equine origin, 40 of these patients had abrasions on their hands (4).

Evidence for *B. pseudomallei* infection by cutaneous inoculation comes from studies showing an increased incidence amongst rice farmers in endemic areas who are commonly exposed to wet soil in flooded rice fields, and often do not wear foot protection (110, 145, 223, 241-242). In addition the prescience of cutaneous lesions following a penetrating injury or at the site of a previous wound suggests cutaneous infection (293-295).

Mucous membrane infection including respiratory infection with *B. mallei* is thought to occur less frequently. Of 156 glanders cases, 17 cases were thought to be acquired through the nasal or oral mucosa, and 7 were thought to be respiratory infections (4). Evidence for respiratory infection in melioidosis comes from an increased rate of infection amongst helicopter pilots from the Vietnam War, supposedly acquired following aerosolization of soil bacteria by rotor blades (130, 225, 296-297). In addition, extreme weather events are thought to aerosolize *B. pseudomallei* resulting in respiratory disease (155, 298-299).

Oral infection is a well described route of infection for glanders in horses, although it is thought to occur less frequently in humans (4, 9, 16, 161, 300). One case in Robin's report of 156 cases was attributed to ingestion, and in a first person account of glanders the disease was thought to be acquired following ingestion (4, 300).

Evidence for oral infection of humans with *B. pseudomallei* includes isolation of the bacterium from gastric fluids, intestinal contents, and feces (21, 86, 172, 301). Human infection has been attributed to ingestion of contaminated lake or pond water, and contaminated drinking water was responsible for two melioidosis outbreaks in Australia (94, 96, 302). Ulcers have been observed in the stomach, small intestine and colon of human melioidosis patients, and infants have developed melioidosis following consumption of culture positive breast milk (153, 239, 303-306). Therefore, enteric infection may be more common than previously realized.

Direct human to human transmission is rare but is thought to occur in about 10% of glanders cases (4). Only two documented cases of melioidosis have occurred due to direct human to human transmission. One case was a sexual transmission from a Vietnam War veteran to his wife, and the other was a transmission between siblings with cystic fibrosis (307-308).

Less frequent routes of transmission include both suspected and confirmed vertical transmission of melioidosis from mother to child (309-311). In addition, 3 conjunctivitis and 1 vaginal glanders case have been reported (4). More recent laboratory exposures to *B. mallei* have resulted in glanders following suspected aerosolization or cutaneous inoculation (43, 158).

1.5(3) Human disease.

Glanders can occur as rapidly as 5 to 6 days after exposure and acute disease is nearly universally fatal; while other patients develop a chronic form of the disease which manifests

weeks to months after exposure (6-7). Studies have reported incubation periods of 10-14 days (43), 2-6 weeks (8), 18 months (7), and in a review of chronic glanders the average duration was 14 ½ months with a range of 6 months to 15 years (4). In other reports the true incubation period was not known, although patients were in contact with *B. mallei* for 1-2 years (158-159). In addition, chronic disease is often associated with episodes of active disease followed by recovery, and recurrent episodes lasting up to 5 years and 9 months have been reported (4, 6-7, 300, 312).

In melioidosis patients most cases are considered to be acute in nature. For instance, studies from Thailand report that about $^{1}/_{3}$ of patients have symptoms for 7 days, $^{1}/_{2}$ report being unwell for 7-28 days, and $^{1}/_{6}$ of the patients have symptoms for more than 28 days (144). In an Australian study 88% of infections were thought to be acute, while 12% were considered chronic, and 13% had a confirmed relapse (313). In a separate study 25% of patients with a known exposure incident had an incubation period of 1-29 days, with a mean incubation time of 9 days (153). Studies investigating if secondary episodes of melioidosis are due to recrudescence or re-infection have shown that the majority of cases are recrudescence (314-316).

Clinical reports demonstrate that melioidosis can be rapidly fatal, and patients are often dead on arrival, die on the day admission, or the day after admission (157). However, melioidosis is not always fatal and patients can recover from pneumonic and systemic disease (317-318). Melioidosis can also manifest as a chronic disease, with or without recovery from an initial episode of melioidosis, with disease developing in one patient 62 years after leaving an endemic area (296, 303, 319-320).

While melioidosis studies have shown that in the 25% of cases with a known exposure event patients had acute disease, the majority of patients (75%) had no known exposure event

and were not included in this analysis (153). Furthermore, reports on human glanders state that symptoms associated with fulminating disease are identical regardless of the acute or chronic nature of the disease (4, 7). Therefore while most melioidosis cases may appear to be acute upon presentation, without a known exposure event the true nature of the disease course is difficult to determine (144). While asymptomatic carriage of *B. pseudomallei* has not been observed in endemic areas, only throat swabs have been tested (144, 215). Further studies will be needed to determine if long term asymptomatic colonization is occurring in endemic areas.

Symptoms associated with glanders include fever which was reported in 46% of cases, skin rash of some form was present in 37% of cases, nasal discharge was observed in 30% of cases, and lesions of the mouth were observed in 20% of cases (4).

The lungs were frequently affected in glanders patients; although amongst patients with respiratory involvement clinical symptoms were rare while pathology was more common. For instance, respiratory symptoms were reported in just 25% of glanders patients, while post mortem investigations demonstrated pulmonary involvement in nearly all cases. The gastrointestinal (GI) tract was affected less frequently; although in contrast to the lung, patents with GI involvement were more likely to have GI symptoms without observable GI lesions. For example, symptoms referable to the GI tract were observed in 24 cases including nausea, vomiting, dyspepsia and diarrhea; although lesions in the stomach and colon were observed in just one case (4). Necropsy findings from 44 glanders cases revealed pulmonary involvement (70%), splenic involvement (25%), brain lesions (16%), liver lesions (14%), and bone lesions (14%) (4). Similar symptoms have been reported in other studies, and lesions of the nose, mouth, and gums were also common. In addition, ingestion of pus draining from nasal lesions, and gastric lesions have also been reported (5-7, 312, 321-322).

Melioidosis is most likely to present as a septicemia and/or pneumonia which occurs in about 50% of cases, while superficial pustules, subcutaneous abscesses and pyomyositis occur in 15-25% of cases. Pulmonary involvement can include the lung parenchyma or pleural cavity, and lesion development may occur. Genitourinary infection is common with 18% of male patients in Australia developing prostatic abscesses, and about 25% of patients in Thailand have positive urine cultures. Osteomyelitis and septic arthritis are well recognized; and liver or spleen abscesses are thought to form in about 25% of patients in Thailand, but only about 6% of patients in Australia. In contrast, neurological symptoms occur in 4% of Australian cases, while in N.E. Thailand central nervous system involvement occurs in only 2% of cases (144, 153, 157, 162, 236, 240-241, 323).

Unique manifestations of melioidosis include acute suppurative parotitis which occurs in 38-40% of children in Thailand, but is rare in adults, and has only been observed in a single case report from Australia (144, 154, 324-327). Additional less frequent symptoms and sites of infection include lymphadenitis, mycotic aneurysm, mediastinal infection including mediastinal lymph nodes, pericarditis, acute otitis media, sinusitis, corneal ulcers, orbital cellulitis, suppurative peritonitis, gastrointestinal, and abscess formation in the neck, adrenal glands, and uterine, breast and scrotal tissues (144, 157).

Although the high rate of pneumonia suggests that respiratory acquisition may be more common, studies have shown that various organs can be affected regardless of the infection route. For example, previous studies have shown that pneumonia can arise from systemic infection (328). In Australia secondary pneumonia is common in patients with genitourinary infection, bacteremia, septic arthritis or osteomyelitis, but unusual in patients with skin infections (157). Conversely, cutaneous lesions may develop from direct inoculation, or following blood

borne dissemination (144). Therefore, the potential for colonization of multiple organs following bacterial dissemination makes identifying an infection route difficult.

1.5(4) Experimental animal models.

A report by Miller, et al. in 1948 revealed that multiple animals could be experimentally infected with either *B. mallei* or *B. pseudomallei*. This study found that hamsters and ferrets were highly susceptible, guinea pigs were more resistant, while rabbits, mice, rats and monkeys were even more resistant (329). Hamsters are known to be highly susceptible to *B. mallei* or *B. pseudomallei* infection, regardless of the route of infection. For instance, in a glanders or melioidosis models LD_{50} values range from 1-16 CFU for intraperitoneal (i.p.) infection (330-333), 3.6-10 CFU for subcutaneous (s.c.) infection (329, 333), and 16-70 CFU for aerosol infection (329, 332). The high susceptibility of hamsters to infection with pathogenic *Burkholderia* spp. makes them a good model for investigation of attenuated bacterial strains or virulence factors (25, 333-339).

Compared to hamster infection, the mouse model of melioidosis is thought to more accurately model human disease due to similar pathology and immune responses observed in murine and human disease (332-333). Susceptibility differences are known to exist between mouse strains and depend on the mouse strain infected, the route and dose of infection, and the strain of *B. pseudomallei* used to infect. Differential susceptibility of mouse strains, from most to least susceptible, are thought to be: CBA > 129/SvEv > BALB/c > DBA/2 > C3H/HeN >> C57BL/6 or TO (333). Due to the differential susceptibility of BALB/c and C57BL/6 mice to acute *B. pseudomallei* infection, these strains have been proposed to be good models of acute and chronic infection, respectively (340). In addition, altering the dose of *B. mallei* or *B.*

pseudomallei within the same mouse strain can also result in acute or chronic disease (341-343). In general, the least to most virulent routes of infection with *B. pseudomallei* are subcutaneous (s.c.) > intraperitoneal (i.p.) > intravenous (i.v.), while intranasal (i.n.) infection is less consistent and LD_{50} values are typically 1-2 logs less than i.p. infection (333).

Perhaps the greatest source of variance in experimental infection comes from the use of different *B. mallei* and *B. pseudomallei* strains. For instance in an i.v. model of infection testing 40 different *B. pseudomallei* strains varied by 6 logs, with LD_{50} values ranging from 10 to > 10^6 CFU (344). Furthermore, some *B. pseudomallei* strains have been shown to be more virulent following respiratory infection, but less virulent following systemic infection; while other strains are more virulent systemically, and less virulent following respiratory infection (333, 345).

Further complicating animal models is that serial passage of either bacterium *in vivo* can alter virulence. Alteration of virulence has been well described for *B. mallei*, but appears to occur less frequently with *B. pseudomallei* (4, 8-9, 15, 329, 344, 346-349). For instance, serial passage of *B. mallei* within an animal species increases virulence in mice (15, 350), and guinea pigs (15, 351). Differential virulence has also been observed, whereby passage in one animal can affect the virulence of that strain in other animals (15, 352). For instance, *B. mallei* isolated from a horse and serially passaged in guinea pigs increased the virulence in guinea pigs, but reduced the virulence in mice and rabbits (15, 348). In contrast, studies have shown that passage in rabbits, and more recent studies in equines, have shown that serial passage can also attenuate *B. mallei* (4, 336).

In *B. pseudomallei* studies have demonstrated that serial passage of brain homogenate from mice infected cranially but the i.p., i.n., or s.c. route in guinea pig increased bacterial virulence, and a mouse-passaged strain was more virulent in both C57BL/6 and BALB/c mice

(347, 349, 353). In contrast, in a more recent study only 1 of 8 *B. pseudomallei* strains increased in virulence following serial passage of a spleen-liver homogenate passaged i.v. (344). Therefore serial passage of *B. mallei* can result in highly virulent or highly attenuated strains, and similar results can also be obtained with certain *B. pseudomallei* strains. One possible mechanism for altered virulence following animal passage is mutations in short sequence repeat (ssr) regions. Studies in *B. mallei* have shown that changes in ssr occur during *in vitro* passage as well as following mouse, horse and human infections. Mutations in ssr regions can result in phase and structural changes to proteins potentially altering bacterial virulence (354). To summarize, disease severity in the mouse model of glanders or melioidosis is dependent on the dose and route of infection, as well as the genetic background of the mouse strain and bacterial strain used. The ability to alter virulence also has implications for use of either bacterium as a biological weapon. Although natural infection with either *B. mallei* or *B. pseudomallei* is rare; highly virulent strains can be isolated, and would likely be highly infectious following an intentional release.

The effect of diabetes on infection with *B. pseudomallei* has been investigated using streptozotocin treatment which results in death of pancreatic β cells, and reduced insulin production (355). A separate murine model of diabetes involves BKS.Cg-*Dock7*^{m} +/+ *Leprb*^{db}/J mice, and is a model of type 2 diabetes. These mice contain a genetic mutation in the leptin receptor which results in weight gain, resistance to insulin and hyperglycemia (279). The BKS.Cg-*Dock7*^{m} +/+ *Leprb*^{db}/J model may provide advantages over the streptozotocin model which is a model of type 1 diabetes, as 90% of melioidosis patients have type 2 diabetes (356).

1.5(5) Antibiotic treatment and resistance mechanisms.

Rapid and appropriate administration of antibiotics to patients with melioidosis is critical as any delay can greatly reduce the chances for recovery (357). Current recommendations for treatment of melioidosis include an initial intensive phase with systemically administered antibiotics. The current drug of choice for intensive therapy is ceftazidime, 20 mg/kg/dose delivered i.v. every 6-8 hours; or in the case of ceftazidime failure, treatment with meropenem 25 mg/kg/dose i.v. every 8 hours. The intensive phase of treatment should be administered for at least 10-14 days, and for deep seeded infection at least 4 weeks (144).

Following the intensive therapy phase an oral eradication phase is required to prevent relapse. The oral treatment phase consists of treatment with trimethoprim/sulfamethoxazole (TMP/SMX) at a dose of 8/40 mg/kg/dose twice a day. Treatment with doxycycline (DOX) 2.5 mg/kg/dose can be used in conjunction with TMP/SMX. The oral eradication phase should be administered for at least 3-6 months, or longer if necessary. In patients unable to use TMP/SMX, amoxicillin/clavulanic acid (AMC) can be used at a dose of 20/5 mg/kg every 8 hours. However, treatment with AMC is associated with an increased risk for relapse, if used in either the initial eradication or the oral maintenance phase (358-359). Completion of the entire antibiotic course is critical as this is the most important factor related to relapse (144, 157).

B. pseudomallei contains a number of intrinsic antibiotic resistance mechanisms, including resistance to the standard combination of penicillin and gentamicin for treatment of community acquired sepsis (306, 357, 360). A survey of the *B. pseudomallei* genome reveals the presence of seven β-lactamase genes in Ambler classes A, B and D, and early case reports demonstrate the ineffectiveness of penicillin in treating melioidosis (361-363). β-lactamase studies have shown that the class A enzyme cleaves ampicillin, carbenicillin, cefoperazone,

cefotaxime, cefuroxime, cefepime, cefpirome, and cephalothin, but not cefoxitin, ceftazidime, cloxacillin, imipenem or meropenem (364-365); and the class D enzyme cleaves ampicillin, penicillin G, cloxacillin, oxacillin, piperacillin, cephalothin and cephaloridine (366). The identification of ceftazidime as an effective melioidosis treatment reduced the mortality rate by 50%, although an 11-17% failure rate is still observed (306, 367-369).

Ceftazidime resistant *B. pseudomallei* strains have been isolated which typically contain single amino acid substitutions in the class A β-lactamase (*penA*), resulting in increased resistance to ceftazidime (370-373). A more recent study using targeted gene deletion has shown that the *penA* gene is responsible for ceftazidime resistance, and that the β-lactamase is secreted via the twin-arginine transport system (374). Recent studies have also exposed a completely new resistance mechanism involved with large scale genomic deletion. Multiple *B. pseudomallei* strains were found to have genomic deletions of up to 150 Kb, and penicillin binding protein 3 was lost in all strains, conferring resistance to ceftazidime (369).

An additional major mechanism of antibiotic resistance in *B. pseudomallei* is efflux. The major family of efflux pumps involved with drug resistance in *B. pseudomallei* is the resistance-nodulation-division (RND) family of pumps. RND pumps are proton/drug antiporters and are tripartite pumps composed of a cytoplasmic membrane pump, a periplasm spanning membrane fusion protein, and an outer membrane channel protein (375). *B. pseudomallei* is known to expresses 7-10 RND pumps (376-377). The BpeEF-OprC pump has been shown to efflux chloramphenicol and trimethoprim (378). Trimethoprim efflux has relevance to clinical treatment as this drug is used to treat melioidosis, and 13% of strains are known to be resistant (379-380). Chloramphenicol is currently only used in cases of neurological melioidosis when ceftazidime, carbapenems or TMP/SMX can't be used (144, 381). However, chloramphenicol

was previously part of the "conventional therapy" and was used in conjunction with DOX and TMP/SMX (306). Chloramphenicol has been used successfully to treat melioidosis (382-385), although due to toxicity issues it is currently only used in cases of neurologic melioidosis (144, 303, 383, 385-386).

RND pumps are also responsible for resistance to aminoglycosides and macrolides. For example the AmrAB-OprA pump is involved with aminoglycoside and macrolide resistance (377, 387). In some *B. pseudomallei* strains the BpeAB-OprB is also involved, while in other strains only macrolides are a substrate for this pump (388-389). Inclusion of gentamicin in Ashdown's selective medium highlights how ineffective aminoglycosides are at treating melioidosis (167, 390). Although rare, gentamicin sensitive strains have been isolated from melioidosis patents at a rate of ~1/1000, and this susceptibility has been shown to be due to reduced expression, or the lack of AmrAB-OprB efflux pump (377, 391). Kanamycin has been used in some melioidosis cases, although often in conjunction with other more effective drugs such as chloramphenicol, tetracycline or sulfonamides (164, 384). Similar to chloramphenicol, toxicity issues have resulted in termination of kanamycin treatment (164).

Finally, efflux of fluoroquinolones, chloramphenicol and tetracyclines has been shown to be dependent on the BpeAB-OprB efflux pump. This study also demonstrated that there is substrate overlap between the AmrAB-OprA pump and BpeAB-OprB pump, in that either pump could efflux macrolides, acriflavine, fluoroquinolones, and tetracyclines (388). In addition to efflux, mutation of the DNA gyrase gene (*gyrA*) has also been seen in laboratory induced fluoroquinolone resistance in *B. pseudomallei* (392-393).

Additional, efflux independent antibiotic resistance mechanisms include resistance to cationic peptides such as polymyxin B. Resistance was shown to be mediated by the envelope

structure of *B. pseudomallei*, including both the outer core and O-antigen moieties of *B. pseudomallei* lipopolysaccharide (LPS), and low membrane permeability (394).

B. mallei is susceptible to similar antibiotics as *B. pseudomallei*, although minimum inhibitory concentrations (MIC) are typically lower in *B. mallei* (390, 395-396). Similar to *B. pseudomallei*, *B. mallei* contains an Ambler class A β-lactamase (*penA*), resulting in β-lactam resistance patterns similar to those observed in *B. pseudomallei* (371, 397). Furthermore, *B. mallei* is also resistant to cationic peptides such as polymyxin B (9). In contrast to *B. pseudomallei*, aminoglycosides and macrolides are effective against *B. mallei* (390, 395-396, 398). This susceptibility is likely due to a 50 kb deletion on chromosome 1 of *B. mallei* which corresponds to the location of the AmrAB-OprA efflux pump in *B. pseudomallei* (397, 399). Evidence for the effectiveness of aminoglycosides comes from the successful treatment of a human case of glanders with streptomycin (159).

In addition to the report of successful treatment of human glanders with streptomycin, additional antibiotics used in human glanders include sulfadiazine which successfully treated 6 patients following a laboratory exposure in 1944, and sulfamides in a French report (9, 43, 160). A more recent exposure in 2001 treatment with imipenem and DOX resulted in recovery, although an extended course of antibiotics was needed to clear the infection in this case (158).

In summary, *B. pseudomallei* contains multiple intrinsic mechanisms for antibiotic resistance. Treatment with i.v. ceftazidime or meropenem for 2 weeks followed by 3-6 months of oral TMP/SMX is the current recommended treatment. The susceptibility pattern of *B. mallei* is similar to that of *B. pseudomallei*, although aminoglycosides or macrolides are also effective treatment options. Both pathogens require an extended course of treatment to avoid relapse. In the case of accidental laboratory exposure to *B. pseudomallei* or *B. mallei*, treatment with

TMP/SMX for 3 weeks is recommended as a prophylactic treatment, while exposure resulting in active disease should be treated as described above for clinical cases of melioidosis (400).

1.6 Bacterial genetics and virulence factors.

1.6(1) Bacterial genetics.

Genomic sequences are now available for *B. mallei* strain ATCC23344 (397), *B. pseudomallei* strain K96243 (362), and the avirulent soil bacterium *B. thailandensis* strain E264 (401). These studies have revealed that all of these species contain two circular chromosomes of varying size (Chr. 1/Chr. 2): *B. mallei*: (3,510,148 bp/ 2,325,379 bp) (5.84 Mbp) and 5,535 predicted open reading frames (ORFs); *B. pseudomallei* (4,074,542 bp / 3,173,005 bp) (7.25 Mbp) and 5,855 predicted ORFs; *B. thailandensis* (3,809,201bp /2,914,771 bp) (6.72 Mbp) and 5,645 predicted ORFs. In both *B. mallei* and *B. pseudomallei* chromosome 1 contains most of the genes for basic biosynthesis, while chromosome 2 is more variable and contains more virulence genes. Analysis of both the *B. mallei* and *B. pseudomallei* genomes reveals the high potential for genetic alteration in both species. For example, in *B. mallei* a large number of insertion sequences are thought to be responsible for genome reduction from *B. pseudomallei*. *B. pseudomallei* contains about 16 genomic islands which are thought to allow for genetic mutations. In addition, both *B. mallei* and *B. pseudomallei* contain a large number of simple sequence repeats which provide the potential for slight genetic mutations, potentially resulting in phase variation and slight alteration of protein products (362, 397).

Subsequent experiments have confirmed the potential for genetic variation and have demonstrated that genetic variants occur during human infection with either *B. mallei* or *B. pseudomallei*. For instance, in *B. mallei* studies have shown that in blood and liver isolates from

a single human glanders infection were genetically distinct (354). In melioidosis patients, variable number tandem repeat (VNTR) analysis was used to demonstrate as many as 10 unique genotypes in one patient (402). In a more recent study of acute melioidosis multiple genetic variants were isolated from patients, and results suggested that there may be micro-evolution of strains within certain tissues (403). In addition there appears to be a "core" and "accessory" genome in *B. pseudomallei*. Transcripts from multiple *B. pseudomallei* strains were analyzed and revealed that about 86% of the genome is expressed in all strains, while 14% of the genome is variable (404). Therefore multiple genetic variants can develop during human glanders or melioidosis, and variants may arise due to the ability of *B. pseudomallei* to alter the "accessory" genome without affecting bacterial fitness.

Evolutionary studies in *B. pseudomallei* have demonstrated that *B. pseudomallei* likely originated in Australia. Introduction of a single *B. pseudomallei* strain into S.E. Asia from Australia is thought to have happened via an ice bridge. All S.E. Asian strains have subsequently evolved from this one strain, resulting in the ability to cluster S.E. Asian and Australian isolates by analysis of SNPs (405). Evolutionary studies have also demonstrated that *B. mallei* appears to have evolved from a clone of *B. pseudomallei* (32, 406-408), and has undergone only slight alteration since divergence from *B. pseudomallei* (122, 406).

1.6(2) Virulence factors.

A number of virulence factors have been identified in both *B. mallei* and *B. pseudomallei*, and both pathogens are facultative intracellular bacteria (407). Following infection bacteria escape from the endosomal compartment, polymerize host cell actin, and spread to neighboring cells resulting in formation of multinucleated giant cells (MNGC). A number of virulence

factors have been identified, although the presence of nearly all of these virulence factors in the avirulent bacterium *B. thailandensis* complicates analysis of virulence factors **(Table 1.3)**. The role of virulence factors in animal models of infection will be discussed. Although, a number of virulence and mechanistic experiments have been performed in tissue culture, including: capsule (409), type IV pili (410), flagellin (411-412), type III secretion systems (T3SS) (413-415), *Burkholderia* intracellular motility protein A (BimA) (416-418) and type VI secretion systems (T6SS) (417, 419-421).

All three *Burkholderia* species are known to invade a number of phagocytic and non-phagocytic cells (264, 418). The type IV pili of *Burkholderia* spp. may be involved with cell adhesion, and type IV pili mutants are attenuated in animal models (410). Flagellin are known to enhance adherence in other bacterial models may be necessary for cell adhesion following *Burkholderia* infection (451). Conflicting results in animal models have been obtained, while flagellin (*fliC*) mutants were avirulent following i.n. or i.p. infection of mice (411), no difference in virulence was observed following i.p. infection of diabetic rats or hamsters (447).

After entering the cell *Burkholderia* spp. all escape from the endosomal vesicle following expression of the *Burkholderia* secretion apparatus (Bsa) T3SS, which is similar to T3SS of *Salmonella* spp. and *Shigella* spp. (407, 452). Both *B. pseudomallei* and *B. mallei* T3SS mutants are attenuated in mice and hamsters (337, 436-437), and *B. thailandensis* mutants are also attenuated in mice (413).

T3SS translocators (BipB, BipC and BipD; Bip = *Burkholderia* invasion protein) and effectors (BopA, BopC and BopE; Bop = *Burkholderia* outer proteins) in *Burkholderia* spp. were determined by their homology to those of *Salmonella* spp. and *Shigella* spp. (407, 453-454). Translocators are thought to localize to the tip of the needle and form a pore in the host cell

Table 1.4. Virulence factors in *B. pseudomallei*, *B. mallei,* and *B. thailandensis*
Adapted from (407)

	Necessary for virulence in animal models[a]					Presence	
Virulence factor	***B. p.***	**Reference**	***B. m.***	**Reference**		***B.t.***	**Reference**
Capsule	Yes	(339, 409, 422-423)	Yes	(424-425)		No / Rare	(25-26, 339, 408, 426-428)
Quorum sensing	Yes	(429-430)	Yes	(431)		Yes	(432-433)
LPS O-Antigen	Yes	(338, 409, 434)	Yes	(434)		Yes	(435)
T3SS	Yes	(337, 436)	Yes	(437)		Yes	(401, 413, 438)
T6SS	Yes	(420-421)	Yes	(335)		Yes	(335, 439)
BimA	Yes	(440)	Yes	(335)		Yes	(441-442)
MviN	Yes[b]	(443)	Unknown[f]	(407)		Yes	(401)
Phospholipase C	Yes	(444)	Unknown[g]	(397)		Yes	(401, 428)
Type IV pili	Minor/No[c]	(410)	Unknown	(407)		Yes	(428)
CHBP	Yes	(445)	NE	(445)		No	(445)
LfpA	Yes	(446)	NE	(446)		No	(446)
Flagellin	Yes/No[d]	(411-412, 447)	NE	(397)		Yes	(412)
Type III OPS	Minor[e]	(448)	NE	(397)		Yes	(448-449)
Type IV OPS	Minor[e]	(448)	NE	(397)		Yes	(448-449)
T2SS	Minor	(450)	NE	(397)		Yes[h]	

[a] Animal models used: *B.p.* = Hamster, mouse guinea pig, infant diabetic rat; *B.m.* = Hamster, mouse and horse.
[b] *mviN* mutants also had an *in vitro* growth defect.
[c] *pilA* mutants were attenuated following i.n., but not i.p. infection of mice.
[d] Attenuated in mice, but not hamsters or diabetic rats.
[e] Increased mean time to death.
[f] *B. mallei* locus: BMA0378 (407).
[g] Plc-3 was not upregulated in a *B. mallei* hamster model, and virulence was not tested.
[h] *B. thailandensis* E264 loci BTH_I0007 to BTH_I0018 (Similar to *B.p.* genes described in (450)).

NE = Not Expressed in *B. mallei*

Abbreviations: ***B.p.*** *B. pseudomallei*; ***B.m.*** *B. mallei*; ***B.t.*** *B. thailandensis*; **T3SS** Type III Secretion System; **T6SS** Type VI Secretion System; **BimA** *Burkholderia* intracellular motility protein A; **CHBP** Cif homologue in *Burkholderia pseudomallei* (cif = cycle inhibitory factor); **LfpA** Lactonase family protein A; **LPS** Lipopolysaccharide; **OPS** outer polysaccharide; **T2SS** Type II Secretion System.

cytoplasm allowing for secretion of effector proteins into the host cell cytoplasm (407). Effector functions include avoidance of autophagy mediated by BopA, and reduced entry observed in bacteria lacking BopC or BopE (452, 455-457). In animal models, mutant *B. pseudomallei* strains unable to produce BopA, BopB or BopE were all still virulent, but mutant bacteria were attenuated compared to WT *B. pseudomallei* following i.p. infection of mice. In addition, a *B. mallei* strain lacking BopA was attenuated following i.n. infection of mice (436, 458).

Another potential effector of the T3SS may be the cycle inhibitor factor (Cif) homologue in *B. pseudomallei* (CHBP). CHBP is a homologue to the Cif protein from *E. coli* (Cif_{Ec}) which modulates the eukaryotic cell cycle (459). Recent studies have shown that expression of CHBP in *E. coli* can disrupt the eukaryotic cell cycle halting the cell at G2 / M phase (445, 460).

Following escape from the endosome all three *Burkholderia* spp. are capable of polymerizing host cell actin by expressing BimA (441-442, 461). Actin polymerization results in mobilization within the cell, similar to infection with *L. monocytogenes*, *Shigella flexneri*, enteropathogenic *E. coli*, and *Rickettsia* spp. (462). *bimA*$^{-}$ strains remained virulent in a hamster model of *B. mallei* (335), but were avirulent in a mouse model of *B. pseudomallei* (440). Interestingly, recent studies suggest that BimA independent intracellular mobility occurs in *B. thailandensis* following expression of a second flagellar system (*fliC2*) (417).

Another virulence factor expressed following escape from the endosome is the T6SS. T6SSs are a newly discovered secretion system used by many pathogens including *Vibrio cholerae*, *P. aeruginosa*, *Yersinia pestis*, *Francisella tularensis*, *Salmonella typhimurium*, *E. coli* and others, to secrete effector proteins across the bacterial membrane (463). T6SS mutants of both *B. mallei* and *B. pseudomallei* are avirulent in an i.n. mouse model, or an i.p. hamster model

(335, 420-421). Effector molecules of the T6SS are still unknown, and further studies will be needed to identify effectors.

In addition to the virulence factors needed for cell to cell spread there are a number of additional factors involved with virulence. One of these is the capsular polysaccharide which is present in *B. mallei* and *B. pseudomallei* but absent from *B. thailandensis* (25, 339, 408, 426, 428). Capsule is involved with adhesion and evasion of innate and adaptive immunity in bacterial infections, including *Y. pestis*, *K. pneumoniae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *S. pyogenes* (464-465). The capsular polysaccharide in *B. pseudomallei* was determined to be a 1,2-linked 2-O-acetyl-6-deoxy-β-heptane (466). In *B. mallei* and *B. pseudomallei* capsule is known to mediate resistance to complement due to decreased deposition of C3b (467). In addition both *B. mallei* and *B. pseudomallei* capsule mutants are attenuated in multiple animal models including the mouse, hamster and horse (339, 409, 422-425). Furthermore, in a comparative study capsule mutants were more attenuated than LPS or flagellin mutants (409). Although the capsular polysaccharide of *B. pseudomallei* has been shown to be absent in *B. thailandensis*, a recent study demonstrated that 5 of 92 *B. thailandensis* strains could express capsular polysaccharide (427). Interestingly 3 of the 5 capsule expressing strains were isolated from mammalian hosts (2 human, 1 horse). Although this suggests a subset of virulent *B. thailandensis* strains exists, none of the 5 strains were virulent in a mouse model of infection (427). Therefore additional factors are likely required for full virulence.

Another well studied virulence factor is lipopolysaccharide (LPS). The lipid A portion of LPS is immunostimulatory, and pathogens such as *Y. pestis*, *F. tularensis* and *S. enterica* express an altered lipid A molecule allowing for immune evasion (468-469). The lipid A molecule of both *B. pseudomallei* and *B. mallei* is known to be recognized by Toll-like receptors (TLR),

although some studies suggest that *B. pseudomallei* LPS is recognized by TLR2 rather than TLR4 (470-472). LPS from both bacteria is known to protect against complement mediated killing (338, 434). In addition both *B. mallei* and *B. pseudomallei* LPS mutants are avirulent in mice, hamsters, guinea pigs, and infant diabetic mice (338, 409, 434, 473). Differences in lipid A structure between *B. pseudomallei* and *B. thailandensis* were shown to result in an increased stimulatory capability of *B. thailandensis* LPS (435). Therefore in addition to protecting against complement mediated killing, LPS of *B. mallei* and *B. pseudomallei* may allow the bacteria to avoid immune detection.

Quorum sensing is a system which allows bacterial populations to control gene expression based on population density, and is involved in multiple processes including biofilm formation. The activation of *luxI* genes results in the production of acyl-homoserine lactone (AHL) signaling molecules, which bind to the luxR transcription regulator and result in gene activation (474). *B. pseudomallei* contains three *luxI* homologues that can produce 7 AHLs, and has five *luxR* homologues to detect AHLs. *B. mallei* contains two *luxI* homologues that can produce 4 AHLs, and has four *luxR* homologues (407). Mutations in the *luxI* or *luxR* genes results in attenuation in both B. *mallei* or *B. pseudomallei* models of hamster and mouse infection (429-431), although the genes that are controlled by quorum sensing systems are still unknown (407).

B. pseudomallei is also known to modulate the immune response. The *tssM* gene encodes a putative ubiquitin-specific protease and was recently shown to be a bacterial factor capable of suppressing NF-κB expression and type I IFN pathways, resulting in an attenuated immune response. Interestingly a *tssM* mutant bacterium was hypervirulent compared to WT bacteria, suggesting an overactive immune response may be detrimental (475). Other studies have shown

that *B. pseudomallei* can activate the suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homologue 2-containing protein (CIS), resulting in reduced IFN-γ production in RAW macrophages (476-477). Recent studies have demonstrated that activation of SOCS3 is performed through the intracellular nucleotide binding oligomerization domain-containing protein 2 (NOD2) (478). Further studies will be needed to determine the effects of SOCS3 induction on virulence *in vivo*.

Additional virulence factors include lactonase family protein A (LfpA), phospholipase C, MviN, Type-III outer polysaccharide (OPS) and Type IV OPS, and the type II secretion system (T2SS) (General Secretory Pathway) (407, 448, 450). All of these virulence factors have only a modest effect on virulence in animal models, and most of them are not expressed in *B. mallei* (397, 407).

Interestingly the only major virulence factor identified to this point that is present in *B. mallei* and *B. pseudomallei* but absent from *B. thailandensis* is the capsule polysaccharide. Recent studies have demonstrated that even this virulence factor is occasionally identified in avirulent *B. thailandensis* strains, suggesting additional factors are necessary for virulence. Both CHBP and LfpA are absent from *B. thailandensis*, and may account for some of the increased virulence seen in *B. pseudomallei*. A number of virulence factors present in *B. pseudomallei* are not expressed in *B. mallei* including flagellin, T2SS, CHBP, LfpA, and both Type III and IV OPS. Therefore although disease symptoms are similar in glanders and melioidosis patients, there may be differences in how each bacterium causes disease.

1.7 Immune response to *Burkholderia* infection.

1.7(1) Innate immunity.

Effective innate immunity against *B. mallei* or *B. pseudomallei* is critical given the potential for acute disease following infection, while adaptive immunity is essential to prevent the development of chronic disease. The mouse model of melioidosis and glanders has been used to identify cell types required to protect against acute disease. For instance, depletion of macrophages and monocytes with clodronate, or carrageenan has demonstrated that macrophages and monocytes are important in response to acute disease (479-480).

In contrast, other studies have demonstrated an essential role for neutrophils, but not monocytes, in response to *B. pseudomallei* infection (481). In this study the necessity of neutrophils was investigated following neutrophil depletion using the anti-Gr-1 antibody, which also resulted in a transient depletion of monocytes and $CD8^+$ T cells. Neutrophils were also shown to be necessary following *B. mallei* infection, although this study also used the Gr-1 antibody to deplete neutrophils (482). Because the Gr-1 antibody depletes both neutrophils and monocytes, these studies are difficult to interpret.

Recognition of pathogenic infection by the innate immune system is performed following ligation of pattern recognition receptors (PRR) which have evolved to identify conserved pathogen associated molecular patterns (PAMP) unique to pathogenic organisms. Toll-like receptors (TLR) are PRRs located at the surface of the cell or in endosomal compartments. Individual TLRs recognize molecules with unique characteristics from viral or bacterial infections, and the TLRs (ligand) involved with gram-negative bacterial infection include TLR2 (lipoteichoic acids), TLR4 (LPS), TLR5 (flagellin) and TLR 9 (unmethylated CpG DNA) (483). Following TLR ligation a signal cascade is initiated resulting in cellular activation and cytokine

production. A common adapter protein used to signal following ligation of all gram-negative TLRs is myeloid differentiation factor 88 (MyD88), although TLR4 can also signal through the TIR-domain-containing adapter-inducing interferon-β (TRIF) adapter protein (483). Therefore mice lacking MyD88 are unable to signal through multiple TLRs, while mice lacking individual TLRs allow for more precise investigation of TLR signaling.

Previous studies have shown that mice lacking MyD88 had reduced neutrophil recruitment and TNF-α production and were susceptible to *B. pseudomallei* or *B. thailandensis* infection (484-485). In contrast, MyD88$^{-/-}$ mice were protected against infection with *B. cepacia*, and the attenuated inflammatory response observed in MyD88$^{-/-}$ mice was shown to be protective (486). Therefore a wide range of responses have been observed in MyD88$^{-/-}$ mice following *Burkholderia* spp. infection, and the role of MyD88 following *B. mallei* infection has not been investigated.

Individual TLRs required for protection against *Burkholderia* infection have not been identified. Following respiratory *B. pseudomallei* infection a detrimental effect of TLR2 but not TLR4 signaling was observed, and preliminary studies suggest that TL9 is not necessary (470, 485). Following respiratory infection with the avirulent *B. thailandensis* no role for TLR4 or TLR5 was observed (484). Therefore the TLR necessary in response to *Burkholderia* infection is not known.

A different set of PRRs is used to detect pathogens following invasion into the host cell cytoplasm. The nucleotide binding oligomerization domain (NOD) proteins recognize cytosolic infection (487). Bacterial ligands for NOD receptors include breakdown products of peptidoglycan such as muramyl dipeptide (MDP), as well as LPS, flagellin, and components of the T3SS (487-488). Ligation of NOD receptors intracellular signaling results in the assembly of

the inflammasome complex, production of proinflammatory cytokines, and activation of caspase-1. Caspase-1 is necessary for processing of pre-IL-1β and pre-IL-18 into their mature forms, and IL-18 is important for IFN-γ production (487, 489). Mice lacking caspase-1 are more susceptible to *B. pseudomallei* infection, and caspase-$1^{-/-}$ mice were unable to produce IL-18 or IFN-γ (490).

Activation of PRRs results in cellular activation and cytokine production. Investigation of cytokine production in human melioidosis has shown that IFN-γ, IL-8, IL-6, IL-10 and TNF-α are all produced following infection (491-493). Although IFN-γ and TNF-α have been associated with increased risk of mortality, only IL-6 and IL-10 were statistical predictors of lethal disease (492, 494-495). While these studies provide valuable insight into human infection, patients are often very sick upon admission to the hospital, and the increased cytokine production may be associated with high bacterial burden in these patients. Therefore the mouse model has been used to investigate cytokines and chemokines produced following *B. pseudomallei* or *B. mallei* infection, and what factors are required for protection.

Studies have shown that following i.v. or i.p. infection of mice with *B. pseudomallei* upregulation of cytokines including IL-1β, TNF-α, IL-6, as well as the colony simulating factors and chemokines G-CSF, M-CSF, GM-CSF, IP-10, Mig, RANTES, MCP-1, KC and MIP-2 is observed (496-497). Following i.p. *B. mallei* infection increased IL-6, IFN-γ, IL-12p35, IL-27 and MCP-1 were observed (489). Subsequent studies have investigated the necessity of a number of these factors for protection against acute disease.

Genetic knockout animals or antibody depletion in mouse models of glanders and/or melioidosis have been used to demonstrate the essential role of IFN-γ (489, 498-499), TNF-α (480, 498) , IL-12 (489, 498-499) and IL-18 (499-501). While each of these cytokines are necessary for protection, mice lacking IFN-γ are extremely susceptible to infection, succumbing

just 2 days after infection. Studies investigating cytokines necessary for IFN-γ production following *B. mallei* infection have demonstrated that IL-12 is essential, IL-18 enhanced this response, and that IL-27 was not necessary (489).

The cellular sources of cytokine production have also been investigated. In response to i.n. *B. pseudomallei* infection of mice *ex vivo* analysis demonstrated that natural killer (NK) cells and T cells were producing IFN-γ, and monocytes were the major source of TNF-α (481). Further studies have demonstrated that the IFN-γ production by NK cells and T cells is functionally redundant and only IFN-γ production from a population of $F4/80^{+}$ macrophages is necessary for protection against acute infection (499). Following *B. mallei* stimulation of splenocytes IFN-γ was produced mainly by NK cells but also by T cells and γδT cells (489). Therefore IFN-γ production is critical following *B. pseudomallei* or *B. mallei* infection; although only a small amount of IFN-γ is required for protection.

The production of nitric oxide (NO) or reactive oxygen species (ROS) following IFN-γ stimulation is an important mechanism of killing in a number of bacterial pathogens including *L. monocytogenes* (502), *M. tuberculosis* (503-504) and *S. typhimurium* (505). A number of studies in mouse macrophage cell lines have demonstrated that IFN-γ stimulated NO production is critical for bacterial killing following both *B. pseudomallei* and *B. mallei* (506-510). In contrast to *in vitro* studies, i.v. infection of $iNOS^{-/-}$ ($NOS2^{-/-}$) mice on the C57BL/6 background were protected against infection, while mice lacking NADPH oxidase mice ($gp91phox^{-/-}$) were more susceptible to infection (479). Further characterization of the role of NO in mice following i.v. *B. pseudomallei* infection has demonstrated that NO is critical for protection in BALB/c mice, but is detrimental to C57BL/6 mice (511). Following i.p. infection with *B. mallei*, $iNOS^{-/-}$ mice on the C57BL/6 background were more susceptible to chronic disease, although no difference in

acute disease was observed (482). Therefore differences in the requirement of NO for protection appear to vary between mouse strains, infection with *B. mallei* or *B. pseudomallei*, and may also be dependent on the route of infection.

Although the production of proinflammatory cytokines is known to be important for protection against *Burkholderia* infection, a number of studies have shown that overproduction of cytokines can be detrimental. For instance, although TNF-$\alpha^{-/-}$ mice are more susceptible to disease, the role of TNF-α receptors (TNF-αR) was also investigated (480). Previous studies have shown that TNF-αR1 mediates the majority of proinflammatory responses to TNF-α, while TNF-αR2 is involved in feedback control of TNF-α necessary to prevent immune mediated damage (512). Interestingly, TNF-αR2 mice had higher mortality rates as compared to TNF-αR1 mice following *B. pseudomallei* infection, suggesting that control of TNF-α is also important to control of melioidosis (480). Protection due to reduced inflammatory responses have also been observed in $CD14^{-/-}$ and $TLR2^{-/-}$ mouse models, and in diabetic melioidosis patients taking the hypoglycemic control medication Glyburide (470, 513-514). Finally, a recent study has demonstrated that IL-1β is detrimental to infection, and may be the cytokine responsible for immune mediated pathology observed in animal models (501). Therefore, although innate immunity is critical for protection against glanders and melioidosis, overstimulation of the immune response can also be detrimental.

1.7(2) Adaptive immunity.

Studies in human patients have demonstrated that antibodies are developed in response to infection. Indeed, serology studies show that ~ 1-40% of the general populations in endemic areas possess antibodies against *B. pseudomallei* (Table 1.3). While antibodies are produced

they are not protective as patients with IHA titers as high as 1:5120 have re-developed disease (515). In contrast, some patients never develop antibodies despite having septic melioidosis (214). Still other studies have shown that the type of antibody response is critical to the protective effect of antibodies. In this study antibody responses to a certain type of LPS (LPS II) were associated with survival (516).

In mouse models the role of antibody and B cells has been investigated with genetic knockout mice. For instance following i.p. infection with *B. pseudomallei* no difference was observed between WT and $\mu MT^{-/-}$ mice (499). Furthermore, following i.p. infection with *B. mallei* $\mu MT^{-/-}$ and WT mice were equally susceptible to infection (482). Therefore, although B-cells are stimulated by *B. pseudomallei* infection the response is highly variable, and mouse studies suggest that B cells are not necessary for protection.

Cell mediated immunity is also stimulated following exposure to *B. pseudomallei*. Studies in melioidosis patients have shown that T cells are stimulated in response to infection, and that these cells are functional as assessed by the ability of T cells to replicate, increase CD69 expression and produce IFN-γ, following re-stimulation with *B. pseudomallei* (517-518). Recent studies have demonstrated that melioidosis patients develop both $CD4^+$ and $CD8^+$ T cells which have a terminally differentiated effector memory phenotype expressing CD45RA but not CCR7 (519). Although in this study T cell stimulation correlated with antibody production (519), other studies have shown that T cell activation does not always correlate with antibody titer (231, 518). Therefore, similar to antibody production, there can be variability in T cell stimulation in human melioidosis.

In mouse models of infection the role of $CD4^+$ and $CD8^+$ T cells has been investigated by depletion of $CD4^+$ and/or $CD8^+$ T cells following i.p. *B. pseudomallei* infection. These studies

demonstrated that $CD4^+$ T cells and to a lesser extent $CD8^+$ T cells are required for protection, and that depletion of both $CD4^+$ and $CD8^+$ resulted in a more severe phenotype as compared to depletion of either cell type alone (499). $RAG^{-/-}$ (recombination activating gene) mice were significantly more susceptible as compared to WT mice, succumbing at day 10-20 while WT mice succumb from days 20-40. Similar to human studies, T cells from mice infected with *B. pseudomallei* appear to be functionally intact. Both $CD4^+$ and $CD8^+$ splenic T cells re-stimulated with *B. pseudomallei* were capable of producing IFN-γ (499). Following i.p. *B. mallei* infection, depletion of $CD4^+$ and $CD8^+$ T cells also increases susceptibility to infection with depleted mice dyeing 20-30 days after infection. While there was no significant difference of depleting $CD4^+$ or $CD8^+$ T cells alone, depletion of both $CD4^+$ and $CD8^+$ T cells resulted in a significant increase in susceptibility (482).

Therefore these studies demonstrate that both humoral and cell mediated immunity develop in melioidosis patients, and in mice infected with *B. pseudomallei* or *B. mallei*. Mouse experiments suggest that cell mediated immunity is likely more important than antibody production in response to *Burkholderia* infection. Interestingly, despite the importance of cell mediated immunity, there is no association between the development of HIV/AIDS and melioidosis (520-521). This is in contrast to other bacterial pathogens where $CD4^+$ T cells are important for protection, such as *M. tuberculosis*, where HIV/AIDS is a major risk factor for disease development (522). Moreover, chronic recurrent melioidosis develops in some patients despite an apparently functional T cell response. Similar results have been observed in glanders where horses and dogs which recovered from infection were not protected against future disease (8, 15, 352, 523). Further studies will be needed to determine why both humoral and cell mediated immunity fail to clear *B. pseudomallei* or *B. mallei*.

1.7(3) Immunotherapeutics.

Because *B. pseudomallei* and *B. mallei* are resistant to most antibiotics, immune based therapeutics provide an attractive treatment option (144). A limited number of immune based therapeutics have been tested in *B. pseudomallei* or *B. mallei*. While animal studies have suggested that over production of pro-inflammatory cytokines may be responsible for immune mediated pathology; studies testing corticosteroid treatment in mice have shown that corticosteroid treatment does not improve disease outcome, and may be detrimental (524).

Treatment with recombinant human activated protein C (rhAPC) has also been used to treat melioidosis. In a case report of melioidosis in a patient with severe disease following a typhoon was successfully treated with rhAPC and meropenem (525). In addition, low protein C is also a predictor of poor outcome in melioidosis patients (526).

Studies have investigated the ability of unmethylated CpG DNA to provide protection against *B. pseudomallei* infection. Treatment with CpG DNA delivered intramuscularly (i.m.) 2-10 days prior to an i.p. infection induced IL-12 and IFN-γ production, and provided up to 90% protection against *B. pseudomallei* (527). More recent studies have shown that the human stimulatory CpG 10103 administered either i.p. or i.n. is also protective against chronic melioidosis (528). In an aerosol *B. mallei* model treatment with CpG DNA i.p. 24 or 48 hours prior to infection, but not concurrently or 24 hours after infection, was shown to protect mice against a chronic form of glanders. Similar to *B. pseudomallei* studies, mice treated with CpG DNA had increased production of IL-12, IFN-γ, and IL-6 (529).

1.7(4) Vaccines.

Passive immunization experiments have been performed with both *B. pseudomallei* and *B. mallei*. A number of studies have investigated the ability of antibodies to protect against glanders (15, 530-531). For instance, an early study reported treating people with antibodies developed by injecting horses with mallein. Antibodies delivered after infection appeared to cure three human cases, although the lack of disease progression was the only measure of "cure" and the patients may have been chronically infected (531). In contrast other studies where serum from goats treated with mallein was transferred to guinea pigs no protection was observed (15, 346, 532).

In more recent experiments, a number of studies have shown that prophylactic treatment with antibodies developed against whole bacteria, flagellin or capsule can protect mice against *B. mallei* (533) and *B. pseudomallei* (534-536). Although protective when administered prior to infection, antibodies delivered 18 hours after infection were not protective (533). A screen of monoclonal antibodies has demonstrated that antibodies recognizing capsule or LPS are protective, while antibodies recognizing antigens expressed within the bacterium are not protective (537). Therefore although antibody prophylaxis can protect against infection, therapeutic use of antibodies does not appear to be effective.

Cell mediated immunity is also necessary for protection against *Burkholderia* infection. Although early experiments in glanders infection highlight difficulties associated with vaccine development against glanders or melioidosis. For example a number of studies demonstrated that vaccination did not protect horses against infection (4, 15, 300, 312, 538-541), and that recovery from glanders did not protect animals from re-infection (15, 352, 523).

More recent vaccine trials have included heat killed bacteria, live attenuated strains, outer membrane vesicles, antigen pulsed dendritic cells, as well as protein, polysaccharide and lipid based vaccines (542-550). Although all vaccines provide protection against acute disease, the majority of mice ultimately develop chronic disease.

Strategies investigating enhancing vaccine efficacy with adjuvants including cationic liposome DNA complexes (CLDC) or CpG DNA have shown some success (551-552). These studies have shown increased efficacy when antigens are combined with an adjuvant suggesting adjuvant development will be important for *Burkholderia* vaccines. Studies in *B. mallei* have also shown that IL-12 treatment in combination with a heat killed vaccine provides improved protection (553).

B. mallei studies have been performed to determine what components of immunity are required for vaccine protection. In this study mice were vaccinated i.p. with heat killed *B. mallei*, and then challenged i.p. two weeks later with *B. mallei* (554). Genetic knockout and antibody depletion studies demonstrate that, B cell deficient mice were the most susceptible followed by mice lacking $CD4^+$ and $CD8^+$ T cells. Therefore, in contrast to natural infection, B cells appear to be important for vaccine efficacy, and similar to natural infection T cells are also important. These results demonstrate that an effective vaccine will likely need to stimulate both humoral and cell mediated immunity. A number of studies have screened both *B. mallei* and *B. pseudomallei* for protective antigens, and these antigens may improve the ability of future vaccines to induce sterilizing immunity (555-557).

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CHAPTER 2.

RATIONALE FOR RESEARCH AND SPECIFIC AIMS

2.1 Research overview.

The goals of the research described in this dissertation were to gain a better understanding of the pathogenesis of glanders and melioidosis in order to improve treatment options. Chapters 3 and 4 describe investigations of protective innate immune responses following respiratory infection with *Burkholderia mallei*. Chapter 3 describes the necessity of monocytes and their role in cytokine production; and in chapter 4 the effect of myeloid differentiation factor 88 (MyD88) dependent Toll-like receptor (TLR) signaling on cellular recruitment and cytokine production is investigated. Chapter 5 assesses the ability of an immune based therapeutic to protect against respiratory infection with *B. mallei* or *B. pseudomallei*, and identifies correlates of protection. Chapter 6 focuses on chronic melioidosis, and examines the ability of *B. pseudomallei* to persistently colonize the gastrointestinal (GI) tract and disseminate to systemic organs.

2.2 Specific aim 1 (Chapter 3).

The goal of these experiments was to investigate the role of monocytes in response to respiratory *B. mallei* infection. Following bacterial infection, monocytes are mobilized from the bone marrow, migrate to the site of infection and differentiate into dendritic cells or macrophages (1). Monocytes and monocyte derived dendritic cells play an important role in production of key innate immune effectors such as IL-12, TNF-α, and nitric oxide (2-3). In addition, monocytes are necessary for control of a number of intracellular pathogens, including *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Toxoplasma gondii*, and *Cryptococcus neoformans* (2, 4-7). Despite the importance of monocytes in response to bacterial infections the role of monocytes in response to gram-negative infection, specifically *B. mallei* infection, has not been investigated. Therefore, the hypothesis for specific aim 1 was that monocytes are required for protection against respiratory *B. mallei* infection. A mouse model of glanders was used to test this hypothesis by investigating the susceptibility of monocyte deficient mice to respiratory *B. mallei* infection. Cellular recruitment to the lung was investigated, and production of key protective cytokines was measured.

2.3 Specific aim 2 (Chapter 4).

The purpose of specific aim 2 was to determine if signaling through the MyD88 adapter molecule is necessary in protective immunity following respiratory *B. mallei* infection. The MyD88 adapter molecule is a common signaling molecule involved with TLR signaling following recognition of pathogen associated molecular patterns (PAMP) on invading pathogens, resulting in initiation of a proinflammatory response (8). MyD88 signaling is known to control recruitment of neutrophils and monocytes (9-11), as well as the production of multiple cytokines

and chemokines including TNF-α, IL-1β, IL-6, MIP, KC, and IFN-γ (12-19). The attenuated pro-inflammatory response observed in MyD88$^{-/-}$ mice often results in increased susceptibility to bacterial infections including *B. pseudomallei*, but is protective following infection with other bacteria such as *B. cenocepacia* (9, 20-21). Therefore the role of MyD88 signaling in *B. mallei* infection was not clear. The hypothesis was that MyD88 signaling would be necessary for protection against respiratory *B. mallei* infection. To test this hypothesis the susceptibility of MyD88$^{-/-}$ mice to *B. mallei* infection was tested. The necessity of MyD88 signaling for chemokine production and cellular recruitment, as well as production of protective cytokines was investigated.

2.4 Specific aim 3 (Chapter 5).

This chapter focuses on potential immune based therapeutics for glanders and melioidosis. Antibiotic treatment of *B. mallei* and *B. pseudomallei* requires an extended course of treatment with multiple antibiotics, and an 11-17% failure rate occurs in melioidosis patients (22-23). Therefore immune based therapy is an attractive alternative to antibiotic therapy. Cationic liposome DNA complexes (CLDC) are potent stimulators of innate immunity and stimulate high levels of IFN-γ production; a critical protective cytokine in response to *Burkholderia* infection (24-26). The hypothesis was that CLDC would protect against respiratory infection with *B. mallei* and *B. pseudomallei*. A murine model of glanders and melioidosis was used to test this hypothesis. Mucosal administration of CLDC at varying doses delivered both prophylactically and therapeutically was tested.

2.5 Specific aim 4 (Chapter 6).

In specific aim 4 the chronic phase of melioidosis was investigated and studies focused on potential reservoirs of bacterial persistence during asymptomatic phases of disease. While acute melioidosis has been well studied, less work has been done on chronic disease. Melioidosis has been reported to develop 16, 26 and even 62 years following exposure, and chronic infection is recognized as a major complication associated with melioidosis (22, 27-30). Despite multiple reports of chronic disease, the site of bacterial persistence leading to recrudescence is not known. *B. pseudomallei* is an environmental bacterium present in soil and surface water in endemic areas, and infection is thought to occur following inoculation, inhalation or ingestion (22, 31). Therefore the hypothesis for specific aim 4 is that oral infection with *B. pseudomallei* will result in persistent gastrointestinal (GI) colonization and dissemination to systemic organs. To test this hypothesis mice were infected orally with sub-lethal doses of *B. pseudomallei* and the development of chronic infection was monitored. The ability of *B. pseudomallei* to persistently colonize GI organs, and to be shed in the feces were monitored. In addition, studies were performed to localize *B. pseudomallei* within the GI tract.

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CHAPTER 3.

CRITICAL PROTECTIVE ROLE FOR MCP-1 IN PNEUMONIC *BURKHOLDERIA MALLEI* INFECTION

This chapter reports on the role that monocytes play in protection and cytokine production following respiratory *B. mallei* infection. I acknowledge the contribution of Dr. Helle Bielefeldt-Ohmann for analysis of histopathology and for capturing micro photos, Dr. Ryan Troyer for RT-PCR analysis of cytokine production, Abby Jones for cytokine analysis in MCP-$1^{-/-}$ mice, and Dr. Katie Propst for LD_{50} determination of *B. pseudomallei* in BALB/c mice.

3.1 Summary.

Burkholderia mallei is a gram-negative bacterial pathogen of domestic equidae and humans that can cause severe infections and be used as a bioweapon. Little is known regarding the role of chemokines and early cellular immune responses in protective immune responses to pulmonary infection with *B. mallei*. The chemokine monocyte chemoattractant protein-1 (MCP-1) and its receptor chemokine receptor 2 (CCR2) regulate monocyte mobilization and recruitment, but the role of MCP-1 in protective immunity to acute pneumonia caused by gram-negative bacteria such as *B. mallei* has not been previously assessed. Therefore, a mouse model of pneumonic *B. mallei* infection was used to investigate the role of MCP-1 and CCR2. We found that both MCP-$1^{-/-}$ mice and $CCR2^{-/-}$ mice were extremely susceptible to pulmonary infection with *B. mallei*, compared to wild type (WT) C57BL/6 mice, with significantly

increased bacterial burdens and severe organ pathology in lung, spleen, and liver. Following *B. mallei* infection in CCR2$^{-/-}$ mice dendritic cell (DC) and monocyte recruitment into the lungs was significantly impaired, while neutrophil recruitment was unaffected. Depletion of monocytes and macrophages prior to infection in wild type mice significantly increased their susceptibility to infection. Moreover, production of IL-12 and IFN-γ in the lungs following *B. mallei* infection was significantly impaired in both MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice. However, treatment of CCR2$^{-/-}$ mice with rIFN-γ restored protection against an otherwise lethal challenge with *B. mallei*. Thus, we conclude that MCP-1 plays a key role in regulating cellular immunity and IFN-γ production following pneumonic infection with *B. mallei* and may also play an important role in other gram-negative pneumonias.

3.2 Introduction.

Burkholderia mallei is an important gram-negative bacterial pathogen that readily infects humans, generally following exposure to infected animals (1-7). Infection in humans can be contracted by inhalation or via infection of wounds or mucus membranes. Horses are the primary animal hosts for *B. mallei* and can develop acute infection involving the lungs and lymph nodes (glanders) or chronic cutaneous infection (farcy) (1, 7-8). Notably, *B. mallei* is highly infectious to humans and other animals when contracted by inhalation (2).

Renewed attention has recently been focused on *B. mallei* and the closely related bacterium *B. pseudomallei*. *B. pseudomallei* is endemic in parts of southeast Asia and northern Australia, where many patients are infected annually (9-11). Though *B. mallei* has been eliminated from most developed countries, it too remains endemic in some parts of the world, including the Middle East, Asia, and South America. In addition, both organisms have high potential for use as bioweapons, due to their ease of aerosol transmission, their resistance to many common antibiotics, and their ability to establish both acute and chronic infections (2, 12). Indeed, *B. mallei* has been previously weaponized and deployed in several wars (13-17). Thus, there are compelling reasons to better understand the pathogenesis of *B. mallei* infection, including early pulmonary immune responses to respiratory infection.

Both IFN-γ and TNF-α have been found to be critical for protection from *B. pseudomallei* after systemic infection (9, 18-21). In addition, neutrophils were found to play an important early protective role in the local control of pulmonary infection with *B. pseudomallei* (22). Macrophages were also found to be important for control of disseminated infection with *B. pseudomallei* following parenteral challenge (21, 23). Much less is known regarding immunological mechanisms responsible for protection from pneumonic *B. mallei* infection,

though an aerosol infection model has been developed in BALB/c mice (24). In that model, pneumonic infection elicited rapidly fatal disease characterized by acute, focal necrotizing alveolitis and pneumonia.

Since little is known regarding innate immune responses and control of *B. mallei* infection, we conducted studies to identify critical protective components of the innate immune response to pneumonic *B. mallei* infection. In particular, we focused on the role of the chemokine MCP-1 in controlling acute pulmonary infection with *B. mallei*. Previously, it was found that MCP-1 and inflammatory monocytes played an important role in controlling infection with the gram-positive bacterium *Listeria monocytogenes* (25). For example, mice lacking the MCP-1 receptor ($CCR2^{-/-}$ mice) were much more susceptible to infection with *L. monocytogenes* than wild type mice (26-27). However, the role of MCP-1 in controlling bacterial infections in general has not been systematically examined and may not be readily predictable. For example, MCP-1 was found to not be necessary for controlling low-dose pulmonary infection with *Mycobacterium tuberculosis,* but was necessary following a high dose challenge (28-31). Thus, the role of MCP-1 in controlling bacterial infections may vary depending on the pathogen, the route of exposure, and the challenge dose.

To address the role of MCP-1 and monocytes in controlling *B. mallei* infection, we used a model of pneumonic *B. mallei* infection in C57BL/6 mice. The kinetics of pulmonary infection, bacterial dissemination, and cytokine responses were investigated in wild type mice and in MCP-$1^{-/-}$ and $CCR2^{-/-}$ mice. In addition, liposomal clodronate depletion studies were used to assess the role of monocytes and macrophages in controlling pneumonia induced by *B. mallei* infection. The link between MCP-1 and production of IFN-γ was also explored. Our findings indicated that both MCP-1 and its receptor CCR2 played critical roles in generating protective immunity to

pneumonic *B. mallei* infection and that their effectiveness was mediated at least in part through regulation of IFN-γ production.

3.3 Materials and methods.

3.3(1) Mice.

C57BL/6 mice and MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice on the C57BL/6 background used in these studies were purchased from Jackson Laboratories (Bar Harbor, ME) or bred in-house. BALB/c mice were purchased from Jackson Laboratories, 129S6/SvEv and Black Swiss mice were purchased from Taconic Laboratories (Germantown, NY). All mice used in experiments were 6-8 weeks of age at the time of infection, and the mice were housed under pathogen-free conditions.

3.3(2) Bacterial strains and infections.

Burkholderia mallei strain ATCC23344 was used in these studies. This strain was kindly provided by Dr. Herbert Schweitzer, Colorado State University. Bacterial cultures were grown at 37°C with shaking at 250 RPM in Luria-Bertani broth with 4% glycerol (LB4G) (BD Biosciences, San Jose, CA) or Brucella broth with 4% glycerol (BB4G) (Remel, Lenexa, KS). Prior to use for *in vivo* infections, *B. mallei* ATCC23344 was serially passaged three times in BALB/c mice, then stocks were prepared and frozen at -80ºC. Animal passage of *B. mallei* has been reported previously to increase virulence, which we also noted in our studies (6-8, 32-35). Prior to each challenge study, fresh broth cultures of *B. mallei* were grown in BB4G broth until bacteria reached the log phase of growth, then titers were determined based on optical density values, and appropriate bacterial dilutions were prepared in sterile phosphate buffered saline (PBS). Inoculum titers for each experiment were confirmed by plating the inoculum on BB4G agar plates (Remel). Challenge studies were also done with *Burkholderia pseudomallei* (strain 1026b), which was also provided by Dr. Herbert Schweitzer. Stocks of *B. pseudomallei* were

thawed immediately prior to animal inoculation and the challenge dose was confirmed by plating after infection.

For intranasal (i.n.) infection, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) (Vedco, Saint Joseph, MO) and xylazine (10 mg/kg) (Ben Venue Labs, Bedford, OH). In preliminary experiments animals were also anesthetized by i.p. injection of 2.5% avertin (375 mg/kg 2,2,2, Tribromethyl alcohol, 2.5% Tertiary amyl alcohol in PBS) (All reagents from Sigma-Aldrich, St. Louis, MO). Mice were then infected i.n. with a total volume of 20 µl of bacterial inoculum (10µl per nostril).

All procedures involving *Burkholderia* were performed in a Biosafety Level 3 (BSL3) facility, in accordance with approved BSL3 and Select Agent protocols. All animal studies were approved by the Colorado State University Institutional Animal Care and Use Committee. In survival experiments mice were euthanized upon reaching one of the following pre-determined euthanasia endpoints: (1) hunched posture with decreased movement or response to stimuli; (2) development of respiratory distress (tachypnea, open-mouthed respirations); or (3) loss of > 15% body weight.

3.3(3) Determination of bacterial burden.

Lung, liver and spleen tissues from infected mice were collected separately and placed in 5 ml sterile PBS. Organs were homogenized using a Stomacher 80 Biomaster (Seward, Bohemia, NY) and supernatants were serially diluted in saline and plated on BB4G agar plates (Remel). Agar plates were incubated at 37°C for 48 hours and colonies were counted. The limit of detection for determination of bacterial burden in organ homogenates was 100 CFU/organ. For quantification of bacteremia, blood was collected in lithium heparin microtainer tubes (BD

Biosciences) and serial dilutions were prepared in saline and plated on BB4G agar plates. The limit of detection for determination of bacterial burden in the blood was 10 CFU/ml.

3.3(4) Collection and preparation of BAL and lung cells for flow cytometry.

Airway cells were obtained by bronchoalveolar lavage (BAL) as previously described (36-37). Immediately after euthanasia, an 18-guage catheter was inserted into the trachea of each mouse and the lungs were then insufflated with approximately 1 ml of PBS containing 1 mM EDTA (Sigma-Aldrich), which was then immediately withdrawn from the lungs by suction. This procedure was repeated 6 times for each mouse and the fluid collected was pooled. Cells were recovered from the BAL by centrifugation. Following lavage, lung digestion was performed as previously described (38). Briefly, lungs were minced and digested in Hank's buffered salt solution (HBSS) containing 2.5 mg/ml collagenase, 10U/ml DNase, and 10 μg/ml soybean trypsin inhibitor (all reagents from Sigma-Aldrich). After digestion cells were triturated through an 18 gauge needle, and passed through a 70 micron cell strainer (BD Biosciences). Red blood cells were lysed using ammonium chloride, cells were washed twice in HBSS, immunostained, and then stored on ice until analyzed. All cells were re-suspended in FACS buffer (PBS with 2% FBS and 0.05% sodium azide) before immunolabeling and flow cytometric analysis.

3.3(5) Flow cytometry.

Directly conjugated antibodies for flow cytometry were purchased from eBioscience (San Diego, CA) or BD Biosciences. The following eBioscience antibodies were used for flow cytometry staining: anti-CD11b (APC conjugated; clone M1/70), anti-CD11c (PE conjugated;

clone N418), anti-CD45 (Pacific blue conjugated; clone 30-F11), anti-NK-1.1 (biotin conjugated; clone PK136), anti-CD4 (PE conjugated; clone GK1.5), anti-CD8 (APC conjugated; clone 53-6.7). The following BD Biosciences antibodies were used: anti-Ly6C (biotin conjugated; clone AL-21), anti-Ly6G (FITC conjugated; clone 1A8). Pacific orange and Alexa 488 steptavidin conjugates were purchased from Invitrogen (Carlsbad, CA). Before staining, nonspecific antibody binding was blocked by addition of FACS block consisting of normal mouse serum (Jackson ImmunoResearch, West Grove, PA), human IgG (Jackson ImmunoResearch) and unlabeled anti-mouse CD16/32 (clone 93) (eBioscience) for 5 minutes at room temperature. Staining was performed in FACS buffer for 30 minutes on ice, followed by washing with FACS buffer. In the case of biotinylated antibodies, the streptavidin-fluorochrome conjugate was added next for 20 minutes on ice. After a final wash, the cells were fixed in 1% paraformaldehyde in PBS for 24 hours at 4^{o}C, washed once, resuspended in FACS buffer and stored at 4^{o}C until analyzed. Flow cytometry was performed using a Cyan ADP flow cytometer using Summit software (Beckman Coulter, Fullerton, CA). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR). Samples were gated on forward and side scatter characteristics for viable cells. For investigation of monocytes (Mono), neutrophils (PMN), dendritic cells (DC) and alveolar macrophages (AVM), live cells were then gated on $CD45^{+}$ cells to identify leukocytes for analysis. Alveolar macrophages were defined as $CD11c^{hi}$ and $CD11b^{-}$ cells. Dendritic cells were defined as $CD11c^{+}$ and $CD11b^{+}$. Neutrophils were defined as $CD11b^{+}$ and $Ly6G^{+}$ cells, while monocytes were defined as $CD11b^{+}$ and $Ly6C^{+}$ but $Ly6G^{-}$. For investigation of NK and T cells live cells were gated on lymphocytes based on their low forward and side scatter characteristics. NK cells were identified as $CD45^{+}$ and NK 1.1^{+} cells.

3.3(6) Assessment of cytokine and nitric oxide concentrations in BAL fluid and lung homogenate.

The BAL supernatant was separated from the cells by centrifugation, sterile filtered and frozen at -80°C prior to cytokine analysis. Lavaged lungs were harvested and placed into 4 ml PBS. Lungs were homogenized using a Stomacher 80 Biomaster (Seward). Homogenate was centrifuged at 3000g for 15 min at 4ºC, supernatants were sterile filtered and frozen at -80ºC until analyzed.

BAL fluid and lung homogenates from $CCR2^{-/-}$ and concurrently infected WT mice were assayed for cytokine production of IFN-γ, TNF-α and IL-12p70 using the cytometric bead array (CBA) (BD Biosciences), and KC was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) DuoSet kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. BAL fluid from $MCP\text{-}1^{-/-}$ and concurrently infected WT mice was assayed for the presence of IFN-γ, IL-12p40, KC and TNF-α by ELISA, using DuoSet kits (R&D Systems). Samples were assayed for the presence of nitric oxide by using a commercially available Griess Reagent System (Cayman Chemicals, Ann Arbor, MI).

Data acquisition from CBA experiments was performed using a Cyan ADP flow cytometer, with data analysis done using Summit software (Beckman Coulter, Fullerton, CA) and FlowJo software (Tree Star, Ashland, OR). For ELISA and Griess reaction assays, optical density readings were determined using a Multiskan Ascent ELISA plate reader and Ascent software (Thermo Scientific, Waltham, MA).

3.3(7) Determination of MCP-1 concentrations.

Organs were harvested and half of each lung and spleen was homogenized in 4 ml saline using a Stomacher 80 Biomaster (Seward). The organ homogenate was centrifuged at 3000g for 15 minutes at 4ºC, supernatants were sterile filtered and then stored frozen at -80ºC prior to analysis. Plasma was prepared from blood collected via cardiac puncture and also frozen at -80ºC prior to cytokine analysis. MCP-1 concentrations in organ homogenates and plasma were determined using a CBA kit (BD Biosciences), according to the manufacturer's directions. Data acquisition was performed using a FACScan flow cytometer, with data analysis done using Cell Quest software (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

3.3(8) Cytokine and chemokine analysis by quantitative real-time PCR.

Lung tissues were placed in 1 ml Trizol Reagent (Invitrogen), homogenized using a Tissue Tearor (BioSpec Products, Bartlesville, OK), and then frozen at -80°C. Total RNA was extracted following storage in Trizol Reagent using the manufacturer's instructions (Invitrogen). Any remaining DNA was then eliminated by treatment with Amplification Grade DNase I (Invitrogen) and RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) RNA clean-up protocol.

Reverse transcription to generate cDNA was carried out with 4 μg total lung RNA using the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen). Cytokine and chemokine cDNA was quantified using specific primers and FAM dye-labeled TaqMan probes designed by Applied Biosystems (Foster City, CA). Reactions were prepared using TaqMan Universal PCR Master Mix (Applied Biosystems) and run in 96-well format on an iCycler (BioRad, Hercules, CA). Cytokine and chemokine gene expression was normalized to

abundance of the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) gene and the ΔΔCt method was used to calculate fold change in relative gene expression.

3.3(9) Clodronate depletion.

Clodronate liposomes (CL) were prepared as described previously and were used within a week of preparation (39). Control PBS liposomes (PL) were prepared according to the same protocol used for CL, but liposomes were re-hydrated in a molar-equivalent concentration of PBS instead of clodronate. For depletion of monocytes and macrophages, mice were injected intravenously (i.v.) with 0.2 ml CL 24 hours prior to infection with *B. mallei*. Control mice received 0.2 ml PL i.v. 24 hours prior to infection.

3.3(10) Histological analysis.

Liver and spleen tissues were collected immediately after euthanasia and placed in 10 ml 10% neutral buffered formalin (NBF) (Sigma-Aldrich) for 24 hours. For lung histology, the lung lobe was first inflated with NBF via the trachea for 5 minutes prior to removal, then placed in NBF for 24 hours. After 24 hours, organs were transferred into a solution of 70% ethanol for 7 days. Tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissues were examined and photographed by a veterinary pathologist experienced in mouse pathology.

3.3(11) *In vivo* treatment with recombinant IFN-γ.

Mice were injected i.p. with 10^5 units recombinant murine IFN-γ (rIFN-γ) (Peprotech, Rocky Hill, NJ) or diluent (PBS + 0.1% bovine serum albumin (BSA)). rIFN-γ treatments were

initiated at the time of infection and continued once daily for 5 days following infection. This treatment protocol was adapted from a protocol used previously in a *Salmonella* infection model (40).

3.3(12) Statistical analysis.

Statistical analyses were performed using Prism 5.0 software (Graph Pad, San Diego, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. A two-tailed Student's t-test or Mann Whitney test was used to determine differences between two groups, and a one-way ANOVA followed by a Tukey's multiple mean's comparison test was used for more than two groups. Comparisons between two groups over time were performed with a two-way ANOVA followed by a Bonferroni post test. Correlation analysis was performed using a two-tailed Pearson's correlation. Differences were considered statistically significant for $p < 0.05$, and statistical trends were considered for $p < 0.1$.

3.4 Results.

3.4(1) Mouse models of respiratory glanders and melioidosis.

A mouse model of respiratory glanders was developed for the investigation of monocytes in response to *B. mallei* infection. *B. mallei* is an obligate mammalian pathogen, and the majority of animal models with *B. mallei* use freshly grown cultures for infection (41-42). Initial attempts to infect mice with *B. mallei* prepared directly from glycerol freezer stocks were unsuccessful; and even infections with overnight cultures yielded unpredictable results (data not shown). Therefore growth kinetics experiments were performed to identify optimal growth conditions for infection.

Preliminary studies comparing cultures grown in sealed 50 ml conical tubes or 125 ml Erlenmeyer flasks equipped with a 0.2 μm filter for aeration showed that no differences in growth were observed up to the stationary growth phase. Although, upon reaching the stationary phase we observed a significant die-off of bacteria in cultures grown in sealed 50 ml conical tubes (21-24 hours, data not shown) ($p < 0.05$; 2 way-ANOVA). Therefore 125 ml Erlenmeyer flasks were used in all subsequent experiments.

Based on previous studies showing that Brucella broth was an effective medium for the growth of *B. mallei* we compared growth of *B. mallei* in Luria Bertani broth + 4% glycerol (LB4G) and Brucella broth + 4% glycerol (BB4G) (43). These studies showed that growth of *B. mallei* in BB4G was both more rapid and more consistent as compared to growth in LB4G **(Figure 3.1(A))**. Growth kinetics of *B. mallei* in BB4G media were then investigated and demonstrated that within the log growth phase there were variable regions (VR) (Labeled as 1, 2 and 3 in Fig. 3.1B), where CFU/ml titers varied between samples. Interestingly these variable phases of growth appeared to occur at consistent stages of bacterial growth in multiple

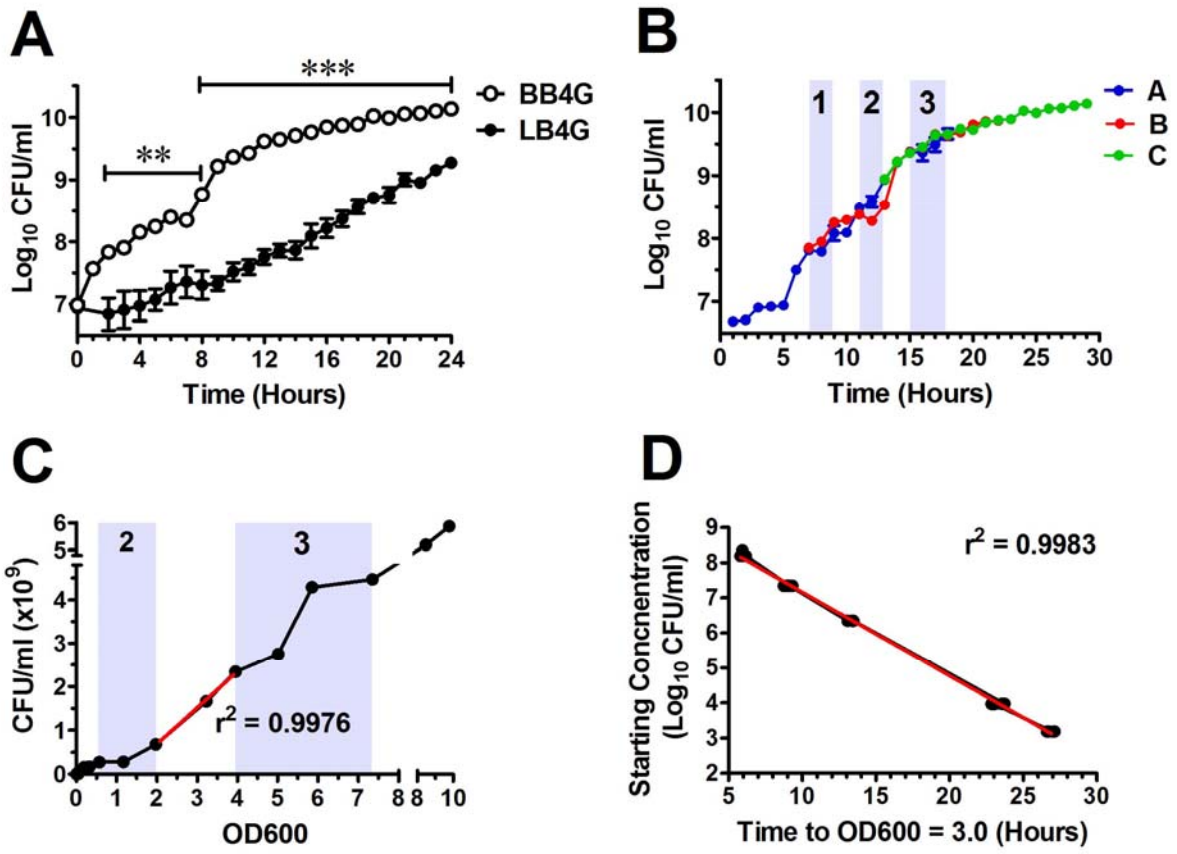

Figure 3.1. Optimization of culture methods for *B. mallei*. (A) Comparison of growth in Luria Bertani + 4% glycerol (LB4G) medium or Brucella broth + 4% glycerol (BB4G) (n = 4 to 12 cultures per time point). Cultures were grown at 37°C and at 1 hour intervals aliquots were taken for agar plating which was performed on the same medium used for broth culture. Data are plotted as $\log_{10}$ CFU/ml ± SEM. Statistical differences between LB4G and BB4G at each time point were determined by a 2-way ANOVA followed by a Bonferroni post test (** $p < 0.01$, *** $p < 0.0001$). Data were pooled from 3 independent experiments. (B) Growth kinetics of *B. mallei* in BB4G medium. Cultures were grown at 37°C and $\log_{10}$ CFU/ml titers were determine by agar plating at 1 hour intervals. Data are graphed as mean ± SEM, and 3 independent experiments are presented (labeled as A, B and C). Areas where variable $\log_{10}$ CFU/ml titers were observed are highlighted with gray bars, and labeled 1-3. (C) Correlation of CFU/ml and OD600 values following *B. mallei* culture in BB4G. Average OD600 and CFU/ml values from the three cultures in (B) were used for correlation analysis. The areas corresponding to variable regions 2 + 3 from (B) are shown for reference. The linear regression between variable regions 2 + 3 is shown by a red line, and the r^2 value is reported. (D) Correlation between the starting concentration of *B. mallei* cultures grown in BB4G and time to reach OD600 = 3.0 (n = 36 cultures). The linear regression line is shown in red with the r^2 value.

independent culture experiments. Correlation of CFU/ml data with OD600 values revealed that although a significant correlation was observed over the entire log phase of growth (5-20 Hrs) ($p < 0.0001$, $r^2 = 0.98$; two-tailed Pearson's correlation), areas of non correlation existed (Fig 3.1C). Correlation analysis was not possible for all individual regions as 4 values are required for statistical analysis, and most variable regions were only 3 hours long; therefore only regression values (r^2) are reported for these regions. Poor correlation of CFU/ml and OD600 values occurred in VR1 ($r^2 = 0.95$), VR2 ($r^2 = 0.83$) and VR3 ($p = 0.08$, $r^2 = 0.84$; two-tailed Pearson's correlation). While similar values were obtained for values before VR1 ($r^2 = 0.96$) and between VR1 and VR2 ($r^2 = 0.92$), high levels of correlation were observed between VR2 and VR3 ($r^2 = 0.998$) and above VR3 ($r^2 = 0.996$). Therefore we selected a target OD600 of 3.0 for infections, which correlated to a titer of 1.4×10^9 CFU/ml, and based on the growth curve (Fig 3.1B), was a late log phase culture (~ hour 16).

A number of difficulties are associated with working under BLS-3 conditions. Therefore we optimized the culture system to ensure the proper growth stage could be obtained for scheduled animal infections. There was a linear correlation between the culture starting concentration and the time at which the culture reached an OD600 value of 3.0 (Fig 3.1D). Therefore by altering the starting concentration, bacteria in the optimal growth phase could be obtained following culture for a pre-determined amount of time.

Although this system resulted in highly reproducible inoculum preparation, intranasal (i.n.) infectious doses were still higher as compared to previously reported LD_{50} values of $\sim10^3$ CFU (data not shown (24, 44)). Previous studies have demonstrated that animal passage can increase the virulence of *B. mallei* (6-8, 32-35). Therefore, serial passage of lung homogenate from i.n. infected BALB/c mice was performed by culturing lung homogenate from *B. mallei*

infected mice on BB4G agar plates, followed by growth in broth media, and i.n. infection of a new set of mice (repeated 3 times). Serial passage in mice significantly increased the virulence of this *B. mallei* strain **(Figure 3.2)**. In addition, following animal passage the mean time to death (MTD) was reduced from 4.0 days (n = 23) to 3.4 days (n = 31) ($p < 0.05$; two-tailed Student's t-test, data not shown). Furthermore, the acute i.n. LD_{50} value (euthanasia on or before day 7) prior to animal passage was 8.4×10^4 CFU, and following animal passage the LD_{50} was 8.2×10^2 CFU. MTD and LD_{50} values obtained with the animal passaged *B. mallei* strain are much closer to previously reported values (24, 44). In addition to serial passage of *B. mallei*, the anesthesia used for i.n. infections was changed from 2.5% avertin to ketamine/xylazine (100/10 mg per kg), and resulted in more consistent i.n. infections. Because animal passage and anesthesia changes were implemented concurrently, either could be responsible for the observed increase in virulence; although it is unlikely that changing the anesthesia would have such a large effect.

The intranasal infection procedure was further characterized using the animal passaged *B. mallei* strain. A comparison of infections performed in a total volume of 20 µl or 40 µl demonstrated that 3 hours after infection there were no differences in the number of bacteria reaching the lung ($p = 0.81$) or bacterial titers in the nasal associated lymphoid tissue (NALT) ($p = 0.35$) (data not shown; two-tailed Student's t-test, pooled from 3 experiments). Therefore an infection volume of 20 µl was chosen as it was easier for mice to inhale. Analysis of lung titers from mice infected in a volume of 20 µl demonstrated that 40.9% of the original inoculum reached the lungs (data not shown, pooled from 2 experiments).

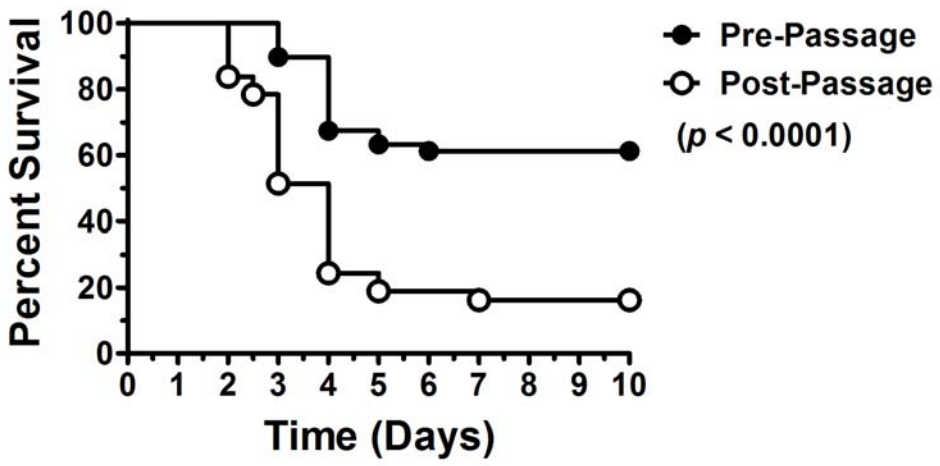

Figure 3.2. Animal passage increases the virulence of *B. mallei* ATCC23344. BALB/c mice were infected i.n. with a *B. mallei* strain ATCC23344 strain prior to animal passage (Pre-passage) (n = 49), or after animal passage (Post-passage) (n = 37), and survival was monitored. The average infectious dose for pre-passage infections was 3.2×10^6 CFU (Range: 2.3×10^3 to 4.3×10^6 CFU). The average infectious dose for post-passage infections was 1.0×10^4 CFU (Range: 4×10^2 to 4.3×10^4 CFU). Data were pooled from 9 experiments for pre-passage and 6 experiments for post-passage. Statistical analysis was performed by a log-rank analysis.

The respiratory glanders model was also developed in a number of other mouse strains and revealed differential susceptibility between mouse strains. For instance, following i.n. infection with the animal passaged *B. mallei* strain LD_{50} values determined by the Reed-Muench method for different mouse strains included 8.8×10^3 CFU in Black Swiss mice, 8.4×10^2 CFU in C57BL/6, and just 17 CFU in 129 Sv/Ev mice (45). The equivalent susceptibility of BALB/c and C57BL/6 mice is in contrast to previous studies demonstrating C57BL/6 mice are typically more resistant to *B. mallei* infection than BALB/c mice (46-48). The use of a mouse-passaged strain of *B. mallei* may be responsible for the increased virulence observed in C57BL/6 mice, as similar results have been reported for mouse-passaged *B. pseudomallei* strains (49). The high susceptibility of 129SvEv mice is similar to previous studies in *B. pseudomallei*, and susceptibility of this mouse strain is equivalent to the high susceptibility of hamsters (20, 41, 50).

Finally, LD_{50} values were also determined for infection with *B. pseudomallei* strain 1026b with inoculums prepared directly from glycerol freezer stocks. For acute disease the LD_{50} in BALB/c was determined to be 9.5×10^2 CFU, and in C57BL/6 the LD_{50} was 3.9×10^3 CFU. These values are in agreement with previous studies, and in contrast to *B. mallei*, with regard to differences in mouse strain susceptibility (46-48). In addition to acute disease, chronic disease was also modeled by lowering the infectious dose in either BALB/c or C57BL/6 mice. Results of chronic infections will be discussed in the relevant sections of this dissertation. In summary, the intranasal models of glanders and melioidosis resulted in consistent pneumonic infection of mice, and were used for all subsequent experiments.

3.4(2) MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice are highly susceptible to inhaled *B. mallei* infection.

Prior studies in a systemic *L. monocytogenes* infection model demonstrated a critical role for CCR2, but not MCP-1, in controlling infection (26-27, 51). However, a role for MCP-1 or its receptor has not been previously investigated in animal models of acute pneumonia due to gram-negative bacterial infection. To address these questions we established a respiratory infection model of acute pneumonic infection with *B. mallei* in C57BL/6 mice. In this model, infection of wild type C57BL/6 mice with a high-dose challenge (5×10^3 CFU by i.n. administration) led to lethal infection within 3-4 days of inoculation (data not shown). To investigate factors that might regulate susceptibility to pulmonary challenge with *B. mallei*, we also developed a low-dose challenge model in C57BL/6 mice, in which mice were challenged i.n. with 5×10^2 CFU (~$0.5\times LD_{50}$) *B. mallei.* In this model, infected mice developed early signs of pneumonia, but most recovered 2-3 days after inoculation **(Figure 3.3).** It should also be noted that the majority of mice that recovered from low-dose challenge later developed chronic infection of the spleen

and liver, typically 45 to 60 days after infection. For example, in addition to mice euthanized due to progressive and clinically apparent chronic disease, determination of bacterial burdens revealed that 33.3% of asymptomatic mice surviving to day 60 had chronic infection of the spleen (Fig 3.3, and data not shown). Therefore 55% of mice surviving acute infection developed some form of chronic disease by day 60.

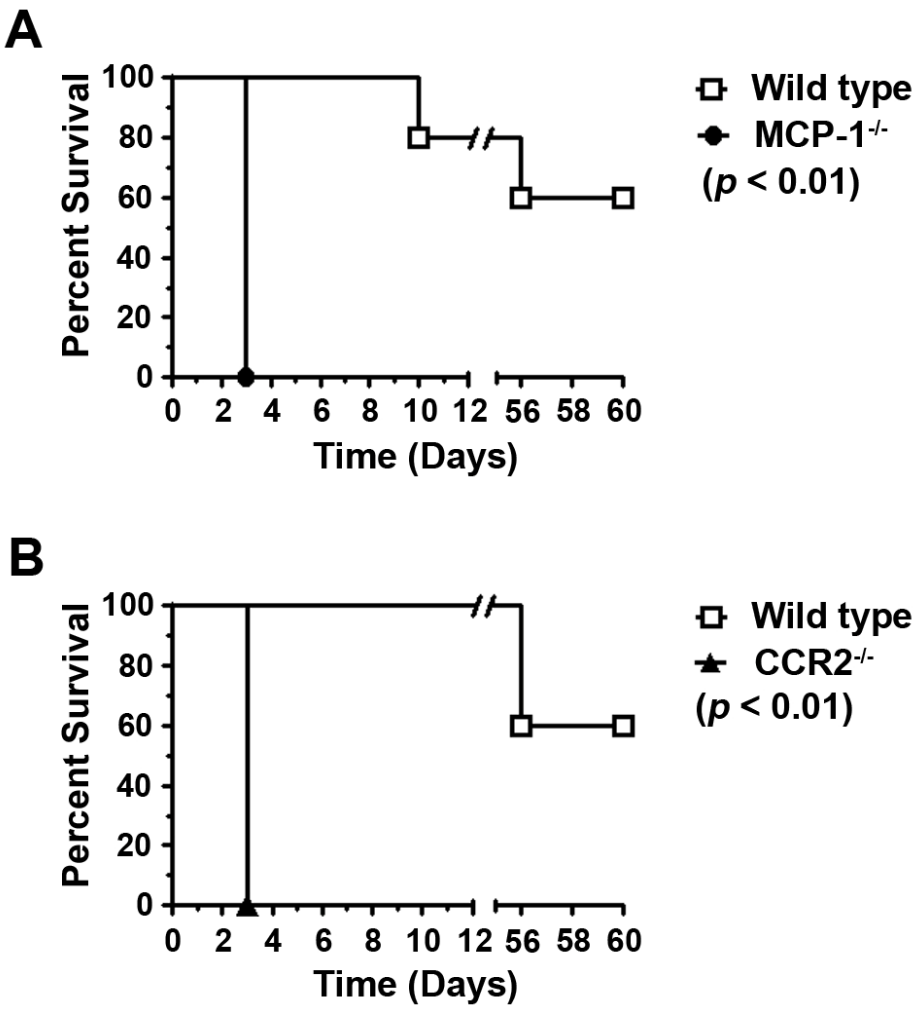

Figure 3.3. Increased susceptibility of MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice to lethal infection with *B. mallei*. Mice (n = 5 per group) were subjected to low-dose (5×10^2 CFU) i.n challenge with *B. mallei* and survival times were determined. In (A), survival times in wild type C57BL/6 mice and MCP-1$^{-/-}$ mice were compared, while in (B) survival times in wild type and CCR2$^{-/-}$ mice were compared. Both MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice were significantly ($p < 0.01$) more susceptible to low-dose pulmonary infection with *B. mallei*, as determined by were compared by means of Kaplan-Meier curves and log rank analysis. Data are representative of two independent experiments.

To determine whether MCP-1 played a role in regulating susceptibility to *B. mallei* pneumonia, we subjected wild type and MCP-1$^{-/-}$ mice (n = 5 per group) to low-dose respiratory challenge with 5×10^2 CFU *B. mallei*. We found that MCP-1$^{-/-}$ mice all developed rapid and overwhelming infection within 3-4 days of challenge, whereas 90% of wild type mice survived the same challenge dose (Fig 3.3). These results indicated clearly that MCP-1 played a critical role in generating protective immunity to *B. mallei* pulmonary infection.

We wished next to determine whether the chemokine receptor CCR2 also played an important role in regulating immunity to *B. mallei* challenge. Though CCR2 is considered to be the primary biologically relevant receptor for MCP-1, it is known that MCP-1 can also bind to other receptors in addition to CCR2, including CCR3 and CCR4 (52-55). In addition, CCR2 can bind ligands other than MCP-1, including MCP-2, MCP-3 and MCP-5 (56-57). The relevance of this chemokine and receptor complexity was demonstrated clearly in the *Listeria* infection model, where it was found that CCR2$^{-/-}$ mice were significantly more susceptible to infection than MCP-1$^{-/-}$ mice (51). Furthermore, increased numbers of monocytes were retained in the bone marrow of CCR2$^{-/-}$ mice when compared to MCP-1$^{-/-}$ mice (26).

Thus, it was important to determine in the *B. mallei* infection model whether in fact CCR2 was the key MCP-1 receptor regulating resistance to infection. Therefore, we compared the susceptibility of MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice to infection with a low-dose challenge with *B. mallei*. Compared to wild type mice, CCR2$^{-/-}$ mice were also significantly ($p < 0.01$) more susceptible to infection (Fig 3.3). Importantly however, and unlike the case with *Listeria* infection, MCP-1$^{-/-}$ mice were equally susceptible to low-dose *B. mallei* infection as were CCR2$^{-/-}$ mice (Fig 3.3). Thus, in the *B. mallei* pneumonia model, MCP-1 and CCR2 expression were equivalent in terms of regulating resistance to infection.

B. mallei and *B. pseudomallei* are genetically similar and the pathogenesis of disease caused by infection with these two organisms is thought to be very similar. Therefore, we also assessed the susceptibility of CCR2$^{-/-}$ mice to low-dose respiratory infection with *B. pseudomallei*. However, when CCR2$^{-/-}$ mice (n = 5 per group) were challenged i.n. with 1.5×10^3 CFU (~$0.5\times LD_{50}$) of *B. pseudomallei*, we found that all of the challenged mice survived (data not shown), whereas an equivalent infectious dose (~$0.5\times LD_{50}$) was rapidly lethal in 100% of *B. mallei* challenged CCR2$^{-/-}$ mice (Fig. 3.3).

3.4(3) MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice are unable to control bacterial replication in the lungs or systemic tissues.

To investigate the mechanisms underlying the extreme susceptibility of MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice to infection with *B. mallei*, bacterial burdens in lung, liver, and spleen were assessed in mice 72 hours after low-dose i.n. infection. Compared to wild type mice, bacterial burdens were significantly increased in both MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice compared to wild type mice **(Figure 3.4)**. Notably, even within 3 days of infection, bacterial burdens were extremely high in the spleens and livers of MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice. However, bacterial burdens in MCP-1$^{-/-}$ mice were not significantly different from those in CCR2$^{-/-}$ mice ($p = 0.39$ for lung, $p = 0.78$ for liver, and $p = 0.20$ for spleen; two-tailed Student's t-test). Thus, MCP-1 production and signaling via the CCR2 receptor both markedly increased the resistance of mice to *B. mallei* infection in the lungs and to dissemination and replication in extrapulmonary sites.

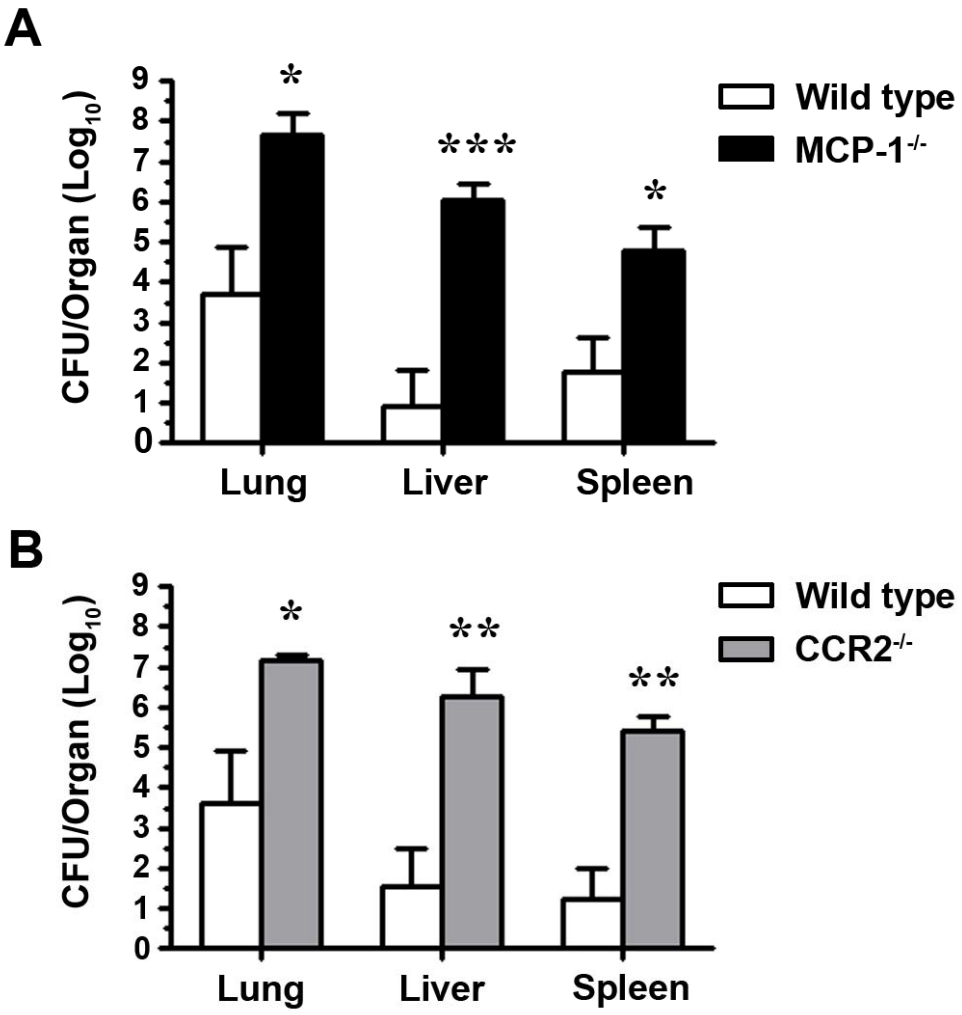

Figure 3.4. MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice are significantly impaired in their ability to control replication and dissemination of *B. mallei* following respiratory challenge. Seventy-two hours after i.n. low-dose (5×10^2 CFU) challenge with *B. mallei*, mice (n = 5 per group) were euthanized and organs (lungs, spleen, and liver) processed as described in Methods for determination of bacterial burden. The bacterial burden in all 3 organs examined was significantly higher in MCP-1$^{-/-}$ mice (A) and CCR2$^{-/-}$ mice (B) than in wild type mice, as determined by a two-tailed Students t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data were plotted as means (± SEM). Data are representative of two independent experiments.

3.4(4) MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice develop marked organ pathology following low-dose challenge with *B. mallei*.

Burkholderia mallei has been shown previously to elicit marked pulmonary pathology when inhaled at high doses in mice (24). However, we wished in this case to compare organ

pathology between wild type and MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice following a low-dose respiratory challenge. Therefore, mice (n = 5 per group) were infected i.n. with 5×10^2 CFU *B. mallei* and tissues were collected for histological evaluation 72 hours after infection.

In *B. mallei* infected wild type mice, multifocal lesions were observed in the lungs, consisting of moderate to severe alveolar infiltration of neutrophils and macrophages, along with mild fibrin transudation and mild parenchymal and leukocytoclastic necrosis with variable occlusion of alveolar spaces **(Figure 3.5)**. Occasional bronchioles with luminal accumulation of leukocytes and cellular debris were observed, but the respiratory epithelium remained intact. There was a mild increase in neutrophils in the splenic red pulp, and in the liver there were mild multifocal sinusoidal infiltrates of neutrophils and macrophages with accompanying piecemeal hepatocyte necrosis.

Much more severe lesions were noted in the lungs of MCP-1$^{-/-}$ mice. There were multifocally coalescing and extensive areas of severe bronchopneumonia with pronounced neutrophil and macrophage infiltration, along with severe fibrin transudation and parenchymal and leukocytoclastic necrosis (Fig. 3.5). Also noted were severe accumulations of cellular debris and degenerate leukocytes within the lumina of bronchi and bronchioles with scattered areas of epithelial degeneration, necrosis and exfoliation. There was also marked perivascular edema, severe endothelial hypertrophy and trans-endothelial leukocyte migration and frank vasculitis in multiple regions of the lung. In the spleen, the white pulp was mildly to moderately lymphocyte depleted and there was moderate accumulation of neutrophils in the red pulp. In the liver, there was multifocal moderate hepatocyte necrosis accompanied by mild to moderate neutrophil and macrophage infiltration.

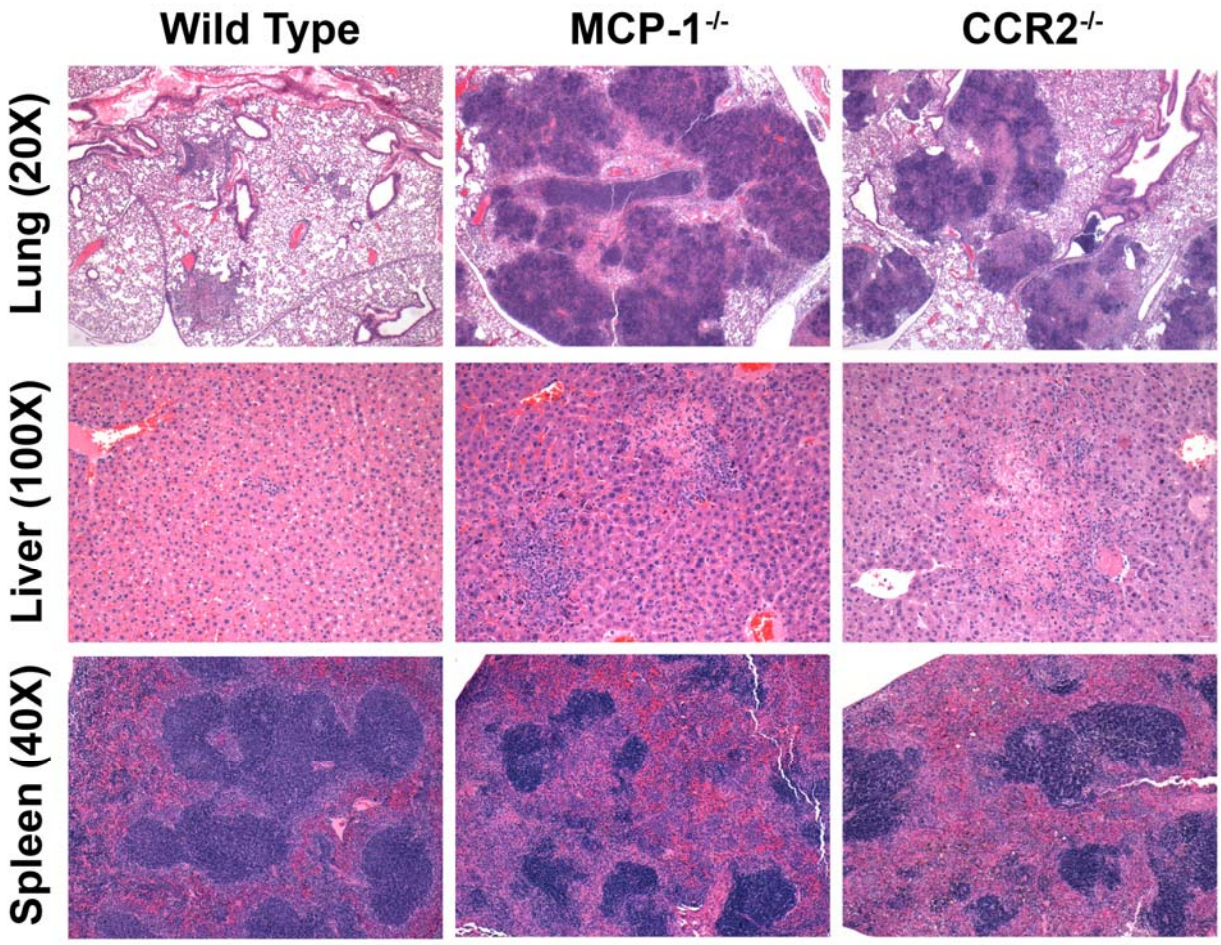

Figure 3.5. Organ pathology following respiratory *B. mallei* challenge in wild type and MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice. Mice (n = 5 per group) were subjected to i.n. challenge with approximately 5×10^2 CFU *B. mallei* and organs were collected for histological examination 72 hours later. Representative images from the lungs (top row; 20X magnification), livers (middle row; 100X magnification), and spleens (bottom row; 40X magnification) of infected wild type, MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice are presented.

Lung lesions in CCR2$^{-/-}$ mice were similar in severity and extent to those noted in MCP-1$^{-/-}$ mice (Fig. 3.5). The spleens of CCR2$^{-/-}$ mice contained a marked accumulation of neutrophils in the red pulp, causing expansion of the organ. Lesions in the liver of CCR2$^{-/-}$ mice were similar to those noted for MCP-1$^{-/-}$ mice.

Thus, the overall histological picture in MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice infected with *B. mallei* was one of much more extensive formation of inflammatory lesions compared to wild

type mice, characterized by infiltration of large numbers of neutrophils and in some cases macrophages, with tissue destruction and necrosis.

3.4(5) Effects of *Burkholderia* infection on production of MCP-1.

The preceding results indicated that MCP-1 production was critical for generating protection from *B. mallei* infection. Therefore, we conducted experiments to investigate the kinetics of MCP-1 production in the bloodstream, lungs and spleen of infected mice. Wild type mice (n = 5 per group) were infected with *B. mallei* (5×10^2 CFU *B. mallei* delivered i.n.), then sacrificed at 24 hours, 48 hours, and 72 hours after infection to assess MCP-1 concentrations. Concentrations of MCP-1 in plasma increased rapidly after infection, with significant increases noted by 24 hours of inoculation, then decreased as the infection was cleared and the mice recovered **(Figure 3.6)**. Production of MCP-1 in the lungs and spleen increased more slowly following infection, with high concentrations first detected at 48 hours and returning to baseline concentrations by 72 hours (Fig. 3.6). Thus, pneumonic infection with *B. mallei* rapidly triggered circulating concentrations of MCP-1 in the bloodstream, followed 24 hours later by MCP-1 production in the lung and spleen.

The amount of MCP-1 produced in response to B. *mallei* infection was also related to the challenge dose. For example, in the low-dose challenge model, transient production of low amounts of MCP-1 was observed, followed by a drop in MCP-1 concentrations as the infection was controlled. However, MCP-1 concentrations in WT mice subjected to high-dose *B. mallei* challenge (10^4 CFU i.n.) were significantly higher than in low-dose infected mice, and remained elevated until the mice were euthanized due to progressive infection (Appendix I, Table A1.2).

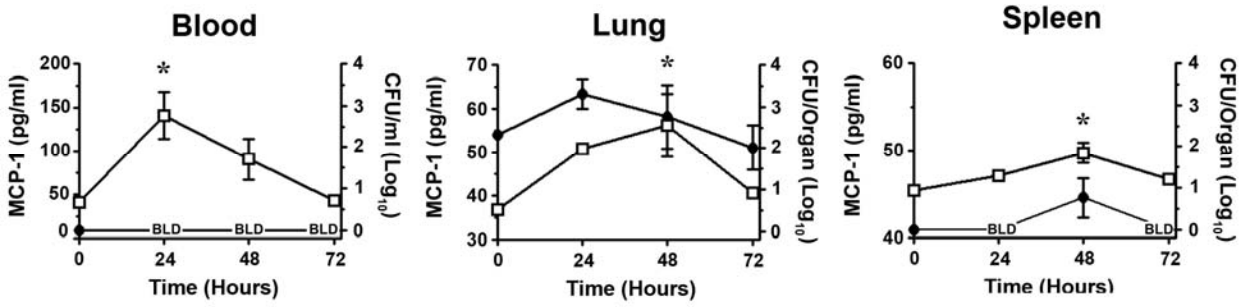

Figure 3.6. Kinetics of MCP-1 production in wild type mice following low-dose *B. mallei* infection. Mice (n = 5 per group) were subject to i.n. challenge with 5×10^2 CFU *B. mallei*. Mice were euthanized at 24, 48 or 72 hours after infection, organs were harvested and processed for determination of bacterial burden and MCP-1 concentrations, as described in Methods. Mean MCP-1 concentrations are represented by open squares and concentrations are shown on the left y-axis, while mean bacterial $\log_{10}$ CFU are represented by filled circles and titers are shown on the right y-axis. Data points are expressed as mean ± SEM. Statistical differences in MCP-1 concentrations at 24, 48 or 72 hours were determined by comparison with pre-infection concentrations using one-way ANOVA followed by Tukey's multiple means comparison test (* $p < 0.05$) (BLD = below limit of detection; limits of detection = 10 CFU/ml for blood and 100 CFU/organ for lung and spleen). Data are representative of two independent experiments.

Experiments were also done to assess the kinetics of replication of *B. mallei* following respiratory infection. Bacterial burdens were determined in the blood, lungs and spleen following i.n. infection with 5×10^2 CFU *B. mallei*. As expected, *B. mallei* replicated rapidly in the lungs following respiratory challenge, but by 48 hours the infection was controlled and bacterial counts began to decline (Fig. 3.6). Bacteria were undetectable in the bloodstream at this time point, and only low numbers of bacteria were detectable in the spleen.

Notably however, in animals subjected to high-dose challenge, *B. mallei* replicated much more rapidly in the liver and spleen, with bacterial titers increasing by a factor of almost 7-fold from the time of challenge until euthanasia 3 days later (data not shown). Interestingly, the magnitude of *B. mallei* bacteremia was less than expected, given the rapid dissemination to liver and spleen. In fact, *B. mallei* was virtually undetectable in the bloodstream until 48 hours after infection, despite the fact that the liver and spleen already contained large numbers of bacteria by

24 hours after inoculation (data not shown). These results indicated that high-level bacteremia was not necessary for efficient dissemination of *B. mallei* to extrapulmonary organs. This data suggests that *B. mallei* may be disseminating through lymphatic vessels, and infection of lymphatic tissue has been previously reported in glanders cases in horses and humans (6, 8, 35, 58). Moreover, once the organism reached organs such as the liver and spleen, replication was essentially unchecked in the first few days after infection, even in immunologically intact animals.

3.4(6) Airway inflammatory cell responses to *B. mallei* infection in wild type and CCR2$^{-/-}$ mice.

Given that MCP-1 is a key regulator of monocyte mobilization and recruitment, cellular inflammatory responses to *B. mallei* infection in the lungs were assessed next. Wild type and CCR2$^{-/-}$ mice (n = 5 to 6 per group) were subjected to low-dose challenge with *B. mallei* and 48 hours after infection, airway cells were collected by BAL and lung cells were obtained by enzymatic digestion of lung tissue. BAL and lung cells were immunostained and analyzed by multicolor flow cytometry to evaluate monocytes, neutrophils, DC, alveolar macrophages, T cells, and NK cells.

Infection with *B. mallei* led to a marked inflammatory response in the lungs of infected mice, both in wild type and CCR2$^{-/-}$ mice. Infection induced a large influx of neutrophils, myeloid DC, and monocytes into the lungs **(Figure 3.7)**, whereas there was little effect on numbers of natural killer (NK) cells, T cells and alveolar macrophages (**Figure 3.8** and Fig. 3.7). In addition, no differences in total cell counts in the airways ($p = 0.19$) or lung tissue ($p = 0.81$) were observed (data not shown; two-tailed Student's t-test). We observed a striking decrease in

monocytes in the airways and lung parenchyma of CCR2$^{-/-}$ mice compared to wild type mice (Fig. 3.7). Moreover, there were significantly fewer DC (CD11b^{+}/CD11c^{+}) in the BAL and lungs of *B. mallei* infected CCR2$^{-/-}$ mice compared to infected wild type mice (Fig. 3.7).

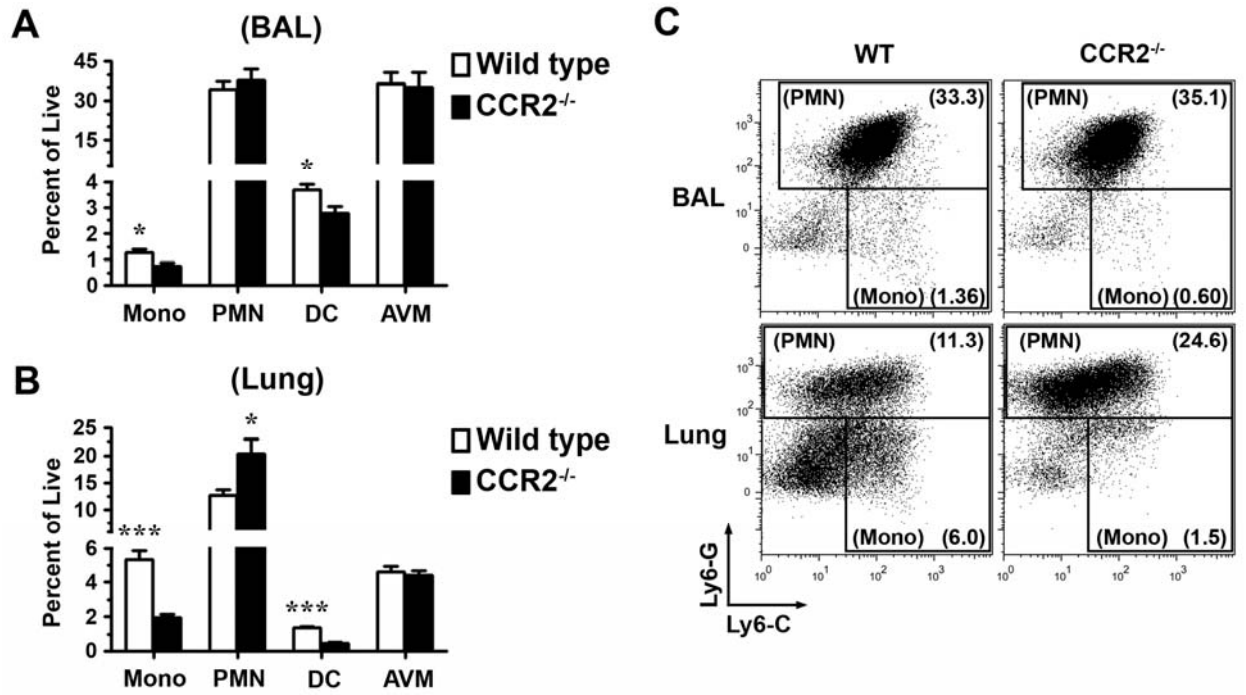

Figure 3.7. Inflammatory cell responses in the airways and lung parenchyma of wild type and CCR2$^{-/-}$ mice following *B. mallei* infection. Wild type and CCR2$^{-/-}$ mice (n = 5 per group) were subjected to low-dose respiratory challenge with *B. mallei*, as described in Methods. After 48 hours, the mice were sacrificed and BAL and lung cells were collected, counted, and immunostained as described in methods. Cell populations were identified as follows: Monocytes (Mono): CD45^{+}/CD11b^{+}/Ly6-C^{+}/Ly6G^{-}; Neutrophils (PMN): CD45^{+}/CD11b^{+}/Ly6-G^{+}; Dendritic cells (DC): CD45^{+}/CD11b^{+}/CD11c^{+}; Alveolar macrophages (AVM): CD45^{+}/CD11c^{+}/CD11b^{-}. (A) and (B) Graphical representation of cell populations in the BAL (A) and lung parenchyma (B). Data are plotted as the mean ± SEM of each cell population's percentage of all live cells. Data are representative of two independent experiments. Statistical differences between WT and CCR2$^{-/-}$ mice were determined using a two-tailed Student's t-test (* $p < 0.05$, *** $p < 0.001$). (C) Representative flow plots illustrating monocyte (Mono) and neutrophil (PMN) populations. BAL plots were generated by using FlowJo software to pool all 5 BAL .fcs files from each group. 50,000 events from each pooled BAL sample were then used to generate representative dot plots. Lung flow plots are individual representative samples from each group. The percentage of total live cells is listed in each gate. Data are representative of two independent experiments.

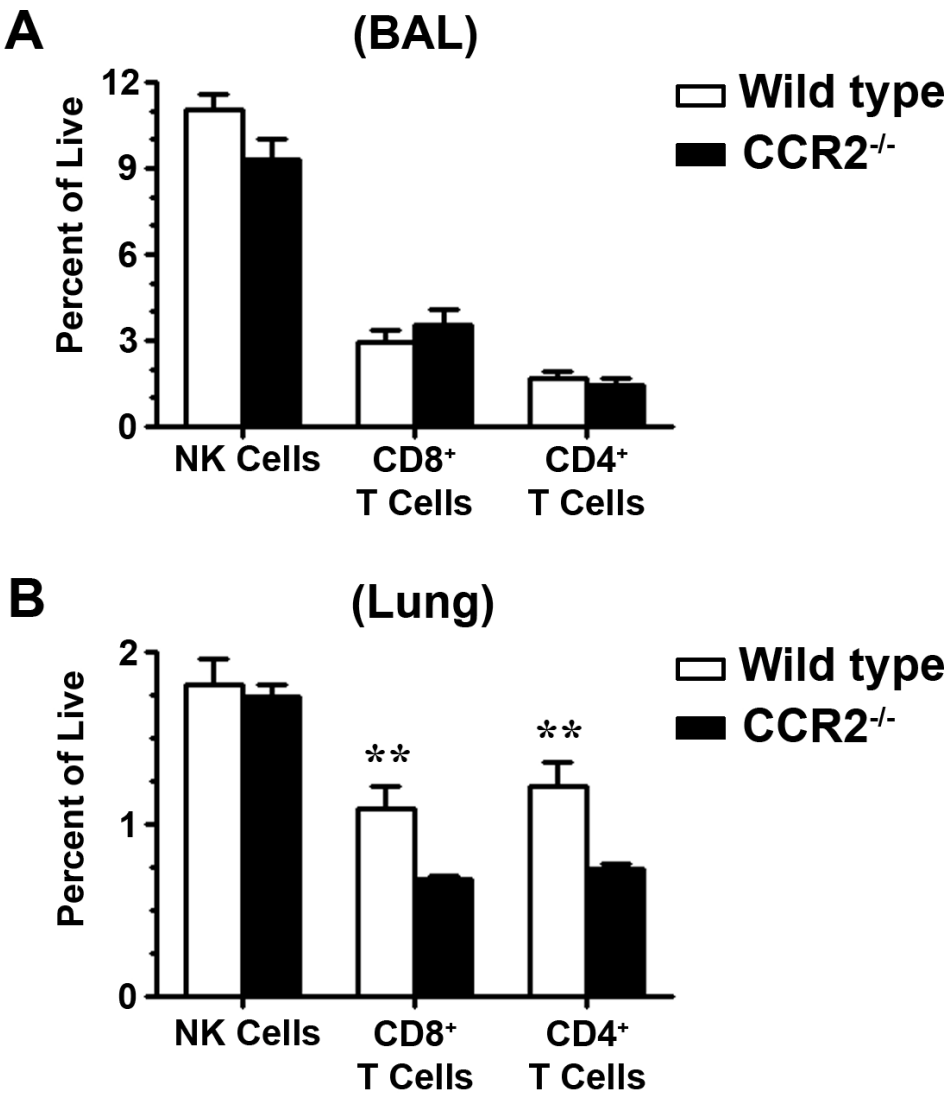

Figure 3.8. Lymphocyte responses in the airways and lung parenchyma of wild type and CCR2$^{-/-}$ mice following *B. mallei* infection. Wild type and CCR2$^{-/-}$ mice (n = 5 per group) were subjected to low-dose respiratory challenge with *B. mallei*, as described in Methods. After 48 hours, the mice were sacrificed and BAL and lung cells were collected, counted, and immunostained as described in methods. Cell populations were identified as follows: NK Cells CD45^{+}/NK-1.1^{+}; CD8^{+} T cells: Low FSC and SSC/CD8^{+}; CD4 T Cells: Low FSC and SSC/CD4^{+}. (A) and (B). Graphical representation of cell populations in the BAL (A) and lung parenchyma (B). Data are plotted as the mean ± SEM of each cell population's percentage of all live cells. Data are representative of two independent experiments. Statistical differences between WT and CCR2$^{-/-}$ mice were determined using a two-tailed Student's t-test (** $p < 0.01$).

While there were no differences in alveolar macrophages in the BAL or lung of wild type and CCR2$^{-/-}$ mice, the CCR2$^{-/-}$ mice had significantly more neutrophils in the lung parenchyma

when compared to WT mice (Fig. 3.7). There were no differences in NK cells in the BAL or the lung and while similar levels of T cells were seen in the BAL, there were significant increases in both $CD4^+$ and $CD8^+$ T cells in the lungs of WT mice (Fig. 3.8). Thus, lack of CCR2 expression appeared to primarily affect monocyte and inflammatory DC recruitment and accumulation in the lungs and airways following *B. mallei* infection. Interestingly, neutrophil recruitment was increased in the lungs of $CCR2^{-/-}$ mice compared to WT mice (Fig 3.7). This increase in neutrophils in the lungs of $CCR2^{-/-}$ mice most likely represents an intact neutrophil response due to the increased bacterial burden present in the lungs of $CCR2^{-/-}$ mice (Fig 3.4). While both $CD4^+$ and $CD8^+$ T cells were reduced in the lungs of $CCR2^{-/-}$ mice, there were no differences in NK cells, which are the main producers of protective cytokines during the early innate immune response to acute *Burkholderia* infection (18-19, 22).

3.4(7) Effects of monocyte depletion on protection from *B. mallei* infection.

MCP-1 plays a key role in regulating release of monocytes from the bone marrow and directing their recruitment to sites of inflammation (25). The preceding experiments revealed decreased DC and monocyte recruitment to the lungs of *B. mallei* infected mice. However, the reduction in monocyte and DC recruitment to the airways may not have been directly responsible for the inability of $CCR2^{-/-}$ mice to control *B. mallei* infection, since these mice may have other defects in innate immune responses. To address this question in a different manner, we directly depleted monocytes from the bone marrow of wild type mice, using i.v. injection of clodronate liposomes (CL) (59-61). Treatment with CL has been shown previously to transiently deplete inflammatory monocytes from the bone marrow and blood of mice (60, 62). Thus, the CL

depletion technique allowed us to assess the effects of transient monocytopenia on the ability of mice to control pulmonary infection with *B. mallei*.

Wild type mice (n = 9 or 10 per group) were pre-treated by i.v. administration of either CL or control PBS liposomes (PL). Injection of CL resulted in a transient 60 to 70% reduction in numbers of circulating monocytes (data not shown). Twenty-four hours after injection, mice were subjected to low-dose *B. mallei* i.n. challenge. We found that the mice pretreated with CL had significantly decreased survival times, compared to mice pretreated with PL **(Figure 3.9)**. In addition, pre-treatment with CL also resulted in significantly increased bacterial counts in the lungs, spleens, and livers of CL treated mice compared to mice receiving PL (Fig. 3.9). These data therefore provided additional evidence for a critical role for monocytes in generating early protective immunity against pneumonic *B. mallei* infection and in controlling dissemination from the lungs to extrapulmonary sites.

3.4(8) CCR2$^{-/-}$ mice are impaired in their ability to produce critical anti-bacterial cytokines.

The preceding experiments indicated that MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice were both markedly impaired in their ability to mount effective innate immune responses against *B. mallei* infection. To determine whether the extreme susceptibility of these mice to infection could also have resulted from dysregulation of key antibacterial effector mechanisms, we compared pro-inflammatory cytokine and nitric oxide responses in the lungs of wild type and MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice following low-dose infection with *B. mallei*. Wild type mice, MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice were subjected to low-dose i.n challenge and BAL and lung tissues were collected 48

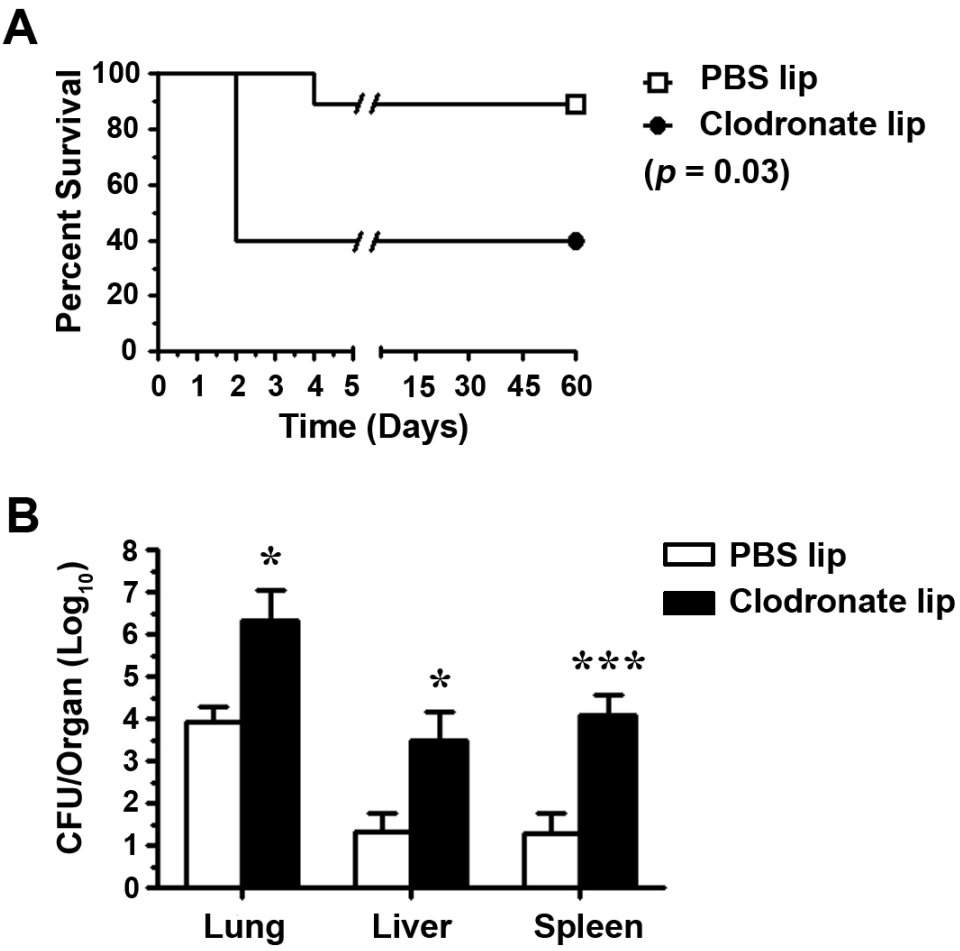

Figure 3.9. Effects of monocyte and macrophage depletion using liposomal clodronate on susceptibility of wild type mice to *B. mallei* infection. Mice (n = 9 or 10) were treated with clodronate liposomes (CL) or PBS control liposomes (PL) and then infected with approximately 5×10^2 CFU *B. mallei* by the i.n, route, as described in Methods. In (A), survival times were determined and Kaplan Meier analysis followed by a log-rank test was used to determine statistical differences. Survival times were significantly decreased ($p = 0.03$) in CL treated mice compared to PL treated mice. In (B), bacterial burdens in lung, liver, and spleen were determined in mice treated with CL or PL that were euthanized 48 hours after infection. Data are presented as means ± SEM. Statistical differences were determined using two-tailed Student's t-test (* $p < 0.05$, *** $p < 0.001$). Data were pooled from two independent experiments.

hours later. BAL fluid and lung homogenates were analyzed by CBA, ELISA, quantitative RT-PCR, and biochemical assays.

Cytokine responses (IFN-γ, TNF-α, and IL-12) and nitric oxide responses to *B. mallei* infection were compared in the BAL and lung homogenates of infected WT and $CCR2^{-/-}$ mice 48

hours after infection **(Figure 3.10)**. Interestingly, CCR2$^{-/-}$ mice actually mounted a significantly stronger TNF-α and KC responses in the airways and lung parenchyma than wild type mice following *B. mallei* infection (Fig. 3.10). In contrast to the findings in CCR2$^{-/-}$ mice, TNF-α and KC levels in the BAL fluid of MCP-1$^{-/-}$ mice were not significantly different when compared to WT mice **(Figure 3.11)**.

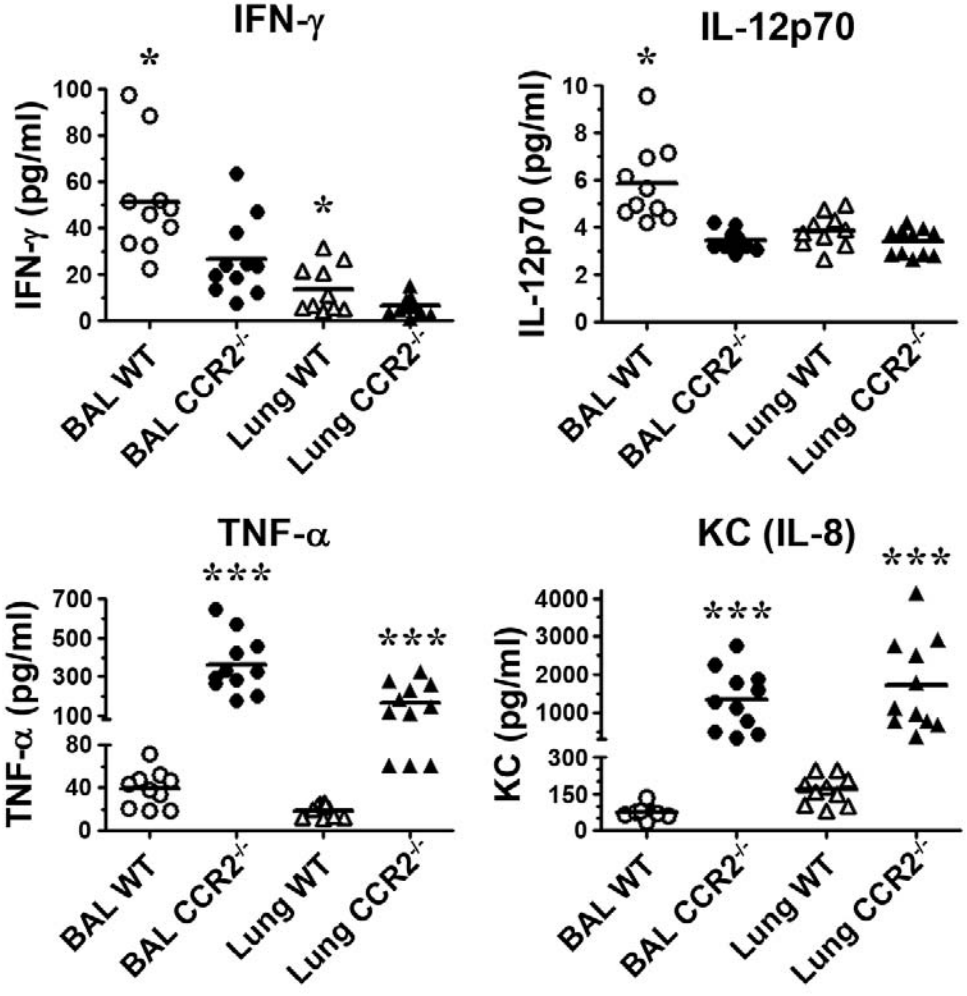

Figure 3.10. Cytokine responses in the BAL and lungs of *B. mallei* infected wild type and CCR2$^{-/-}$ mice. Mice (n = 10 per group) were subjected to low-dose respiratory challenge with *B. mallei* (~5×10^2 CFU), then sacrificed at 48 hours after infection. Airway lavage samples and lavaged lung tissue were collected for analysis of TNF-α IFN-γ IL-12p70 and KC concentrations, as described in Methods. Data are graphed as individual values with bars representing mean values. The mean cytokine concentrations were compared statistically between groups using a two-tailed Student's t-test (* $p < 0.05$, *** $p < 0.001$ between WT and CCR2$^{-/-}$ values). Data were pooled from two independent experiments.

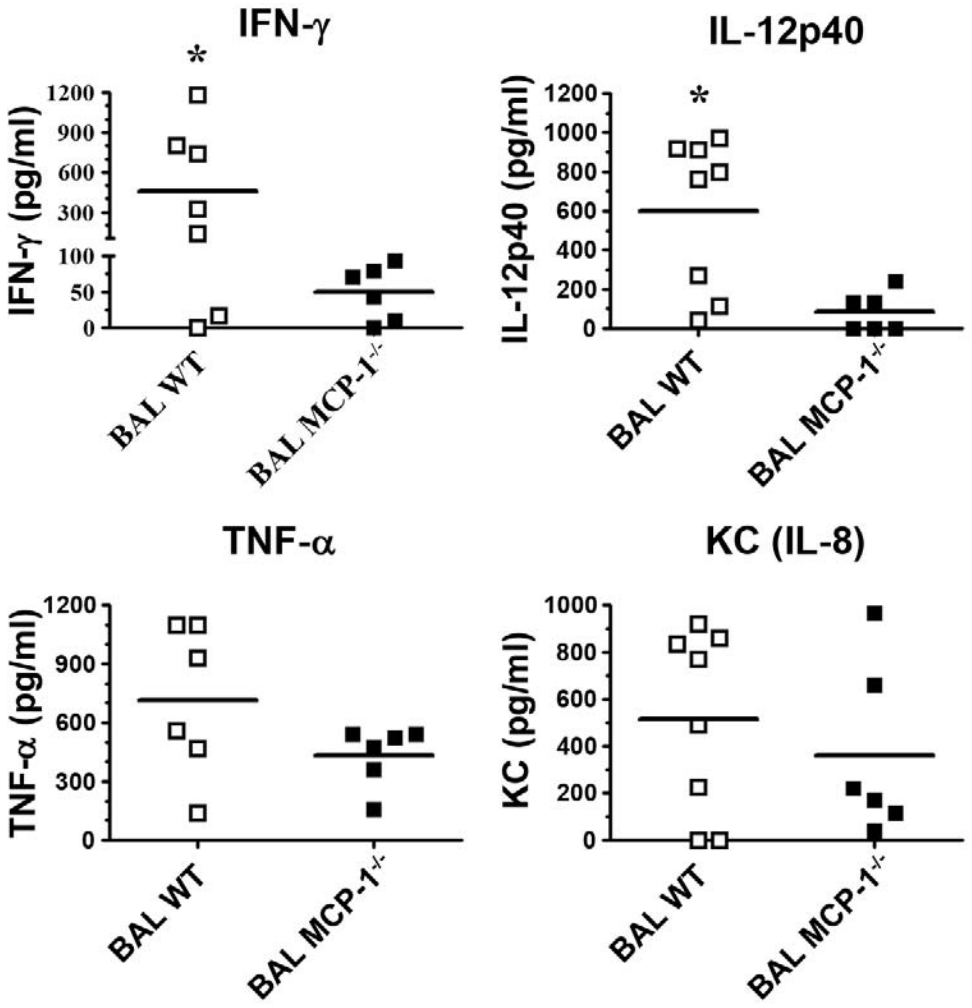

Figure 3.11. Cytokine responses in the BAL of *B. mallei* infected wild type and MCP-1$^{-/-}$ mice. Mice (n = 6-7 per group) were subjected to low-dose respiratory challenge with *B. mallei*, and then sacrificed at 48 hours after infection. Airway lavage samples were collected for analysis of TNF-α IFN-γ IL-12p40 and KC concentrations, as described in Methods. Data are graphed as individual values with bars representing mean values. The mean cytokine concentrations were compared statistically between groups using a two-tailed Student's t-test (* $p < 0.05$ between WT and CCR2$^{-/-}$ values). Data were pooled from two independent experiments.

The most dramatic cytokine differences were observed when IFN-γ and IL-12 responses in the airways and lungs were compared. Here, both MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice were markedly impaired in their ability to produce IFN-γ in response to infection, compared to wild type mice **(**Fig 3.10 and Fig 3.11**)**. Moreover, the IL-12 responses in the airway of both MCP-1$^{-/-}$ and

CCR2$^{-/-}$ mice were also significantly impaired. While IL-12 responses in the lungs of CCR2$^{-/-}$ mice were reduced, the difference did not reach the level of statistical significance ($p = 0.1$; two-tailed Student's t-test) (Fig 3.10). Thus, the inability to produce or respond to MCP-1 resulted in markedly attenuated IFN-γ responses to *B. mallei* infection, while TNF-α and KC responses were either increased (CCR2$^{-/-}$ mice) or unchanged (MCP-1$^{-/-}$ mice).

The ability of CCR2$^{-/-}$ mice to mount a nitric oxide (NO) response to *B. mallei* infection was also assessed. Samples of BAL and lung homogenate collected 48 hours after *B. mallei* infection were analyzed to assess total NO production. Despite the differences in IFN-γ production, we did not observe differences in NO production in either the BAL ($p = 0.47$) or lung homogenates ($p = 0.77$) in CCR2$^{-/-}$ mice compared to WT mice **(Figure 3.12)**.

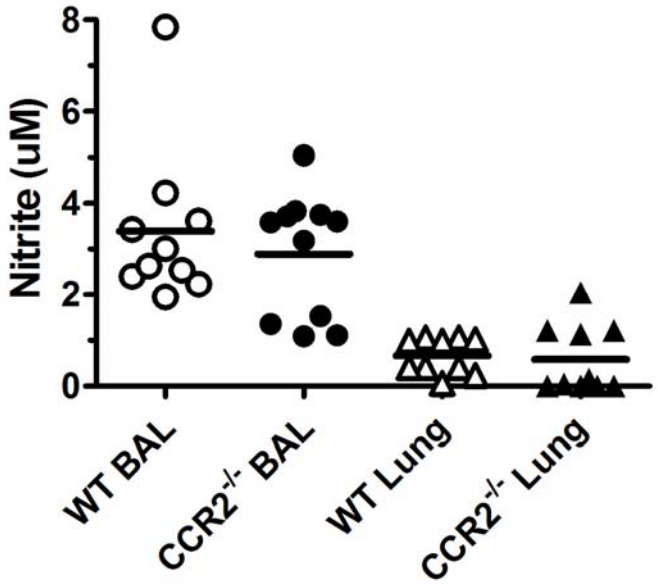

Figure 3.12. Nitric oxide responses in the BAL and lungs of *B. mallei* infected wild type and CCR2$^{-/-}$ mice. Mice (n = 10 or 11 per group) were subjected to low-dose respiratory challenge with *B. mallei* (~5×10^2 CFU) and sacrificed at 48 hours after infection. Airway lavage samples and lavaged lung tissue were collected for analysis of nitric oxide concentrations, as described in Methods. The mean nitrite concentrations were compared statistically between groups using a two-tailed Student's t-test. Data were pooled from two independent experiments.

The effects of pneumonic *B. mallei* infection on expression of other relevant cytokines and chemokines in the lungs were also examined 48 hours after infection using qRT-PCR. Levels of gene expression in lung tissues of the following cytokines and chemokines were compared in wild type and MCP-$1^{-/-}$ and $CCR2^{-/-}$ mice (n = 4 mice per group): IFN-β, IFN-α, MIP-2α, CXCL1, and MIP-1. A significant decrease in the expression of MIP2α (RANTES) mRNA in the lungs of $CCR2^{-/-}$ mice was noted, whereas there were no significant differences in other cytokines measured (data not shown). Thus, the lack of MCP-1 expression or inability to respond to MCP-1 affected certain components of the innate immune response to *B. mallei* infection, most notably IFN-γ, while leaving other innate immune responses intact.

3.3(9) *In vivo* administration of IFN-γ restores resistance to infection in $CCR2^{-/-}$ mice.

Experiments were conducted next to further elucidate the role that lack of IFN-γ production played in conferring the extreme susceptibility to infection exhibited by $CCR2^{-/-}$ mice. $CCR2^{-/-}$ mice (n = 3 per group) were subjected to lethal, low-dose i.n. challenge with *B. mallei* and then treated i.p. daily for 5 days with recombinant murine IFN-γ (rIFN-γ) (10^5 IU per mouse per day), beginning at the time of challenge. We found that $CCR2^{-/-}$ mice treated with rIFN-γ were significantly protected from lethal infection compared to sham-treated mice **(Figure 3.13)**. Moreover, the resistance to infection conferred by rIFN-γ treatment was long-lived, in that mice surviving beyond day 5 were able to survive to at least day 30. Thus, providing an exogenous source of IFN-γ was able to complement the defect in innate immunity present in $CCR2^{-/-}$ mice. This result illustrated clearly the critical role that the defect in IFN-γ production played in conferring the extreme susceptibility of $CCR2^{-/-}$ mice to *B. mallei* infection.

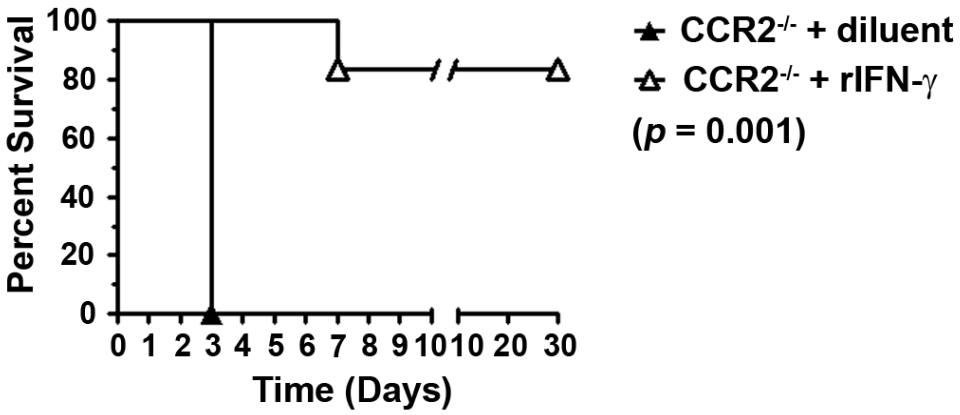

Figure 3.13. Effects of treatment with rIFN-γ on resistance to *B. mallei* infection in CCR2$^{-/-}$ mice. CCR2$^{-/-}$ mice (n = 6 per group) were subjected to low-dose i.n. challenge with *B. mallei* (~5×10^2 CFU). Treated mice were administered 10^5 IU recombinant murine rIFN-γ at the time of infection, and then daily for 5 consecutive days by i.p. injection, for a total of 6 rIFN-γ injections. Sham treated mice were injected i.p. with PBS diluent. Survival times were determined and compared statistically by means of Kaplan-Meier analysis, followed by log rank test. Survival times were significantly longer ($p = 0.001$) in rIFN-γ treated mice than in sham-treated mice. Data were pooled from two independent experiments.

3.5 Discussion.

Despite the importance of *B. mallei* as a zoonotic pathogen and potential bioweapon agent, little is known regarding key early protective immune responses to infection with this organism. The studies presented here therefore provide important new information regarding the pathogenesis of pneumonic *B. mallei* infection and identify a critical protective role for MCP-1 in the early innate immune response to this pathogen. It is also noteworthy that the antibacterial effects of MCP-1 appeared to be mediated through regulation of production of IFN-γ, as well as by stimulating monocyte and DC recruitment to the airways. In addition, MCP-1 and CCR2 were found to be important for controlling the dissemination of *B. mallei* from the lungs to extrapulmonary sites following initial pneumonic infection.

The role of MCP-1 and CCR2 in immunity to bacterial infection has been investigated previously in three different infection models. In the *L. monocytogenes* parenteral infection model, CCR2 expression was a critical regulator of early protective innate immune responses to infection, whereas MCP-1 expression had a less important role (26-27, 51). In the *L. monocytogenes* model, the infection route was i.p. and the primary target organ for bacterial infection was the spleen (27, 63). In mice infected with *Listeria*, CCR2 expression was found to be necessary for recruitment of a population of DC to the spleen that produced high levels of TNF-α and iNOS and generated anti-bacterial immunity (27). In contrast to the *Listeria* infection model, in a low-dose inhalational *M. tuberculosis* challenge model, CCR2 expression was found to play no role in regulating resistance to low-dose challenge (28, 31). However, in a high-dose inhalational *M. tuberculosis* challenge model and in an i.v. challenge model, CCR2 expression was found to be important for protective immunity (29-30). Finally respiratory infection with *Streptococcus pneumoniae* in MCP-$1^{-/-}$ mice also resulted in increased bacterial

burden and lung pathology. Similar to the findings in our study MCP-1$^{-/-}$ mice were found to have decreases in DCs and increases in neutrophils. In addition TNF-α and KC production was equivalent or increased in MCP-1$^{-/-}$ mice when compared to WT mice, although IFN-γ production and cytokine levels in CCR2$^{-/-}$ mice were not investigated (64).

The studies presented here are the first to our knowledge to assess the role of MCP-1 in a model of acute pulmonary infection with a gram-negative bacterium. In contrast to results obtained in *Listeria* studies where TNF-α responses were significantly diminished in the spleens of *L. monocytogenes*-infected CCR2$^{-/-}$ mice, the TNF-α responses in the lungs of CCR2$^{-/-}$ mice infected with *B. mallei* were preserved. Although increased TNF-α concentrations in *B. mallei* infected CCR2$^{-/-}$ mice could be due to the increased bacterial burdens seen in these mice, TNF-α levels were still significantly increased in CCR2$^{-/-}$ mice compared to WT mice when cytokine levels were normalized to bacterial burden ($p < 0.0001$ for BAL and Lung; two-tailed Student's t-test, data not shown). Despite the increased bacterial burden in CCR2$^{-/-}$ mice, we found that IFN-γ responses were markedly reduced in the lungs of CCR2$^{-/-}$ mice infected with *B. mallei*. This is in contrast to mice infected with *L. monocytogenes*, where IFN-γ responses were maintained in the spleens of infected CCR2$^{-/-}$ mice (27). Thus, we postulated that the loss of IFN-γ production in the lungs of *B. mallei*-infected CCR2$^{-/-}$ mice was one of the key immunological mechanisms underlying the extreme susceptibility of these mice to infection. In support of this idea, we found that treatment with exogenous rIFN-γ could significantly restore resistance to *B. mallei* infection in CCR2$^{-/-}$ mice.

The differing roles of MCP-1 and CCR2 in regulating resistance to infection have also been observed previously in a *Leishmania* infection model (65-66). MCP-1 has been shown to also play an important protective role in protozoal and fungal infections. For example, CCR2$^{-/-}$

mice were found to be more susceptible to infection with *Toxoplasma gondii* (67). Increased susceptibility to *Toxoplasma* infection correlated with reduced recruitment of Gr-1^{+} monocytes to the peritoneum following infection (67-68). The recruited monocytes were found to contribute to killing of *T. gondii* in a NO-dependent manner. Interestingly however, *Toxoplasma*-infected CCR2$^{-/-}$ mice did not manifest a defect in production of IFN-γ or TNF-α. In a model of pulmonary infection with the fungal organism *Cryptococcus neoformans*, CCR2$^{-/-}$ mice also had increased susceptibility to infection, which was associated with decreased macrophage recruitment to the lungs (69). Thus, CCR2 and MCP-1 appear to play an important role in controlling infection with several different pathogens, all of which share the common feature of being intracellular organisms. However, as noted above, it also apparent that lack of CCR2 expression impairs innate and adaptive immune responses to varying degrees, depending on the infecting organism, the site of infection, as well as the size of the challenge dose and route of infection (70).

While little is known regarding innate or adaptive immune responses to *B. mallei*, more is known about the closely related pathogen *B. pseudomallei.* Control of *B. pseudomallei* infection was shown previously to be dependent on production of IFN-γ and TNF-α (18-21). Interestingly however, and in agreement with our results, *in vivo* control of *B. pseudomallei* infection in C57BL/6 mice was found to be largely independent of iNOS expression (23, 71). Neutrophils were also shown to play an important role in controlling pulmonary infection with *B. pseudomallei*, and *B. mallei*. Although, the use of a Gr-1 antibody to deplete neutrophils in these studies makes interpretation of these results difficult as monocytes also express Gr-1 (22, 72). In this study we observed that CCR2$^{-/-}$ mice had increased susceptibility to respiratory infection with *B. mallei* but not *B. pseudomallei*, and that *B. mallei* infected CCR2$^{-/-}$ mice had significant

increases in lung neutrophils when compared to WT mice. These results suggest there may be a differential role for neutrophils in response to infection with these two closely related bacteria. While neutrophils may be sufficient to protect CCR2$^{-/-}$ mice from *B. pseudomallei* infection, neutrophils recruited in response to *B. mallei* infection of CCR2$^{-/-}$ mice were not capable of controlling infection. Other studies, however, have shown that depletion of monocytes and macrophages using the CL injection technique resulted in increased susceptibility of mice to *B. pseudomallei* infection following i.p. challenge (23). The results of our studies in CCR2$^{-/-}$ mice are therefore consistent with the idea that recruited monocytes and inflammatory DC played a key protective role in the lungs against *B. mallei* infection, possibly by serving as a source of IFN-γ (18).

Monocytes recruited to sites of infection may play several roles in controlling bacterial infections. In the *Listeria* infection model, a subset of DC thought to be derived from inflammatory monocytes were largely responsible for controlling bacterial infection by producing TNF-α, and expression of iNOS (27). In fact, inflammatory monocytes are thought to be the principal precursor for certain types of DC in several sites, including the skin, draining lymph nodes, spleen, and lung (25, 59, 73-74). In the present study we observed that CCR2$^{-/-}$ mice were significantly impaired in their ability to recruit both monocytes and inflammatory myeloid DC, but not neutrophils, into their lungs in response to *B. mallei* infection (see Fig 3.7). Within the lungs and airways, inflammatory monocytes and DC may suppress bacterial replication in infected target cells by producing antibacterial cytokines such as TNF-α and IFN-γ. Inflammatory monocytes may produce IFN-γ directly, as noted previously, or they may indirectly induce production of IFN-γ by other cells in the lung, especially NK cells (18-19, 22, 75).

The lack of IFN-γ production in the lungs of CCR2$^{-/-}$ mice in response to *B. mallei* infection could reflect failure of recruitment of IFN-γ producing cells to the lungs, or may instead reflect a more general impairment in the ability of CCR2$^{-/-}$ mice to mount IFN-γ responses. The latter explanation is less likely however, inasmuch as we found that CCR2$^{-/-}$ mice produced strong IFN-γ responses (equivalent to wild type mice) when challenged *in vivo* with cationic liposome-DNA complexes (CLDC), which are potent inducers of IFN-γ production (data not shown and (76)).

The importance of IFN-γ was also demonstrated in experiments where IFN-γ treatment was sufficient to provide CCR2$^{-/-}$ mice with long term protection against *B. mallei* infection (Fig. 3.13). However, the role of IFN-γ in response to *B. mallei* infection remains unknown as nitric oxide (Fig 3.12) and reactive oxygen species (data not shown) do not appear to be necessary in this model. IFN-γ is known to regulate a large number of genes, with 228 immune related genes containing a gamma activating sequence (GAS), or an interferon stimulated response element (ISRE), suggesting these genes are activated in response to IFN-γ signaling. IFN-γ regulated genes relevant to these studies include expression of cytokines such as IL-12, IL-18, and GM-CSF, monocyte chemoattractants such as MCP-1, MIP-1β (CCL4) and their receptors CCR2 and CCR5, and finally adhesion molecules such as V-CAM, I-CAM, p-selectin and e-selectin (77-78). Therefore, treatment of CCR2$^{-/-}$ mice with IFN-γ mice may have induced monocyte recruitment due to MIP-1β and CCR5 expression and further IFN-γ production due to induction of IL-12 and IL-18 expression. Administration of IFN-γ at the time of infection may have prematurely induced some of these responses, and any secondary responses, as maximal IFN-γ production is not observed in wild type mice until 48 hours after infection (Fig 4.8). Further

studies delaying IFN-γ treatment, or inhibition of additional IFN-γ activated responses may help to identify how IFN-γ production results in *B. mallei* killing.

Although, in contrast to WT mice, where 55% of mice developed chronic disease by day 60; 100% of surviving IFN-γ treated $CCR2^{-/-}$ mice developed chronic disease by day 60 (data not shown). These results suggest that while IFN-γ is sufficient to provide protection against acute disease, monocytes may play additional important roles in the development of the adaptive immune responses necessary for the control of chronic *B. mallei* infection.

We conclude therefore that MCP-1 and CCR2 are key components of pulmonary innate immune responses to infection with a highly virulent gram-negative pathogen such as *B. mallei*. The effects of MCP-1 and CCR2 appear to be mediated through recruitment of key antibacterial effector cells, principally monocytes and DC, to sites of bacterial infection. These findings suggest that therapeutic recruitment of monocytes and DC into the airways may serve as an effective means of generating rapid protection against respiratory exposure to pathogenic bacteria (79-81). Potential therapeutics known to recruit monocytes include innate-defense regulator peptide, recombinant MCP-1 and polyIC (79-80, 82-83). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known to enhance monocyte responses and has been shown to enhance the response to *S. pneumoniae* and *L. monocytogenes* (84-86). Although $MCP\text{-}1^{-/-}$ and $CCR2^{-/-}$ mice were unable to produce IFN-γ, the mechanisms responsible for this defect were not determined. The following chapter investigates how respiratory *B. mallei* infection stimulates recruitment and activation of monocytes and dendritic cells; and identifies cell types responsible for IL-12 and IFN-γ production. More specifically, these studies focused on the role of myeloid differentiation factor 88 (MyD88) dependent Toll-like receptor (TLR) signaling following *B. mallei* infection.

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CHAPTER 4.

MYD88 DEPENDENT RECRUITMENT OF MONOCYTES AND DENDRITIC CELLS REQUIRED FOR PROTECTION FROM PULMONARY *BURKHOLDERIA MALLEI* INFECTION

The studies in this chapter describe the role of myeloid differentiation factor 88 (MyD88) dependent Toll-like receptor (TLR) signaling in cellular recruitment and cytokine production following *B. mallei* infection. I acknowledge Dr. Helle Bielefeldt-Ohmann for analysis of histopathology and for capturing micro photos, Dr. Laurel Lenz for providing the MyD88$^{-/-}$ mice used in these studies, and Dr. Ryan Troyer for screening MyD88 pups for homozygous knockout animals.

4.1 Summary.

The gram-negative bacterium *Burkholderia mallei* causes rapidly fatal illness in equines and humans when contracted by inhalation and also has the potential to be used as a bioweapon. However, little is known regarding the early innate immune responses and signaling mechanisms required to generate protection from pneumonic *B. mallei* infection. We showed previously that monocyte recruitment is required for protection from pneumonic *B. mallei* infection. We have now extended those studies to identify key Toll-like receptor (TLR) signaling pathways, effector cells, and cytokines required for protection from respiratory *B. mallei* infection. We found that myeloid differentiation factor 88 (MyD88) knockout mice were highly susceptible to pulmonary

challenge with *B. mallei* and had significantly shorter survival times, increased bacterial burdens, and more severe organ pathology than wild type mice. Notably, MyD88$^{-/-}$ mice had significantly fewer monocytes and dendritic cells (DC) in lung tissues and airways compared to infected wild type mice, despite markedly higher bacterial burdens. The MyD88$^{-/-}$ mice were also completely unable to produce IFN-γ at any time points following infection. In wild type mice, natural killer (NK) cells were the primary cells producing IFN-γ in the lungs following *B. mallei* infection, while DC and monocytes were the primary cellular sources of IL-12 production. Treatment with recombinant IFN-γ (rIFN-γ) was able to significantly restore protective immunity in MyD88$^{-/-}$ mice. Thus, we conclude that MyD88-dependent recruitment of inflammatory monocytes and DC to the lungs and local production of IL-12, followed by NK cell production of IFN-γ, are the key initial cellular responses required for early protection from *B. mallei* infection.

4.2 Introduction.

Burkholderia mallei, an obligate mammalian pathogen found primarily in equidae, and *B. pseudomallei*, an opportunistic soil bacterium that infects a wide-variety of mammalian species, are both pathogens in mammals (1-5). Both organisms have been classified as category B select agents by the Centers for Disease Control and Prevention (CDC), and respiratory inoculation with either of these bacteria elicits rapidly fatal pneumonia and sepsis (4, 6-8). One unusual feature of *Burkholderia* infection is that acute infection is rarely fully cleared, but often instead results in chronic infection with dissemination to the liver and spleen, which in mice is typically fatal over a period of several months (9-11). Other relatively non-pathogenic species of *Burkholderia* include *B. thailandensis and B. cenocepacia,* both of which are environmental organisms. The former may cause disease in mice after very high-dose inoculation (12), while *B. cenocepacia* causes infections primarily in humans with altered pulmonary defenses, such as patients with cystic fibrosis (13).

The role of Toll-like receptor (TLR) signaling in host defense against *Burkholderia* infection has been previously examined in a limited number of *B. pseudomallei* infection studies (14-15). TLR2 was shown to actually be detrimental to the host response to *B. pseudomallei* infection, and similar results were observed following *B. thailandensis* infection (14, 16). In contrast, TLR4 was found to be non-essential in protection against *B. pseudomallei* or *B. thailandensis* infections, and TLR5 was not essential in protection against respiratory *B. thailandensis* infection (14, 16). Nonetheless, mice lacking myeloid differentiation factor 88 (MyD88) are highly susceptible to infection with either *B. pseudomallei* or *B. thailandensis* (14-15).

Following *B. pseudomallei* infection, it has been reported that IFN-γ production and monocyte recruitment to the lungs was equivalent in wild type and MyD88$^{-/-}$ mice (15). MyD88$^{-/-}$ mice were found to produce less TNF-α, KC and MCP-1 than wild type animals in response to *B. pseudomallei* infection and were unable to recruit neutrophils to the lungs (15). Equivalent numbers of monocytes and neutrophils were recruited to the lungs of wild type and MyD88$^{-/-}$ mice in a *B. thailandensis* infection model (14). In contrast, MyD88$^{-/-}$ mice were actually protected against respiratory infection with *B. cenocepacia* compared to wild type animals. Thus, there appear to be clear *Burkholderia* species-specific differences in the host innate immune response to infection.

Production of IFN-γ is critical for protection from infection with *B. mallei* or *B. pseudomallei*, and TNF-α production is also required for protection from *B. pseudomallei* infection (17-20). Previous studies demonstrated that monocyte chemoattractant protein-1 (MCP-1 / CCL2) plays a key protective role in the innate immune responses to acute pulmonary infection with *B. mallei* (Chapter 3). Both chemokine receptor 2 (CCR2) and CCL2 knockout mice were highly susceptible to pulmonary challenge with *B. mallei*. One key finding from these studies was that while the CCR2$^{-/-}$ mice had the expected defects in monocyte recruitment, CCR2$^{-/-}$ animals actually recruited significantly more neutrophils and produced higher concentrations of TNF-α than wild type animals. These findings are in contrast to previous reports suggesting a key protective role for neutrophils in *B. pseudomallei* and *B. mallei* infection. Although, the use of a Gr-1 antibody for depletion of neutrophils in these studies, suggests that monocytes may also be important, as Gr-1 is expressed on both neutrophils and monocytes (21-22). Indeed, our results in CCR2$^{-/-}$ mice suggest that monocytes are necessary following infection with *B. mallei* but not *B. pseudomallei* (Chapter 3). Therefore, important

differences appear to exist between *Burkholderia* species with regards to the necessity of MyD88 signaling for protective innate immunity. Since the role of TLR signaling in protection from *B. mallei* infection has not been previously examined, we used the *B. mallei* lung infection mouse model to investigate the role of MyD88 and cellular and cytokine responses required to generate early protective pulmonary immunity against this important bacterial pathogen.

4.3 Materials and methods.

4.3(1) Mice.

C57BL/6 mice and TLR2$^{-/-}$, IL-6$^{-/-}$ and IFN-$\gamma^{-/-}$ mice on the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). To investigate the role of TLR4, the C57BL/10ScNJ strain, which contains a natural deletion of the TLR4 gene, and the control strain C57BL/10ScSnJ were used (Jackson Laboratories). MyD88$^{-/-}$ mice on the C57BL/6 background were obtained from Dr. Laurel Lenz (National Jewish Medical, Denver, CO) and were bred in-house. All mice used in experiments were 6-8 weeks of age at the time of infection, and age and sex matched wild type controls were used in all experiments employing knockout mice. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University. In survival experiments mice were euthanized upon reaching one of the following pre-determined euthanasia endpoints: (1) hunched posture with decreased movement or response to stimuli; (2) development of respiratory distress (tachypnea, open-mouthed respirations); or (3) loss of > 15% body weight.

4.3(2) Bacterial strains and infections.

Burkholderia mallei strain ATCC23344 was used in these studies and was kindly provided by Dr. Herbert Schweitzer, Colorado State University. The *B. mallei* organism used in these studies was initially serially passaged three times in BALB/c mice, and then stocks were prepared and frozen at -80ºC, as described previously (Chapter 3). Prior to each challenge study, fresh broth cultures of *B. mallei* were grown in Brucella broth with 4% glycerol (BB4G) (Remel, Lenexa, KS) until bacteria reached the log phase of growth. Titers were determined based on optical density values, and appropriate bacterial dilutions were prepared in sterile phosphate

buffered saline (PBS). Inoculum titers for each experiment were confirmed by plating the inoculum on BB4G agar plates (Remel).

For intranasal (i.n.) inoculation, mice were anesthetized by intraperitoneal (i.p.) injection with ketamine (100 mg/kg) (Vedco, Saint Joseph, MO) and xylazine (10 mg/kg) (Ben Venue Labs, Bedford, OH). Mice were infected i.n. with a total volume of 20 µl of bacterial inoculum (10µl per nostril). The LD_{50} dose following i.n. infection of C57BL/6 mice with *B. mallei* was previously determined to be 8.4×10^2 CFU according to the Reed-Muench method (Chapter 3 (23)). Mice were infected with either ~5×10^2 CFU i.n (~$0.5\times LD_{50}$) or with an experimentally determined LD_{100} dose of 3×10^3 CFU (~$4\times LD_{50}$). In experiments where a consistently lethal infection was necessary, mice were infected with 1×10^4 CFU i.n. (~$10\times LD_{50}$). All procedures involving *B. mallei* were performed in a Biosafety Level 3 (BSL3) facility, in accordance with approved BSL3 and Select Agent protocols.

4.3(3) Determination of bacterial burden.

At 24, 48 or 72 hours after infection mice were euthanized and halves of each lung, liver and spleen were collected separately and placed in 5 ml sterile PBS. Organs were homogenized using a Stomacher 80 Biomaster (Seward, Bohemia, NY) and supernatants were serially diluted in PBS and plated on BB4G agar plates (Remel). Agar plates were incubated at 37°C for 48 hours and colonies were counted. The limit of detection for determination of bacterial burden in organ homogenates was 100 CFU/organ. For quantification of bacteremia, heparinized blood was serially diluted in sterile PBS and plated on BB4G agar plates. The limit of detection for determination of bacterial burden in the blood was 10 CFU/ml.

4.3(4) Histological analysis.

Lungs were inflated with 10% neutral buffered formalin (NBF) (Sigma-Aldrich, St. Louis, MO) via the trachea for 5 minutes prior to removal, and placed in NBF for 24 hours. Livers were collected immediately after euthanasia and placed in NBF for 24 hours. After 24 hours, organs were transferred into a solution of 70% ethanol for 7 days. Tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissues were examined and photographed by a veterinary pathologist experienced in mouse pathology.

4.3(5) Tissue processing and immunostaining for flow cytometry analysis.

Airway cells were obtained by bronchoalveolar lavage (BAL) as previously described (24-25). Cells were recovered from the BAL by centrifugation. Following lavage, lung digestion was performed as previously described (26). Briefly, lungs were minced and digested in Hank's balanced salt solution (HBSS) containing 2.5 mg/ml collagenase, 10U/ml DNase, and 10 μg/ml soybean trypsin inhibitor (all reagents from Sigma-Aldrich). After digestion cells were triturated through an 18 gauge needle, and passed through a 70 micron cell strainer (BD Biosciences, San Jose, CA). Red blood cells were lysed using ammonium chloride, cells were washed twice in HBSS, immunostained, and then stored on ice until analyzed. All cells were re-suspended in FACS buffer (PBS with 2% FBS and 0.05% sodium azide) before immunolabeling and flow cytometric analysis.

Directly conjugated antibodies for flow cytometry were purchased from eBioscience (San Diego, CA) or BD Biosciences. The following eBioscience antibodies were used for flow cytometry staining: anti-CD11b (APC conjugated; clone M1/70), anti-CD11c (PE conjugated; clone N418), anti-CD45 (pacific blue conjugated; clone 30-F11), anti-NK-1.1 (biotin conjugated;

clone PK136), anti-CD4 (PE conjugated; clone GK1.5), and anti-CD8 (APC conjugated; clone 53-6.7). The following BD Biosciences antibodies were used: anti-Ly6C (biotin conjugated; clone AL-21), and anti-Ly6G (FITC conjugated; clone 1A8). Pacific orange and Alexa 488 streptavidin conjugates were purchased from Invitrogen (Carlsbad, CA). Before staining, nonspecific antibody binding was blocked by addition of FACS block consisting of normal mouse serum (Jackson ImmunoResearch, West Grove, PA), human IgG (Jackson ImmunoResearch) and unlabeled anti-mouse CD16/32 (clone 93) (eBioscience) for 5 minutes at room temperature. Staining was performed in FACS buffer for 30 minutes on ice, followed by washing with FACS buffer. In the case of biotinylated antibodies, the streptavidin-fluorochrome conjugate was added next for 20 minutes on ice. After a final wash, the cells were fixed in 1% paraformaldehyde in PBS for 24 hours at 4^{o}C, washed once, resuspended in FACS buffer and stored at 4^{o}C until analyzed. Flow cytometry was performed using a Cyan ADP flow cytometer running Summit software (Beckman Coulter, Fullerton, CA). Analysis of flow cytometry data was performed using FlowJo software (Tree Star Inc., Ashland, OR). Samples were gated on forward and side scatter characteristics for viable cells. For investigation of monocytes (Mono), neutrophils (PMN), dendritic cells (DC) and alveolar macrophages (AVM), live cells expressing CD45 were used for analysis. Alveolar macrophages were defined as $CD11c^{hi}$ and $CD11b^{-}$ cells. Dendritic cells were defined as $CD11c^{+}CD11b^{+}$ cells. Neutrophils were defined as $CD11b^{+}Ly6G^{+}$ cells, while monocytes were defined as $CD11b^{+}Ly6C^{+}$ cells that were $Ly6G^{-}$. For investigation of natural killer (NK) and T cells live cells were gated on lymphocytes based on their low forward and side scatter characteristics. NK cells were identified as $CD45^{+}$ and NK 1.1^{+} cells, and T cells were identified as cells with low FSC/SSC characteristics expressing CD4, CD8 or CD3.

4.3(6) Intracellular cytokine staining.

Intracellular cytokine staining was performed as described previously (21). Lungs were minced and digested in HBSS containing 0.4 mg/ml collagenase (Liberase TL; Roche, Indianapolis, IN) and 10 μg/ml DNase (Sigma-Aldrich) for 30 minutes at 37°C. After digestion cells were triturated through an 18 gauge needle, passed through a 70 micron cell strainer (BD Biosciences), and red blood cells were lysed using ammonium chloride. Following digestion lung cells were cultured at 37°C with 5% CO_2 for 4 hours in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.075% sodium bicarbonate (EMD Science, Gibbstown, NJ), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 50 μM 2-mercaptoethanol (Invitrogen) and 10 μg/ml brefeldin-A (BioLegend, San Diego, CA). Cells were washed in FACS buffer and re-suspended in FACS block to prevent non-specific staining. Cells were surface immunostained for 30 minutes at room temperature. In addition to the surface antibodies described above, anti-CD11c (APC conjugated; clone N418), anti-F4/80 (APC conjugated; clone BM8), anti-CD3 (APC conjugated; clone 145-2C11), and anti-CD4 (FITC conjugated; clone GK1.5) were purchased from eBioscience. Anti-CD45 (pacific orange conjugated; clone 30-F11) was purchased from Invitrogen, and anti-CD11b (Alexa 700 conjugated; clone M1/70) was purchased from BioLegend. For detection of biotinylated antibodies, cells were washed in FACS buffer and incubated with streptavidin PerCP (BD Biosciences) for 20 minutes at room temperature. Next, cells were washed with FACS buffer, re-suspended in fixation-permeabilization buffer (eBioscience) and incubated at 4°C overnight. Cells were washed in permeabilization buffer (eBioscience) and resuspended in anti-cytokine antibodies. From BD Biosciences, anti-IL-12(p40/p70) (PE conjugated; clone

C15.6), anti-IFN-γ (PE conjugated; clone XMG1.2), rat IgG1κ isotype (PE conjugated; clone R3-34), and from eBioscience, anti-TNF-α (PE conjugated; clone MP6-XT22), and rat IgG1 isotype (PE conjugated; clone eBRG1). Next cells were washed in permeabilization buffer and then FACS buffer. Finally cells were fixed in 1% paraformaldehyde for 1 hour at 4°C, washed, and stored, in FACS buffer until analyzed. Flow cytometry was performed and analyzed as described above. Cell populations were differentiated as described above. Additionally, F4/80 macrophages were defined as $CD45^{+}/F4/80^{+}$.

4.3(7) Assessment of cytokine production in plasma and organ homogenate.

Plasma was collected in tubes coated with EDTA (BD Biosciences). Plasma was isolated from the blood by centrifugation of Microtainer tubes at 10,000g for 5 minutes at 4ºC. Plasma was sterile filtered and frozen at -80ºC until analyzed. Lung, liver and spleen tissues were processed as described above for determination of bacterial burden. Half of each organ was placed in 5 ml PBS and homogenized on the Stomacher 80 Biomaster (Seward). Homogenate was centrifuged at 3000g for 15 min at 4ºC, supernatants were sterile filtered and frozen at -80ºC until analyzed.

Plasma and organ homogenates from wild type and $MyD88^{-/-}$ mice were assayed for production of CCL2 (MCP-1), IFN-γ, TNF-α, IL-6 and IL-12p70 using the cytometric bead array (CBA) (BD Biosciences). Concentrations of CXCL1 (KC) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) DuoSet kit (R&D Systems, Minneapolis, MN), according to the manufacturer's directions. Data acquisition from CBA experiments was performed using a FACScan flow cytometer and Cell Quest software (BD Biosciences). Analysis of CBA data was performed using FlowJo software (Tree Star). For

ELISA assays, absorbance readings were determined using a Multiskan Ascent ELISA plate reader and Ascent software (Thermo Scientific, Waltham, MA).

4.3(8) *In vivo* treatment with recombinant IFN-γ.

MyD88$^{-/-}$ mice were injected i.p. with either 1x10^5 units of recombinant murine IFN-γ (rIFN-γ) (Peprotech, Rocky Hill, NJ) or diluent (PBS + 0.1% BSA). rIFN-γ treatments were initiated at the time of infection and continued once daily for 5 days following infection. This treatment protocol was adapted from a protocol used previously in a *Salmonella* infection model (27).

4.3(9) Statistical analysis.

Statistical analyses were performed using Prism 5.0 software (Graph Pad, La Jolla, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. Analyses between two groups were performed using a two-tailed Student's t-test. Comparisons between 3 or more groups were performed using a one-way ANOVA followed by a Tukey's multiple means test. Correlation analysis of bacterial burden and cytokine production was performed using a two-tailed Pearson's correlation. Differences were considered statistically significant for $p < 0.05$.

4.4 Results.

4.4(1) MyD88$^{-/-}$ mice are highly susceptible to *Burkholderia mallei* infection.

Previous studies have revealed that MyD88 signaling plays very different roles in protective immunity to infection with different *Burkholderia* species. For example, MyD88-dependent-immune responses were found to be detrimental in the host response to *B. cenocepacia* infection, whereas MyD88 was critical for protective immunity against infection with *B. pseudomallei* and *B. thailandensis* (14-15, 28). Thus, it was unclear based on the published literature exactly what role MyD88 signaling might play in the innate immune response to acute pneumonic infection with *B. mallei*.

Therefore, wild type (WT) C57BL/6 mice and MyD88$^{-/-}$ mice on the C57BL/6 background (n = 5 mice per group) were inoculated with a low dose i.n. challenge dose of *B. mallei* (0.5×LD_{50}; approximately 5×10^2 CFU *B. mallei*). Infected wild type mice all survived to at least day 60, whereas MyD88$^{-/-}$ mice were euthanized at an average of 3.5 days following challenge **(Figure 4.1).**

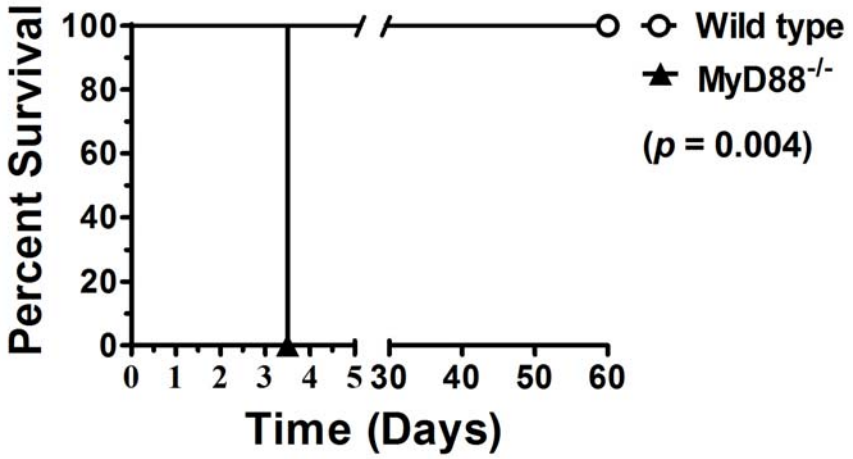

Figure 4.1. Susceptibility of MyD88$^{-/-}$ mice to respiratory *B. mallei* infection. Wild type and MyD88$^{-/-}$ mice (n = 5 per group) were infected intranasally (i.n.) with 5×10^2 CFU *B. mallei*. Mice were monitored for survival and euthanized upon reaching a pre-determined endpoint. Statistical differences were determined using Kaplan-Meier curves and log rank analysis. Data are representative of two independent experiments.

4.4(2) TLR2 signaling increases the susceptibility of mice to lethal *B. mallei* infection.

Previous studies have indicated that TLR2 signaling actually increases the susceptibility of mice to *B. pseudomallei* and *B. thailandensis* infection (14, 16). Therefore, we conducted experiments in TLR2$^{-/-}$ mice to elucidate the role of TLR2 in protection from *B. mallei* infection. Wild type and TLR2$^{-/-}$ mice on the C57BL/6 background were infected with an experimentally determined LD_{100} dose (3×10^3 CFU, ~$4\times LD_{50}$) of *B. mallei*, delivered i.n. As reported previously, following *B. pseudomallei* or *B. thailandensis* infection, we found that TLR2$^{-/-}$ mice were resistant to infection with *B. mallei*, compared to wild type mice **(Figure 4.2)** (14, 16). Protection could, however, be overcome by challenge with a higher dose of *B. mallei*. For example, when TLR2$^{-/-}$ mice were challenged with 4×10^3 CFU *B. mallei*, survival was reduced to 60% (data not shown). In addition, bacterial burdens were determined in wild type (n = 2) and TLR2$^{-/-}$ mice (n = 7) surviving acute disease. At day 30 or 60 following i.n. infection, we found that TLR2$^{-/-}$ mice had significantly fewer bacteria in the spleen ($p = 0.03$) as compared to wild type mice (data not shown; two-tailed Student's t-test). In addition, there were no significant differences in bacterial counts in the blood ($p = 0.50$), and bacterial burdens in the lung and liver were below the limit of detection in all mice (data not shown; two-tailed Student's t-test). Therefore, we concluded that TLR2$^{-/-}$ mice were not only resistant to acute pneumonic infection, but they also were protected from development of chronic disease.

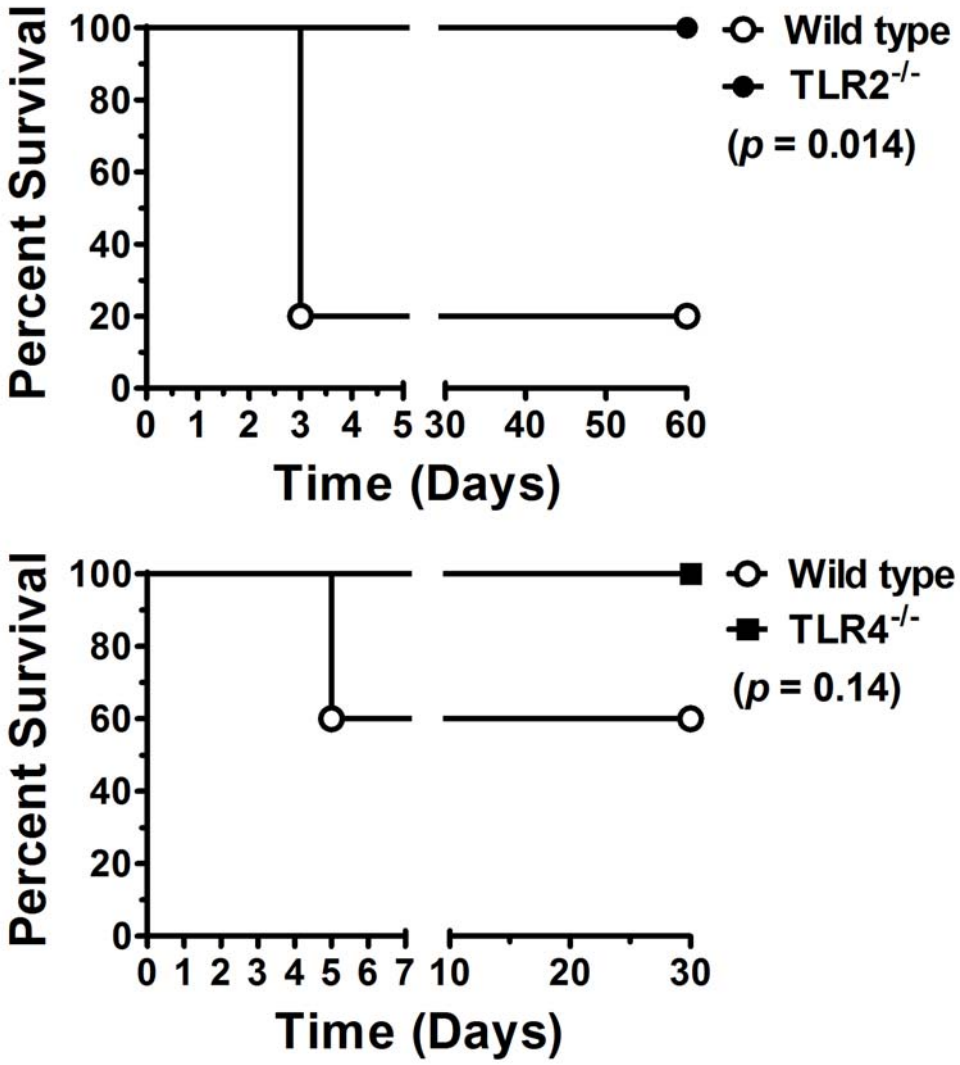

Figure 4.2. Susceptibility of TLR2$^{-/-}$ and TLR4$^{-/-}$ mice to pulmonary *B. mallei* infection. (A) Wild type and TLR2$^{-/-}$ mice were challenged with 3×10^3 CFU *B. mallei* i.n. Survival was monitored and mice were euthanized upon reaching a pre-determined endpoint. Statistical differences were determined using Kaplan-Meier curves and log rank analysis. Data are representative of two independent experiments. (B) Wild type and C57BL/10ScNJ mice containing a natural deletion of TLR4 (n = 5 per group) were infected i.n. with 5×10^2 CFU *B. mallei*. Survival was monitored and mice were euthanized upon reaching a pre-determined endpoint. Statistical differences were determined using Kaplan-Meier curves and log rank analysis. Statistically similar results ($p = 0.42$) were obtained in a separate experiment where wild type and TLR4$^{-/-}$ mice were challenged with 3×10^3 CFU *B. mallei* i.n.

4.4(3) TLR4 does not regulate resistance to pulmonary infection with *B. mallei*.

TLR4 is a key regulator of innate immune responses to gram-negative bacterial infections (29-31). A recent study found that TLR4 expression did not regulate susceptibility to *B. pseudomallei* infection (16). To determine whether TLR4 was required for protection from *B. mallei* challenge, wild type and TLR4$^{-/-}$ mice (n = 5 per group) were inoculated with low-dose i.n. challenge of 5×10^2 CFU of *B. mallei*. We observed that TLR4$^{-/-}$ mice were not more susceptible to *B. mallei* infection than wild type mice (Fig. 4.2). Therefore, wild type and TLR4$^{-/-}$ mice were next subjected to high-dose challenge with *B. mallei* (3×10^3 CFU, ~$4\times LD_{50}$). We found here also that TLR4$^{-/-}$ mice were not more or less susceptible to *B. mallei* infection than wild type animals ($p = 0.42$; log-rank analysis, data not shown). Quantification of bacterial burden in surviving wild type mice (n = 5) and TLR4$^{-/-}$ mice (n = 6) mice 30 days after i.n. infection revealed that chronic infection developed equivalently in wild type and TLR4$^{-/-}$ mice (data not shown). For example, bacteria were undetectable in the lung. In the liver, none of the wild type mice were colonized, while 1 of 6 TLR4$^{-/-}$ mice had a liver titer of 250 CFU/organ. There were no differences in bacterial burden observed in the spleen ($p = 0.87$; two-tailed Student's t-test). Therefore, we concluded that TLR4 signaling did not regulate protective immunity following pulmonary challenge with *B. mallei* in mice.

4.4(4) MyD88$^{-/-}$ mice have increased pulmonary and systemic bacterial burden associated with lung and liver pathology.

The preceding experiments indicated that MyD88$^{-/-}$ mice were highly susceptible to *B. mallei* infection. Therefore, we next assessed bacterial burdens in MyD88$^{-/-}$ mice at several different time points following infection. Wild type and MyD88$^{-/-}$ mice (n = 5 per group) were

infected with a low-dose challenge of 5×10^2 CFU *B. mallei* delivered i.n. Mice were then euthanized at 24, 48 or 72 hours after challenge. At each time point bacterial burdens were determined in blood, lung, liver, and spleen homogenates. MyD88$^{-/-}$ mice had significantly increased bacterial burdens in all organs at all time points after infection, compared to wild type mice **(Figure 4.3)**.

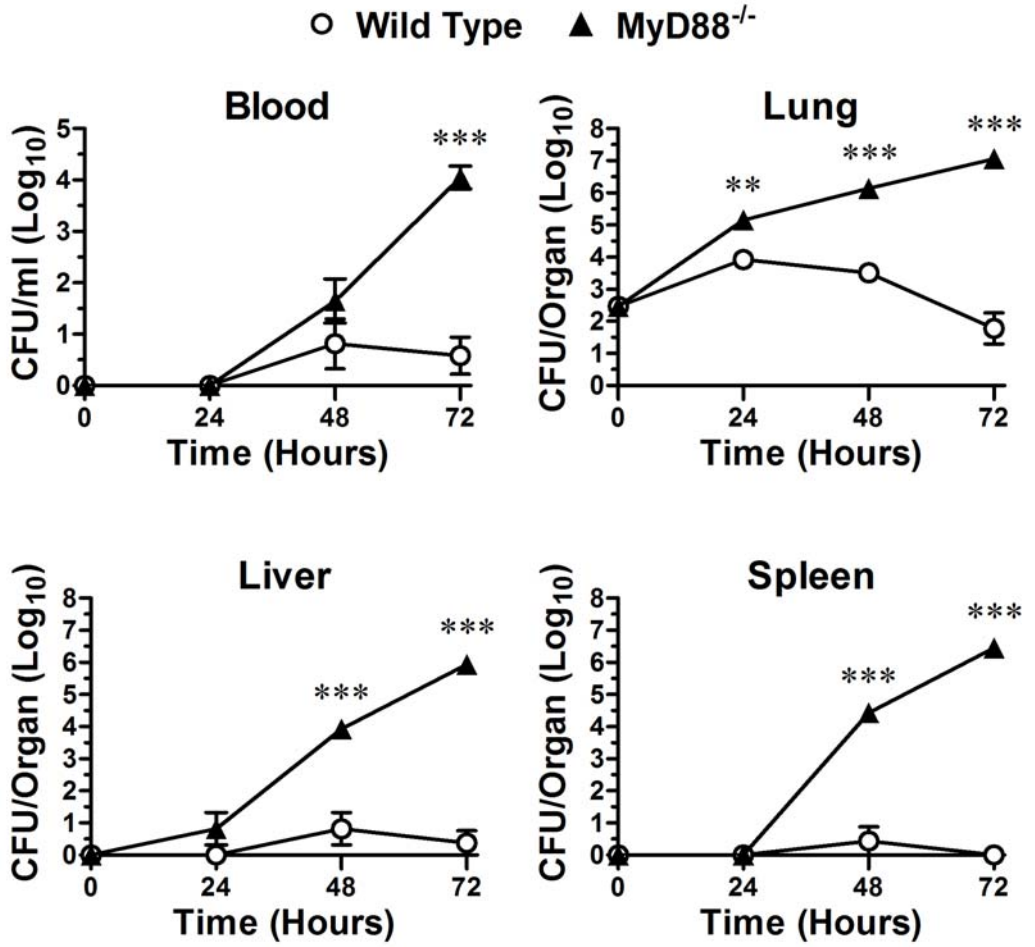

Figure 4.3. Increased bacterial burden in MyD88$^{-/-}$ mice following low dose respiratory *B. mallei* challenge. Wild type and MyD88$^{-/-}$ mice were challenged with 5×10^2 CFU *B. mallei* i.n. Wild type and MyD88$^{-/-}$ mice (n = 3-5 per group) were euthanized at 24, 48 or 72 hours post infection and bacterial burden was determined in blood, lung, liver and spleen as described in materials and methods. Data are expressed as means ± SEM $\log_{10}$ CFU/ml for blood, and means ± SEM $\log_{10}$ CFU/organ for lung, liver and spleen. Statistical differences were determined between wild type and MyD88$^{-/-}$ mice at each time point by two-tailed Student's t-test (** $p < 0.01$, *** $p < 0.001$). Data are representative of two independent experiments.

Next, organ injury in response to *B. mallei* infection in wild type and MyD88$^{-/-}$ mice was investigated using histopathology. Wild type and MyD88$^{-/-}$ mice (n = 5 per group) were euthanized on day 3.5 after infection with 5×10^2 CFU i.n. IFN-$\gamma^{-/-}$ mice used for comparison to MyD88$^{-/-}$, were also infected with 5×10^2 CFU i.n. and were euthanized 2 days after infection. Lung and liver tissues were fixed and routinely processed for microscopic examination **(Figure 4.4)**. Inflammatory responses in the lungs of MyD88$^{-/-}$ mice were more severe than in wild type mice. However, the pathologic changes observed in the lungs of MyD88$^{-/-}$ mice were notably milder compared to lesions observed in other strains of mice that are highly susceptible to *B. mallei* infection, e.g., CCR2$^{-/-}$ or IFN-$\gamma^{-/-}$ mice (Fig 4.4 and Fig 3.5). In addition, the livers of wild type mice had small resolving lesions, whereas the lesions in MyD88$^{-/-}$ mice were multifocal to coalescing and characterized by widespread hepatocyte necrosis and leukocyte infiltration (Fig 4.4). In contrast to the lung pathology, the liver pathology in MyD88$^{-/-}$ mice was much more severe than liver pathology seen in CCR2$^{-/-}$ or IFN-$\gamma^{-/-}$ mice (Fig 4.4 and Fig 3.5). Comparison of bacterial burdens between MyD88$^{-/-}$, CCR2$^{-/-}$ and IFN-$\gamma^{-/-}$ mice revealed that IFN-$\gamma^{-/-}$ mice had significantly higher bacterial burdens in both the lung and liver as compared to MyD88$^{-/-}$ mice ($p < 0.05$), while no significant differences were observed between MyD88$^{-/-}$ and CCR2$^{-/-}$ mice (one-way ANOVA, data not shown). Therefore, we concluded that the increased lung pathology observed in IFN-$\gamma^{-/-}$ mice as compared to MyD88$^{-/-}$ mice may be due to increased bacterial burden in IFN-$\gamma^{-/-}$ mice. However, the dramatic increases in liver pathology observed in MyD88$^{-/-}$ mice appeared to be relatively independent of hepatic bacterial burden, as CCR2$^{-/-}$ mice had equivalent hepatic bacterial burdens to MyD88$^{-/-}$ mice, yet exhibited minimal hepatic pathology (Fig 3.5 and Fig 4.4).

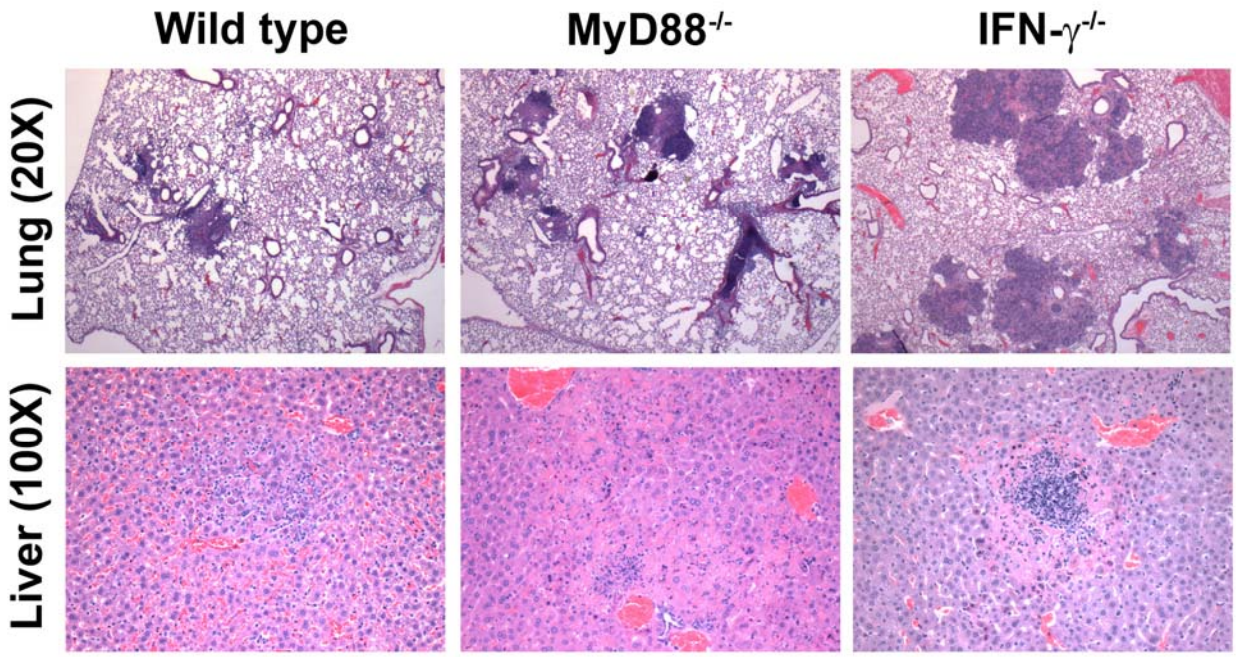

Figure 4.4. Lung and liver pathology following respiratory *B. mallei* challenge in wild type, MyD88$^{-/-}$, and IFN-γ$^{-/-}$ mice. Mice (n = 5 per group) were subjected to i.n. challenge with 5×10^2 CFU *B. mallei* and organs were collected for histological examination 72 hours after infection (WT and MyD88$^{-/-}$) or 48 hours after infection (IFN-γ$^{-/-}$). Representative images from the lungs (top row; 20X magnification), and livers (bottom row; 100X magnification) of infected wild type, MyD88$^{-/-}$ and IFN-γ$^{-/-}$ mice are presented.

4.4(5) Cellular recruitment to the lung is impaired in MyD88$^{-/-}$ mice.

Previous studies have reported that neutrophils were critical for control of *B. pseudomallei* and *B. mallei* infection (21-22). In contrast, we found instead that monocytes were critical for control of *B. mallei* infection, based on the fact that CCR2$^{-/-}$ mice were highly susceptible to infection (Chapter 3). Therefore, in the present study we investigated the effects of TLR signaling on chemokine production and monocyte recruitment to the lungs of mice following *B. mallei* inoculation. Chemokine concentrations in lung homogenates and plasma from wild type and MyD88$^{-/-}$ mice (n = 5 per group) were assessed 24 hours after i.n. inoculation with 5×10^2 CFU *B. mallei*. Production of both CCL2 (MCP-1) and CXCL1 (KC) was significantly reduced in MyD88$^{-/-}$ mice compared to WT animals **(Figure 4.5)**.

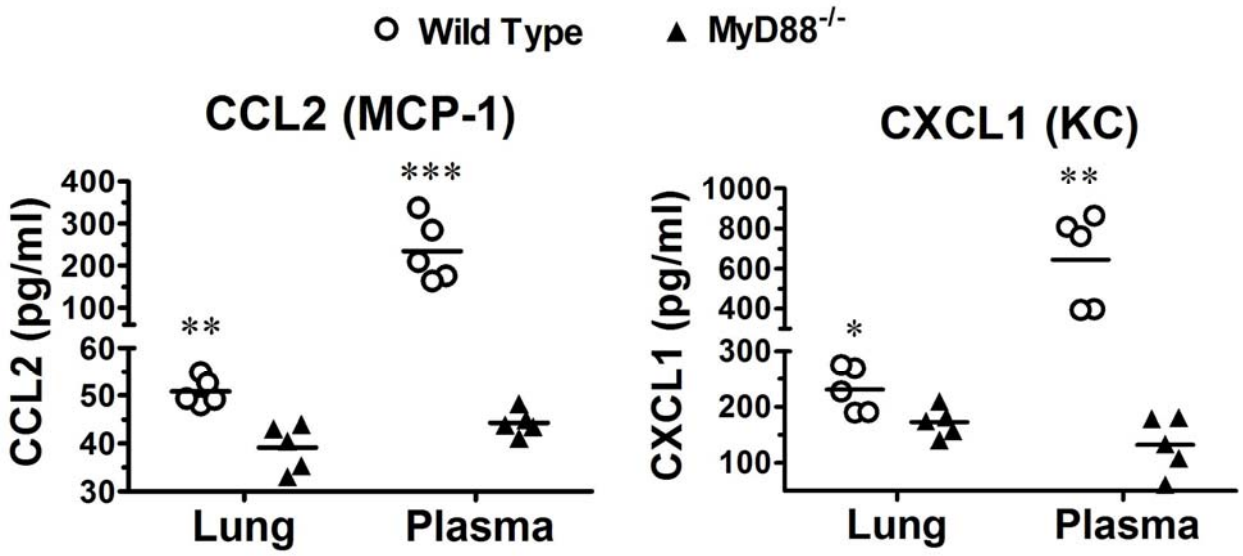

Figure 4.5. Chemokine production following low-dose *B. mallei* challenge. Wild type and MyD88$^{-/-}$ mice (n = 5 per group) were infected i.n. with 5×10^2 CFU *B. mallei*. 24 hours after infection mice were euthanized, and chemokine levels in lung homogenate and plasma were determined as described in materials and methods. Data are presented as individual values with bars representing the mean concentration for each group. Statistical differences between wild type and MyD88$^{-/-}$ mice were determined by a two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are representative of two independent experiments.

Twenty-four hours after inoculation, airway cells were collected by BAL and lung cells were obtained by enzymatic digestion and examined by flow cytometry. In both the airways and lung tissues of MyD88$^{-/-}$ mice, there were significantly reduced numbers of inflammatory cells compared to wild type mice. Notably, the numbers of CD11c^{+}CD11b^{+} DCs, Ly6C^{hi} inflammatory monocytes, and neutrophils were significantly lower in both the airways and lung tissues of infected MyD88$^{-/-}$ mice **(Figure 4.6)**. The lack of monocyte, DC and neutrophil recruitment was particularly pronounced in the airways. Preliminary experiments demonstrated that the NK cell and CD4^{+} T cell populations in lung tissues were not different in MyD88$^{-/-}$ versus wild type mice. However, MyD88$^{-/-}$ mice had increased numbers of CD8^{+} T cells (data not shown) and alveolar macrophages in the lungs (Fig. 4.6). Thus, we concluded that recruitment of key innate immune effector cells, especially inflammatory DC, inflammatory

monocytes, and neutrophils, was MyD88 dependent early after pulmonary infection with *B. mallei*.

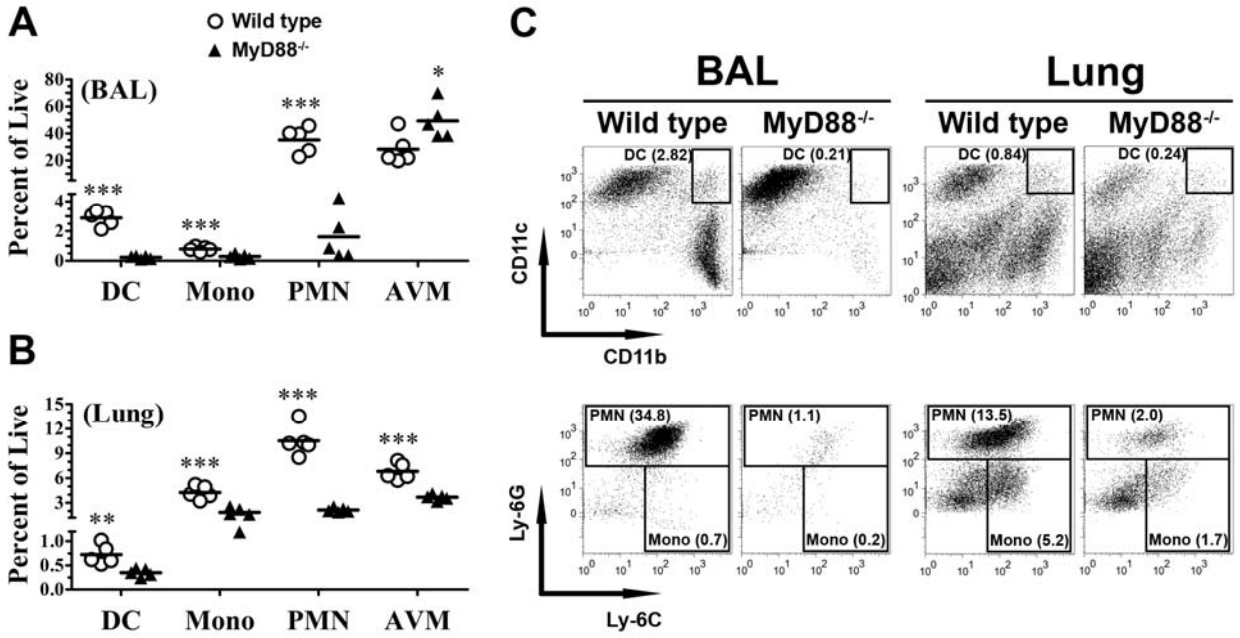

Figure 4.6. Cellular inflammation to the lung is reduced in MyD88$^{-/-}$ mice following intranasal *B. mallei* challenge. Wild type and MyD88$^{-/-}$ mice (n = 5 per group) were infected i.n. with 5×10^2 CFU *B. mallei*. 24 hours after infection mice were euthanized, BAL and lung cells were isolated and immunostained as described in methods. Cell populations were defined as follows: Dendritic cells (DC): CD45$^+$/CD11b$^+$/CD11c$^+$; Monocytes (Mono): CD45$^+$/CD11b$^+$/Ly6-C$^+$/Ly6G$^-$; Neutrophils (PMN): CD45$^+$/CD11b$^+$/Ly6G$^+$; Alveolar macrophages (AVM): CD45$^+$/CD11c$^+$/CD11b$^-$. The percentage of live cells for each cell type are displayed as individual data points with bars representing the mean value for each population isolated from the BAL (A) or lung tissue (B). Data are representative of two independent experiments. Statistical differences between wild type and MyD88$^{-/-}$ mice were determined by a two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (C) Representative dot plots illustrating DCs (top panel) populations, as well as Mono and PMN populations (bottom panel). BAL dot plots were generated by using FlowJo software to pool all 5 BAL .fcs files from each group. 50,000 events from each pooled BAL .fcs file were used to generate representative dot plots. Lung dot plots are individual samples representative of each group. The percentage of total live cells is listed in, or next to each gate. Data are representative of two independent experiments.

4.4(7) Cytokine production by immune effector cells in the lungs in response to *B. mallei* infection.

Next, we conducted intracellular cytokine production studies to identify the cellular sources of key protective cytokines in the lungs early after *B. mallei* infection. Wild type mice (n = 5) were inoculated with 10^4 CFU *B. mallei* i.n. and euthanized 48 hours after infection. Lung tissue was harvested and processed for detection of intracellular cytokine production as described in methods. Cell types producing IL-12, IFN-γ and TNF-α were investigated **(Figure 4.7)**. The major cell type producing IL-12 in the lung following *B. mallei* infection was found to be a population of $CD11b^+CD11c^+$ DC. Smaller amounts of IL-12 were also produced by inflammatory monocytes and resident alveolar macrophages. The majority of IFN-γ was produced by NK cells in the lungs, while small numbers of T cells also expressed IFN-γ.

Unexpectedly, a large population of $CD45^+$/IFN-γ^+ cells that did not express NK cell or T cell markers (i.e., $CD45^+/NK1.1^-/CD3^-$) was also observed (data not shown). We found that within this population of cells, there were IFN-γ^+ DCs, monocytes and neutrophils (Fig 4.7, data not shown). Previous studies have demonstrated that multiple myeloid cell types can produce IFN-γ following stimulation with IL-12 (32-33). However, the remaining IFN-γ^+ cells were not identified, though we did note that they were not γδ-T cells (data not shown).

The cellular source of TNF-α production was investigated next. We found that neutrophils were the major cellular source of TNF-α production in the lungs shortly after *B. mallei* infection (Fig. 4.7). This finding differs from results obtained in a *B. pseudomallei* model, where monocytes were identified as the major source of TNF-α production (21). We also observed that some monocytes, dendritic cells, and alveolar macrophages expressed TNF-α.

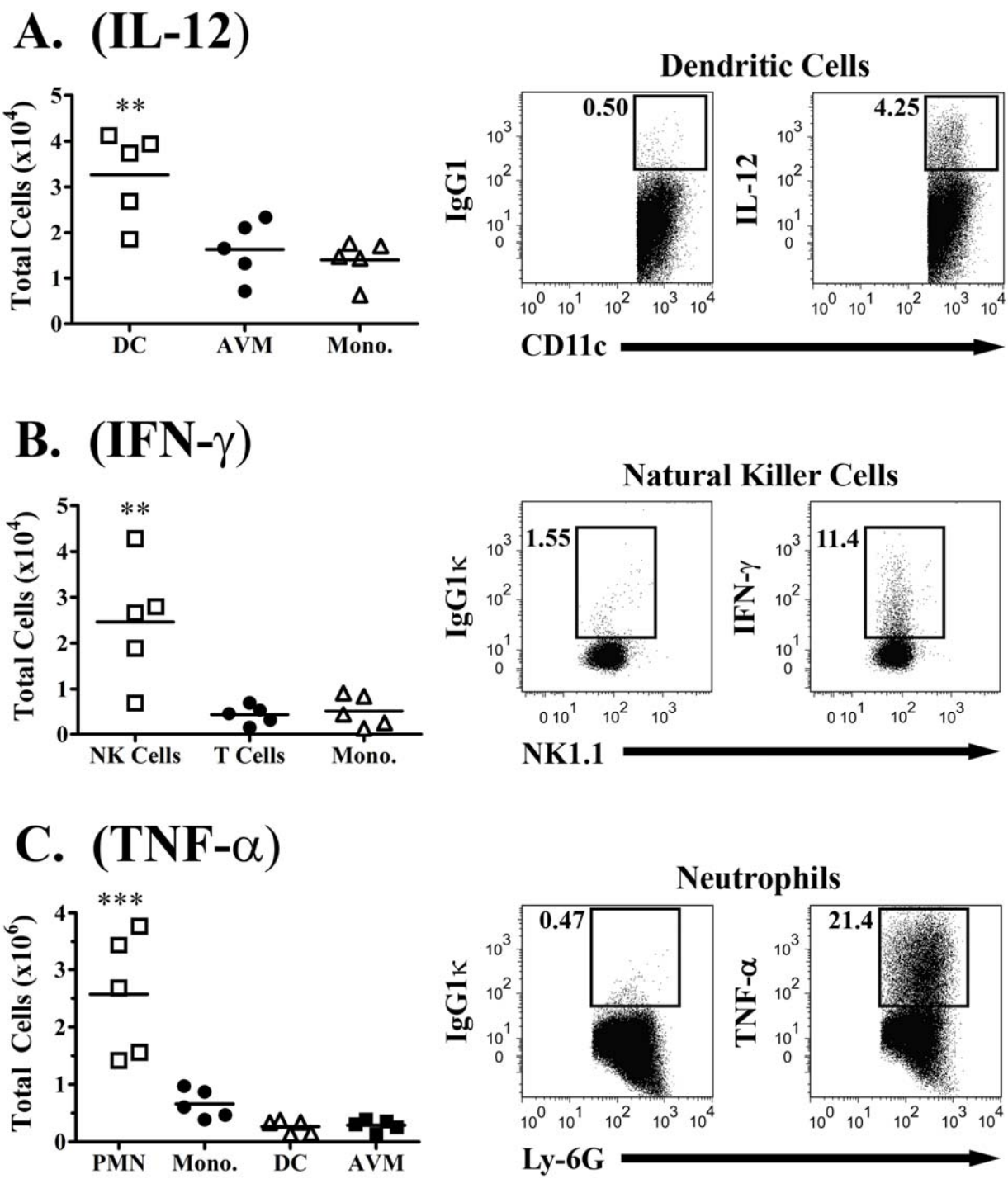
A. (IL-12)
Total Cells (x10^4)
**
DC
AVM
Mono.
Dendritic Cells
0.50
4.25
IgG1
IL-12
CD11c
B. (IFN-γ)
Total Cells (x10^4)
**
NK Cells
T Cells
Mono.
Natural Killer Cells
1.55
11.4
IgG1κ
IFN-γ
NK1.1
C. (TNF-α)
Total Cells (x10^6)
***
PMN
Mono.
DC
AVM
Neutrophils
0.47
21.4
IgG1κ
TNF-α
Ly-6G

Figure 4.7. Cellular sources of IL-12, IFN-γ, and TNF-α following respiratory *B. mallei* challenge. Wild type mice (n = 5) were challenged with 10^4 CFU *B. mallei* intranasally and euthanized 48 hours after infection. Lung tissue was digested and immunostained as described in methods. Cell populations were defined as follows: Dendritic cells (DC): $CD45^+/CD11b^+/CD11c^+$; Monocytes (Mono): $CD45^+/CD11b^+/Ly6\text{-}C^+/Ly6G^-$; Neutrophils (PMN): $CD45^+/CD11b^+/Ly6G^+$; Alveolar macrophages (AVM): $CD45^+/CD11b^-/CD11c^+$; Natural killer cells (NK Cells): $CD45+/NK1.1^+$; T cells: Low FSC-SSC/$CD3^+$. Cellular sources of IL-12 are presented in (A), IFN-γ in (B) and TNF-α in (C). Total cytokine producing cells were calculated by subtracting isotype positive cells from cytokine positive cells. Data are graphed as individual values with bars representing the mean value for each cell type. Statistical differences were determined by a one-way ANOVA, followed by a Tukey's multiple means test to determine differences between cell types (** $p < 0.01$, *** $p < 0.001$). Representative flow plots are presented from the major cell type producing each cytokine. Flow plots were generated by pooling all 5 samples into one .fcs file, and then equalizing the number of events in each pooled flow plot. Values in flow plots represent the percentage of each cell type. Data are representative of two independent experiments.

4.4(8) MyD88-dependent cytokine production following *B. mallei* infection.

Analysis of cytokines production at in multiple organs at multiple time points, and investigation of association with between bacterial burdens resulted in a large data set. Therefore, cytokine production will be summarized in this section; with the entire data set presented in Appendix I tables A1.1 to A1.4. Cytokine concentrations in lung tissues and plasma were investigated in wild type and $MyD88^{-/-}$ mice following i.n. challenge with 5×10^2 CFU *B. mallei*. Mice (n = 5) were euthanized 24, 48 or 72 hours after infection, and cytokine concentrations were measured in lung homogenate and plasma as described in Methods. Compared to WT mice, $MyD88^{-/-}$ mice had significantly reduced concentrations of TNF-α in the lung and plasma at 24 hours after infection, whereas concentrations were not different at later time points **(Figure 4.8)**. IL-12 and IL-10 concentrations in lung tissues were below the limit of detection from both groups of animals (data not shown). IL-6 production in lungs was significantly reduced in $MyD88^{-/-}$ mice compared to WT animals (Appendix I, Table A1.1). Remarkably, we found that $MyD88^{-/-}$ mice were unable to produce detectable IFN-γ in lung tissues or in circulation at any time point examined (Fig 4.8). Thus, we concluded that

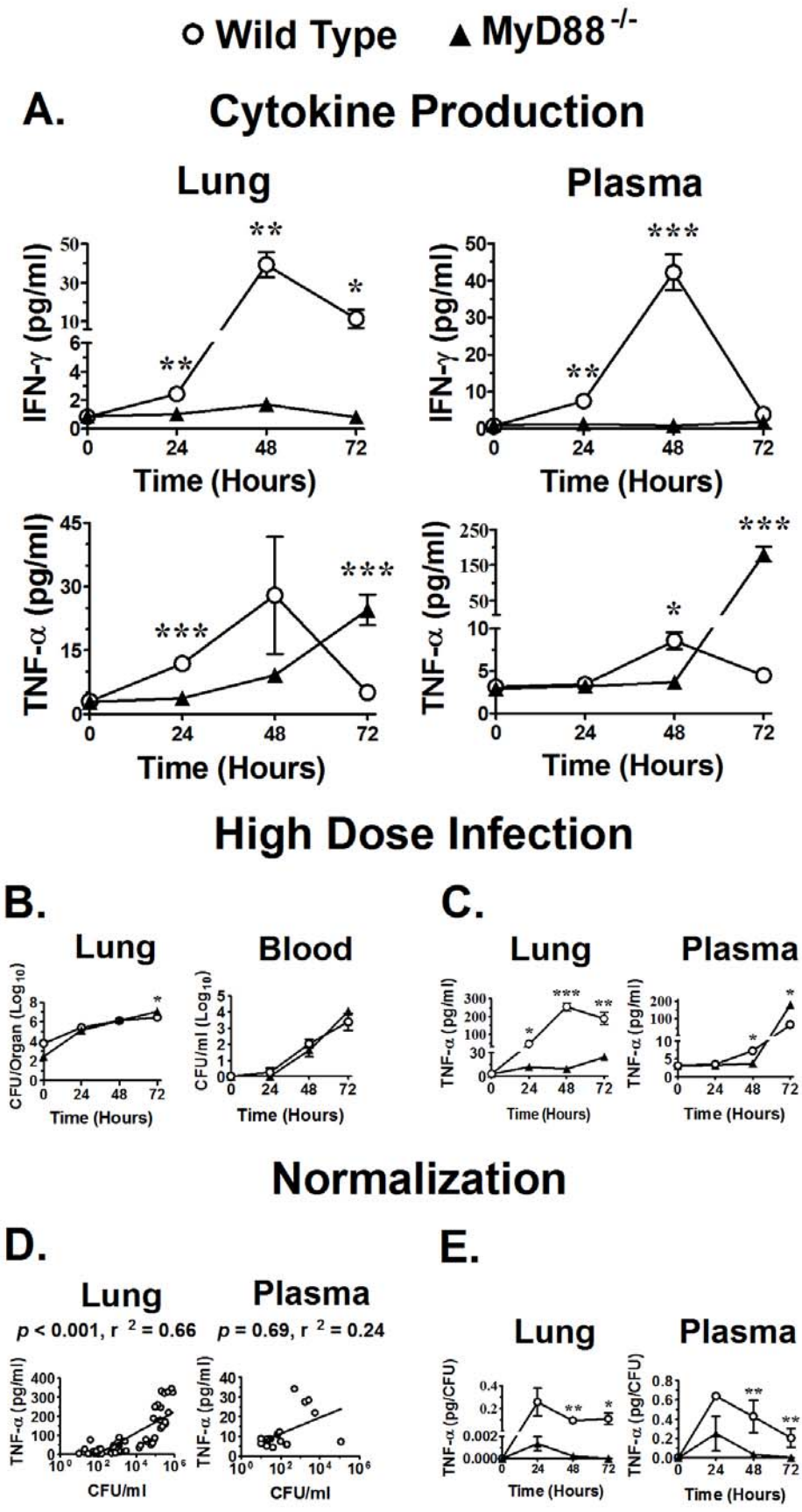
Wild Type
MyD88 -/-
A.
Cytokine Production
Lung
Plasma
IFN-γ (pg/ml)
TNF-α (pg/ml)
Time (Hours)
High Dose Infection
B.
C.
Lung
Blood
Plasma
CFU/Organ (Log10)
CFU/ml (Log10)
Normalization
D.
E.
p < 0.001, r 2 = 0.66
p = 0.69, r 2 = 0.24
CFU/ml
TNF-α (pg/CFU)

Figure 4.8. Cytokine production in wild type and MyD88$^{-/-}$ mice following respiratory *B. mallei* challenge. Wild type and MyD88$^{-/-}$ mice were infected i.n. with *B. mallei*. Mice were euthanized 24, 48 or 72 hours after infection, and both cytokine production and bacterial burden were determined in lung homogenate and plasma, as described in materials and methods. Wild type mice are represented by open circles, and MyD88$^{-/-}$ mice by closed triangles. (A) Cytokine production in lung and plasma of wild type and MyD88$^{-/-}$ mice (n = 5 per group), infected i.n. with 5×10^2 CFU. (B and C) Bacterial burden and cytokine production in lung homogenate and blood of mice infected with *B. mallei* i.n. (wild type 10^4 CFU; MyD88$^{-/-}$ 5×10^2 CFU) (n = 5 per group). (D) Correlation between TNF-α production and bacterial burden in lung homogenate (n = 60) and plasma (n = 18) of wild type mice infected with either 10^4 or 5×10^2 CFU *B. mallei* i.n. (E) TNF-α production mathematically normalized to bacterial burden in the lung homogenate and plasma of wild type and MyD88$^{-/-}$ mice infected with 5×10^2 CFU *B. mallei* i.n. For (A-C, E) data are plotted as mean ± SEM, and graphs are representative of two independent experiments. For (D) individual values are plotted, and data were pooled from 2 independent experiments. Best fit lines in (D) illustrate the semi-log regression for each data set. Statistical differences were determined between wild type and MyD88$^{-/-}$ mice at each time point by two-tailed Student's t-test. Correlation analysis was performed using a two-tailed Pearson's correlation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

production of IFN-γ following pulmonary infection with *B. mallei* was completely dependent on MyD88 signaling, whereas production of other key pro-inflammatory cytokines (TNF-α, IL-6) was only partially MyD88 dependent.

The fact that bacterial burdens were very different in the lungs of MyD88$^{-/-}$ and WT mice could confound interpretation of cytokine response data. Therefore, to eliminate this potential bias, we increased the challenge dose in WT animals to achieve equivalent tissue bacterial burdens in both WT and MyD88$^{-/-}$ animals (Fig. 4.8B). Even after achieving equivalent bacterial burdens, we noted that production of IFN-γ, TNF-α and IL-6 were all significantly reduced in MyD88$^{-/-}$ mice as compared to wild type animal (Fig. 4.8C, Appendix I Table A1.2). Interestingly, plasma concentrations of TNF-α, but not IL-6 or IFN-γ, were increased in MyD88$^{-/-}$ mice at 72 hours compared to wild type mice with equivalent tissue bacterial burdens (Fig. 4.8C, Appendix I Table A1.). Thus, MyD88$^{-/-}$ mice appeared to have a compensatory mechanism for systemic production of TNF-α in response to i.n. *B. mallei* infection.

Since adjusting bacterial challenge doses to achieve equal bacterial burdens is time consuming and expensive, we investigated whether it was possible to simply adjust cytokine concentrations based on bacterial burden. The validity of this assumption was explored by determining whether there was a significant correlation between cytokine concentration and bacterial burden at different bacterial burdens. Bacterial burdens and cytokine concentrations were measured in the lung (n = 60) and plasma (n = 18) of wild type mice infected with either 5×10^2 or 10^4 CFU *B. mallei*. In the lung we found significant correlations with bacterial burden for CCL2, CXCL1, IL-6, and TNF-α concentration ($p < 0.001$). However, a correlation with IFN-γ concentrations was not observed ($p = 0.28$) (Appendix I Table A1.3; two-tailed Pearson's correlation). Cytokine production from wild type and MyD88$^{-/-}$ mice infected with 5×10^2 CFU i.n. (Fig. 4.8A), was mathematically normalized to bacterial burden (Fig. 4.8E, Appendix I Table A1.4). Normalization of TNF-α and IL-6 in the lung produced results consistent with results obtained following a high dose infection presented in figure 4.8C (Appendix I Table A1.4). These results demonstrate that the increased production of TNF-α seen at 72 hours in figure 4.8A was most likely due to increased bacterial burden in MyD88$^{-/-}$ mice. In contrast, plasma concentrations did not correlate with bacterial burden for any of the cytokines investigated in this study (Fig. 4.8D, Appendix I Table A1.3). Therefore, in the lung, but not the blood, normalization of cytokine concentrations to bacterial burden may be valid for evaluation of most inflammatory cytokines, though not for IFN-γ.

In summary, *B. mallei* infection induced early production of IFN-γ, TNF-α and IL-6 in both the lung and plasma of wild type animals, whereas concentrations were significantly reduced in MyD88$^{-/-}$ mice. However, MyD88$^{-/-}$ mice had increased production of TNF-α in the plasma at 72 hours, which was independent of bacterial burden in the blood (Fig. 4.8C). One

explanation for the increased TNF-α production in MyD88$^{-/-}$ mice is reduced expression of regulatory factors such as suppressor of cytokine signaling (SOCS), and Src homology 2-containing protein (CIS) which has been shown to occur in MyD88$^{-/-}$ mice (34). Another possibility is that the increased bacterial burdens in the spleen (Appendix I, Table A1.2) may have resulted in increased TNF-α production in the plasma. MyD88$^{-/-}$ mice were however incapable of producing IFN-γ at any time point, regardless of bacterial burden.

4.4(9) IL-6 production is not detrimental following *B. mallei* infection.

Cytokine analysis demonstrated that MyD88$^{-/-}$ mice were capable of producing TNF-α and IL-6 in response to *B. mallei* infection, and that high levels were observed in mice upon euthanasia. High levels of TNF-α are known to cause immune mediated tissue damage in other animal models, although TNF-α is also known to be necessary for protection following *B. pseudomallei* infection (18, 35). Therefore, we focused on the role of IL-6, since increased IL-6 production generally correlates with decreased survival in gram-negative sepsis (36-38). Additionally, in humans that developed septic melioidosis, increased IL-6, but not TNF-α, concentrations were significantly correlated with increased mortality (39). To address the role of IL-6 in regulating susceptibility to *B. mallei* infection, we infected IL-6$^{-/-}$ and wild type C57BL/6 mice (n = 5 per group) i.n. with 3×10^3 CFU *B. mallei*. We found that IL-6$^{-/-}$ mice were equally susceptible to *B. mallei* infection as wild type mice, though the IL-6$^{-/-}$ mice exhibited markedly few outward signs of infection (**Figure 4.9**). These results suggest that IL-6 production is not detrimental to *B. mallei* infection.

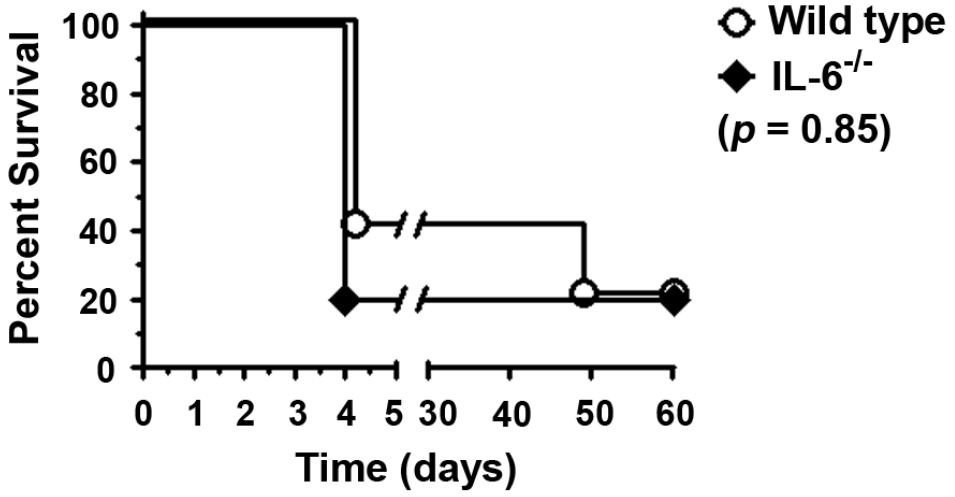

Figure 4.9. IL-6 production is not detrimental to immune protection against *B. mallei* infection. Wild type and IL-$6^{-/-}$ mice (n = 5 per group) were challenged with 3×10^3 CFU *B. mallei* i.n. Survival was monitored and mice were euthanized upon reaching a pre-determined endpoint. Statistical differences were determined using Kaplan-Meier curves and log rank analysis. Data are representative of two independent experiments.

4.4(10) Recombinant IFN-γ treatment protects MyD88$^{-/-}$ mice.

Therefore, we focused our attention next on the IFN-γ production defect in MyD88$^{-/-}$ mice, and whether IFN-γ was indeed the key cytokine required for MyD88-dependent protection from *B. mallei* infection. To address this question, MyD88$^{-/-}$ mice were treated with recombinant murine IFN-γ immediately after *B. mallei* infection. Mice were treated once daily with 10^5 units of rIFN-γ administered intraperitoneally (i.p.) as described previously in a mouse *Salmonella* infection model (27). Mice (n = 5) were treated with rIFN-γ at the time of infection and then once daily for five days, while a separate group of control MyD88$^{-/-}$ mice (n = 5) received i.p. injections of diluent. Treatment with rIFN-γ provided significant, though not complete, protection against respiratory *B. mallei* infection when compared to MyD88$^{-/-}$ mice receiving diluent **(Figure 4.10)**. Additionally, while 80% of MyD88$^{-/-}$ mice treated with rIFN-γ cleared

bacteria from their lungs, all mice ultimately developed disseminated infection in the spleen and liver (data not shown).

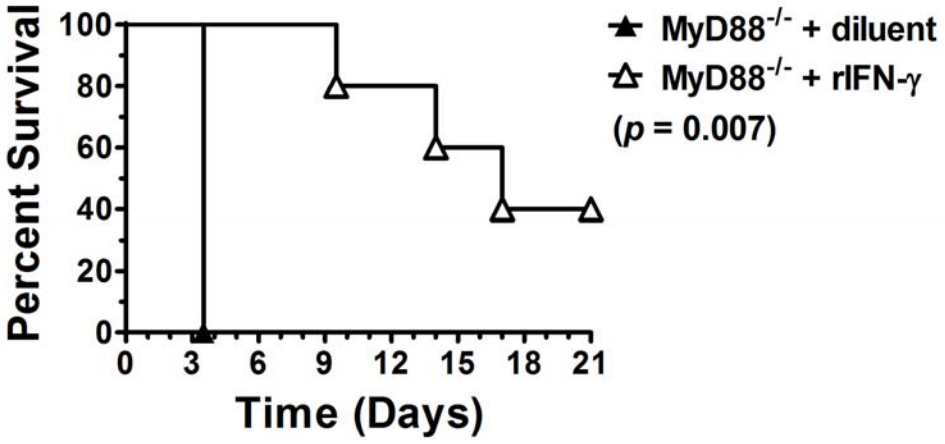

Figure 4.10. rIFN-γ treatment protects MyD88$^{-/-}$ mice against lethal *B. mallei* infection. MyD88$^{-/-}$ mice were treated i.p. with recombinant IFN-γ (rIFN-γ) or diluent (PBS + 0.1% BSA) (n = 5 per group) as described in materials and methods. Mice were challenged with 5×10^2 CFU *B. mallei* i.n. Survival was monitored and mice were euthanized upon reaching a pre-determined endpoint. Statistical differences were determined using Kaplan-Meier curves and log rank analysis. Data were pooled from two independent experiments.

4.5 Discussion.

In the present study the role of MyD88 signaling in generating protective immunity against pulmonary challenge with *B. mallei* was investigated in order to gain a better understanding of the role of innate immunity in controlling pulmonary infection with this important pathogen. Notably, we found that MyD88$^{-/-}$ mice were highly susceptible to infection with even very low intranasally-delivered doses of *B. mallei*. Along with an inability to control bacterial replication in the lungs, inflammatory responses in the lungs of MyD88$^{-/-}$ mice were significantly attenuated. For example, MyD88$^{-/-}$ mice were unable to recruit inflammatory monocytes, myeloid dendritic cells, or neutrophils to the lungs. In contrast, no differences in pulmonary NK or CD4^{+} T cell populations were observed, while CD8^{+} T cells were increased in MyD88$^{-/-}$ mice. Cytokine analysis revealed that MyD88$^{-/-}$ mice were completely unable to produce IFN-γ, while production of TNF-α and IL-6 was only reduced at early time points. Remarkably, despite the large number of immune defects observed in MyD88$^{-/-}$ mice following *B. mallei* infection, treatment of MyD88$^{-/-}$ mice with only rIFN-γ was able to significantly reverse their susceptibility to *B. mallei* infection.

Some immune responses in MyD88$^{-/-}$ mice following *B. mallei* infection were similar to those observed previously in MyD88$^{-/-}$ mice infected with *B. pseudomallei* (15). For example, neutrophil recruitment was reduced in both infection models in MyD88$^{-/-}$ mice. However, MyD88$^{-/-}$ mice infected with *B. mallei* also exhibited significant defects in monocyte recruitment, which was not observed previously in MyD88$^{-/-}$ mice infected with *B. pseudomallei* or *B. thailandensis* (14-15). The reasons for this difference are unclear, since MyD88$^{-/-}$ mice infected with any of these three *Burkholderia* species produce significantly less CCL2 than wild-type animals (14-15).

Other notable differences between *B. mallei* and *B. pseudomallei* infected mice include differences in cell types producing TNF-α. We found that neutrophils were the major cellular source of TNF-α production in the lungs following *B. mallei* infection, whereas previous studies have found that monocytes were the primary source of TNF-α production following *B. pseudomallei* infection (21). The reasons for these differences are unclear, but could be related to differences in the bacterial envelope (e.g., lipopolysaccharide (LPS) or capsule) or to bacterial burden or timing of sampling. In agreement with previous studies in *B. mallei* and *B. pseudomallei*, IFN-γ was produced mainly by NK cells (19, 21, 40).

Our earlier studies in CCR2$^{-/-}$ mice indicated that IFN-γ was critical for protection against *B. mallei* infection (Chapter 3). Therefore production of IL-12, which is the key molecule regulating NK cell production of IFN-γ, was investigated following *B. mallei* infection. We found that IL-12 in the lungs was produced largely by DCs and monocytes. Since recruitment of both DCs and monocytes to the lungs was significantly impaired in MyD88$^{-/-}$ mice, we postulate that the defect in IFN-γ production observed in the lungs of MyD88$^{-/-}$ mice was due to lack of local IL-12 production. Our results are in accordance with previous findings from a *Listeria monocytogenes* infection model, which also identified monocyte derived DCs as the major cellular source of IL-12. Additionally, IL-12 production by DCs following *L. monocytogenes* infection was found to be dependent on MyD88 signaling (41).

IFN-γ is known to be critical to the immune response to *B. mallei* infection, and inflammatory monocytes appear to be critical for production of IFN-γ (Chapter 3 (19)). Monocytes were also found to be critical for protection following intravenous inoculation with *Francisella tularensis*, where MyD88$^{-/-}$, IFN-γR$^{-/-}$ and CCR2$^{-/-}$ mice all had defects in monocyte recruitment to the spleen (42). Similar to the current study, IFN-γ production following

intravenous *F. tularensis* infection was also found to be MyD88 dependent. Signaling through the IFN-γR was necessary for CCL2 production, and CCL2 production was necessary for monocyte recruitment. A similar TLR dependent signaling circuit may be occurring following *B. mallei* infection of MyD88$^{-/-}$ mice.

Important differences were also identified however, including the fact that in the *B. mallei* infection model TNF-α production remained intact in CCR2$^{-/-}$ mice despite reduced monocyte recruitment to the lung. Neutrophils were the major source of TNF-α production in *B. mallei* infected mice, and TNF-α production was at least partially MyD88-independent. Additionally, although CCR2$^{-/-}$ mice retained the ability to signal through MyD88 and produce IFN-γ in response to TLR3 activating ligands, CCR2$^{-/-}$ mice produced less IFN-γ following *B. mallei* infection (Fig. 3.10). These results demonstrate that additional signals, in addition to MyD88, are necessary for production of IFN-γ following infection with *B. mallei*.

Although administration of exogenous rIFN-γ was sufficient to induce partial but significant protection of MyD88$^{-/-}$ mice from acute disease following *B. mallei* infection, the protection was not complete. This suggests that other cytokines may be necessary for full protection. Indeed, production of a number of other cytokines were significantly reduced in *B. mallei* infected MyD88$^{-/-}$ mice. For example, production of TNF-α, CCL2, CXCL1, and IL-6 were all significantly reduced 24 hours post infection in the lungs of MyD88$^{-/-}$ mice compared to wild type mice (Fig. 4.5, 4.8 and Appendix I Table A1.1). Similar to studies in a *B. thailandensis* model of pulmonary infection, we found that MyD88 independent mechanisms resulted in increased cytokine production in MyD88$^{-/-}$ mice 72 hours after infection, though the extremely high bacterial burdens in MyD88$^{-/-}$ mice made interpretation of cytokine production difficult (Fig 4.3) (14).

It was also noted in this study that MyD88$^{-/-}$ mice infected with *B. mallei* developed unique pathologic changes as compared to wild type animals (Fig. 4.4). A similar effect was observed previously in MyD88$^{-/-}$ mice infected with *B. pseudomallei* (15). Interestingly, pulmonary pathology was only slightly increased as compared to wild type animals despite significantly higher pulmonary bacterial burdens in MyD88$^{-/-}$ mice (Fig4.3). One possible explanation for the relative lack of pulmonary pathology is the reduced chemokine and cytokine production in MyD88$^{-/-}$ mice (Fig. 4.6 and 4.8), which may have resulted in reduced inflammatory cell recruitment and tissue damage. In contrast to MyD88$^{-/-}$ mice, CCR2$^{-/-}$ mice had severe lung pathology (Fig 3.5) and had increased production of TNF-α and KC in the lung as compared to wild type mice (Fig. 3.10).

In contrast to the lung, MyD88$^{-/-}$ mice developed more severe liver pathology as compared to wild type, CCR2$^{-/-}$, or IFN-γ$^{-/-}$ mice. It is possible that the high bacterial burdens in the livers of MyD88$^{-/-}$ mice may account for this dramatic liver pathology. However, previous studies have demonstrated that *B. mallei* infection of CCR2$^{-/-}$ or IFN-γ$^{-/-}$ mice results in equivalent or increased liver bacterial burdens as compared to MyD88$^{-/-}$ mice (data not shown), yet neither CCR2$^{-/-}$ or IFN-γ$^{-/-}$ mice develop the severe liver pathology observed in MyD88$^{-/-}$ mice (Fig. 3.5 and 4.4). Thus, we propose that increased systemic production of TNF-α in MyD88$^{-/-}$ mice may be one explanation for the accentuated liver pathology observed in this study, likely triggered by LPS released from dead or dying bacteria (see Fig. 4.8) (35). Moreover, the adverse effects of LPS and TNF-α on the liver appear to be magnified by the lack of MyD88 signaling. For example, MyD88$^{-/-}$ mice are known to have defects in liver re-generation (43). Additional defects in MyD88$^{-/-}$ mice may also contribute to liver damage, as

other cytokines in addition to TNF-α are known to elicit liver damage and suppress effective regeneration (44-47).

In summary, we report here that MyD88 is a critical molecule required for effective innate immunity against pulmonary infection with *B. mallei*. Interestingly, lack of TLR2 or TLR4 signaling did not recapitulate the effects of MyD88 deficiency in our studies. As TLR9 signaling is also reported to not play a role in protection from *B. pseudomallei* infection, mice lacking individual TLRs do not appear to be as susceptible to *Burkholderia* infection as MyD88$^{-/-}$ mice (14-16). These results suggest that multiple TLRs must be concurrently inactive to reproduce the MyD88 phenotype. Alternatively, TLR independent, but MyD88-dependent signaling through the IL-1βR or IL-18R signaling pathways may be necessary for inducing full protection against *Burkholderia* infection.

Studies from *Mycobacterium tuberculosis* have indicated that MyD88 may be necessary for IL-1R signaling, as TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, or even TLR2/4/9$^{-/-}$ mice are not as susceptible as MyD88$^{-/-}$ mice (48-52). In contrast, IL-1R1$^{-/-}$ mice are also highly susceptible to *M. tuberculosis*, suggesting that the critical role for MyD88 following *M. tuberculosis* infection may be IL-1R signaling (53). Although IL-18 does not appear to be necessary for in response to *M. tuberculosis* infection, MyD88 signaling through the IL-18R is important in response to *Legionella pneumophila* (53-54). In addition, recent studies from *L. monocytogenes* have shown that MyD88 is necessary for IL-18 signaling in NK cells (55). IL-18 is known to be necessary following *B. pseudomallei* infection, and is also known to contribute to IFN-γ stimulation (19, 56). More recent studies have demonstrated that while IL-18 is necessary for protection following *B. pseudomallei* infection, IL-1β is detrimental (57). Therefore, because individual

TLRs do not appear to be essential, and because IL-1β appears to be deleterious, MyD88 may be necessary for IL-18 signaling following *Burkholderia* infection.

The major immune defects in MyD88$^{-/-}$ mice appear to be related to decreased recruitment of inflammatory DCs and monocytes to the lungs, since these are the primary cellular sources of IL-12 production, which in turn regulates IFN-γ production by pulmonary NK cells. This hypothesis is bolstered by the fact that simply replacing the missing IFN-γ by administration of rIFN-γ can largely restore effective short-term immunity to *B. mallei* infection. These findings, as well as the findings from chapter 3, suggest that strategies to enhance DC or monocyte recruitment to the lungs, as well as stimulation of local IL-12 and IFN-γ production, may be effective therapeutic targets for *B. mallei* infection. In the following chapter the ability of the cationic liposome DNA complex (CLDC) therapeutic to protect against *B. mallei* infection is investigated. CLDC is thought to target MyD88 dependent TLRs and is a potent stimulator of IFN-γ (58).

4.6 References.

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CHAPTER 5.

PROTECTION FROM PNEUMONIC INFECTION WITH *BURKHOLDERIA* SPECIES BY INHALATIONAL IMMUNOTHERAPY

The studies in this chapter describe the ability of a mucosally delivered immunotherapeutic to protect against *Burkholderia* infection. I acknowledge Dr. Lisa Kellihan who performed all *in vitro* studies, Dr. Katie Propst for performing protection studies in *B. pseudomallei*, Dr. Ryan Troyer for analysis of pulmonary cytokine production following intranasal therapeutic administration, and Dr. Helle Bielefeldt-Ohmann for analysis of histopathology and for capturing micro photos.

5.1 Summary.

Burkholderia mallei and *B. pseudomallei* are important human pathogens and cause the diseases glanders and melioidosis, respectively. Both organisms are highly infectious when inhaled and are inherently resistant to many antimicrobials, thus making it difficult to treat pneumonic *Burkholderia* infections. We investigated whether it was possible to achieve rapid protection against inhaled *Burkholderia* infection by using inhaled immunotherapy. For this purpose, cationic liposome DNA complexes (CLDC), which are potent activators of innate immunity, were used to elicit the activation of pulmonary innate immune responses. We found that mucosal CLDC administration before or shortly after bacterial challenge could generate

complete or nearly complete protection from inhalational challenge with 100% lethal doses of *B. mallei* and *B. pseudomallei*. While previous studies have demonstrated that monocyte recruitment and myeloid differentiation factor 88 (MyD88) signaling are critical in response to *B. mallei* infection, only MyD88 signaling was necessary for CLDC protection. Protection was found to be dependent on the CLDC-mediated induction of gamma interferon responses in lung tissues and was partially dependent on the activation of natural killer (NK) cells. However, CLDC-mediated protection was not dependent on the induction of inducible nitric oxide synthase, as assessed by depletion studies. We concluded that the potent local activation of innate immune responses in the lung could be used to elicit rapid and non-specific protection from aerosol exposure to both *B. mallei* and *B. pseudomallei*.

5.2 Introduction.

Pathogenic *Burkholderia species*, including *B. mallei* and *B. pseudomallei*, are gram-negative facultative intracellular bacteria. *Burkholderia pseudomallei* is a soil bacterium that causes a disease known as melioidosis, while *B. mallei* is an obligate mammalian pathogen that causes glanders (1-2). The primary host for *B. mallei* is equines, though the organism also can infect other mammals, including humans (2-6). Without antimicrobial therapy, infection with *B. mallei* is nearly 100% fatal (2, 5). Both *B. mallei* and *B. pseudomallei* are classified as category B select agents by the Centers for Disease Control and Prevention (CDC) due to their high potential for use as bioweapons and their high resistance to antibiotics (2, 7-11). Currently, prolonged (up to 6 months) antimicrobial therapy of *Burkholderia* infection is required (12-14). Currently there is no vaccine available for preventing infection with *Burkholderia* organisms.

Proinflammatory cytokines are critical for generating protective immunity to acute *Burkholderia* infection. For example, mice unable to produce interleukin-12 (IL-12) are highly susceptible to infection with *B. mallei*, and treatment with recombinant IL-12 provides partial protection against infection (15-16). TNF-α also plays an important protective role in *B. pseudomallei* infection, as the antibody neutralization of TNF-α increases susceptibility to infection, and TNF-$\alpha^{-/-}$ and TNF-α-receptor$^{-/-}$ (TNF-$\alpha R^{-/-}$) mice are highly susceptible to lethal infection (17-18). IFN-γ also is critical in generating protective immunity to *Burkholderia* infection (16, 18-19). Indeed, even very low concentrations of IFN-γ are sufficient to generate protection against *B. pseudomallei* infection (19).

Given the inherent antimicrobial resistance of *Burkholderia* and the lack of effective vaccines, there is a need for alternative approaches to rapidly protect from aerosol infection. The nonspecific activation of innate immunity by the administration of immunotherapeutics

represents one such approach. For example, it was shown previously that the systemic (intraperitoneal (i.p.)) administration of CpG oligonucleotides prior to infection provided protection against low-dose aerosol *B. mallei* challenge (20). A similar CpG oligonucleotide treatment approach also was shown to be effective against systemic challenge, and a low dose aerosol challenge with *B. pseudomallei* (21). However, an inhaled immunotherapeutic could be more rapidly administered and generate protective immunity more rapidly than a parenterally administered agent. Therefore, we evaluated the potential effectiveness of a mucosally administered immunotherapeutic for protection from inhaled *Burkholderia* infection. The studies described here used cationic liposome-DNA complexes (CLDC) as immunotherapeutics, as our previous investigations have shown CLDC to be potent inducers of innate immunity (22-23). CLDC is thought to activate innate immunity through TLR9 ligation, a MyD88 dependent TLR. Previous studies have demonstrated that MyD88 signaling is critical for protection against *Burkholderia* infection (Chapter 4 (24)), suggest CLDC is likely to be an effective therapeutic for *Burkholderia* infection.

We investigated the effects of the timing of mucosal CLDC administration on the induction of protective immunity and whether CLDC immunotherapy could protect against both *B. mallei* and *B. pseudomallei* infection. These studies also used a rigorous high-dose inhalational *Burkholderia* challenge model and investigated immunological mechanisms of protection. We report here that the intranasal (i.n.) administration of a liposome-based immunotherapeutic was capable of eliciting significant protection from pneumonic *Burkholderia* infection. These results suggest that this approach is an effective strategy for generating rapid and nonspecific protection from the inhalation of acutely pathogenic bacteria.

5.3 Materials and methods.

5.3(1) Mice.

Female BALB/c mice and IFN-$\gamma^{-/-}$ mice on the BALB/c background, as well as C57BL/6 and monocyte chemoattractant protein-1 (MCP-1) knockout mice on the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). MyD88$^{-/-}$ mice on the C57BL/6 background were obtained from Dr. Laurel Lenz (National Jewish Medical, Denver, CO) and were bred in-house. All mice were 6 to 8 weeks of age at the time of infection and were housed under pathogen-free conditions. In all studies involving knockout mice age and sex matched control mice were used.

5.3(2) Preparation and administration of CLDC.

Sterile complexes of cationic liposomes were prepared using equimolar amounts of DOTIM [octadecanoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride] and cholesterol, which were prepared as described previously, except that the liposomes were extruded through a final filter diameter of 200 nm rather than 100 nm (25). Liposome-DNA complexes were formed just prior to injection by gently mixing cationic liposomes with plasmid DNA in 5% dextrose in water at room temperature. The final plasmid DNA concentration in the complexes was 200 μg DNA per ml. Plasmid DNA was isolated from *Escherichia coli* DH5α using the Qiagen Endo-free Giga kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The plasmid used for these experiments was a eukaryotic expression plasmid that contained no transgene insert, as described previously (22). CLDC were administered to mice i.n. in a 20 μl volume (10 μl per nostril).

5.3(3) Preparation of *Burkholderia mallei* and *B. pseudomallei* stocks and animal infections.

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

For *B. mallei* studies, the ATCC 23344 *B. mallei* strain first was serially passaged three times by animal infection in mice. The use of animal passage has been reported previously to increase the virulence of *B. mallei*, and we observed the same phenomenon in our studies (Chapter 3 (2, 4-6, 26-28)). Prior to each *in vivo* or *in vitro* infection with *B. mallei*, fresh broth cultures were grown in Brucella broth supplemented with 4% glycerol (BB4G) (Remel, Lenexa, KS). Bacteria were harvested during the log phase of growth, titers were determined based on optical density values, and bacterial dilutions were prepared in sterile phosphate-buffered saline (PBS). Bacterial titers (in CFU) of each inoculum were determined by plating serial dilutions of the inocula on BB4G agar plates (Remel).

Prior to infection, mice were anesthetized by the i.p. injection of ketamine (100 mg/kg) (Fort Dodge Animal Health, Overland Park, KS) and xylazine (10 mg/kg) (Ben Venue Laboratories, Bedford, OH) solution prepared in sterile water. Mice were infected with *B. mallei* i.n. using a total volume of 20 μl of inoculum (10 μl per nostril). The LD_{50} of *B. mallei* in BALB/c mice by the i.n. route was determined by the Reed-Muench method to be 8.2×10^2 CFU (Chapter 3), and the experimental LD_{100} was 4.2×10^3 CFU. For *in vitro* experiments with *B.*

mallei, frozen stocks of known titers of the animal-passaged *B. mallei* were diluted in cell culture media, added to cells, and incubated at 37°C for the indicated amount of time and at the indicated multiplicity of infection (MOI).

Frozen stocks of *B. pseudomallei* of known titers were prepared from cultures grown in Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA) by freezing the cultures in LB medium containing 20% glycerol. Inocula for *in vivo* infection with *B. pseudomallei* were prepared by thawing and diluting frozen stocks in sterile PBS. The LD_{50} of *B. pseudomallei* in BALB/c mice by the i.n. route was determined by the Reed-Muench method to be 9.5×10^2 CFU (Chapter 3), and the experimental LD_{100} was 4.5×10^3 CFU. In all survival experiments mice were euthanized upon reaching one of the following pre-determined euthanasia endpoints: (1) hunched posture with decreased movement or response to stimuli; (2) development of respiratory distress (tachypnea, open-mouthed respirations); or (3) loss of > 15% body weight.

5.3(4) Determination of bacterial burden in tissues.

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

5.3(5) Histological analyses.

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

5.3(6) Cytokine quantification.

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

5.3(7) *In vitro* infection of macrophages with *B. mallei*.

The mouse alveolar macrophage cell line (AMJ2; American Type Tissue Collection, Manassas, VA) was used to investigate the ability of CLDC supernatants to enhance bactericidal activity *in vitro*. Cells were cultured in 24-well plates in complete medium without antibiotics. Cells were cultured in complete medium that consisted of minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento,

CA), 2 mM L-glutamine (Invitrogen), 1X nonessential amino acids (Invitrogen), and 0.075% sodium bicarbonate (EMD Science, Gibbstown, NJ). Adherent cells were infected with *B. mallei* at a MOI of 2 for 1hour in 250 μl medium at 37°C and 5% CO_2. Extracellular bacteria were removed by washing the cells three times with PBS, followed by treatment with medium plus 350 μg/ml kanamycin (Sigma-Aldrich) for 1 h. After incubation with kanamycin for 1 h, the cells were washed and then cultured in complete medium with 10 μg/ml kanamycin for an additional 24 h. To quantitate intracellular bacterial numbers, the cells were washed three times with PBS and then lysed with sterile double-distilled water containing 0.01% Triton X-100 (Sigma-Aldrich). Serial dilutions of lysate were plated on BB4G agar plates, and plates were incubated at 37°C for 48 h, at which time colonies were counted.

5.3(8) Cytokine inhibition of macrophage infection with *B. mallei*.

The ability of cytokines elicited by CLDC immunotherapy to inhibit the intracellular replication of *B. mallei* in infected macrophages was assessed using AMJ2 alveolar macrophages. AMJ2 cells (1×10^5 per well in triplicate wells of 24-well plates) were pretreated for 24 hours before infection with diluted (1:10 or 1:100) supernatants from spleen cells of mice treated *in vivo* with CLDC or with supernatants from control spleen cells. Briefly, supernatants were generated as described previously by using overnight cultures of spleen cells prepared from mice injected intravenously (i.v.) with 200 μl CLDC three hours prior to sacrifice (22). It was determined by ELISA that the supernatants from spleen cells of CLDC-injected mice contained 3 ng/ml IFN-γ and 140 pg/ml TNF-α (data not shown).

Neutralizing antibodies were used to identify key cytokines that may have been responsible for generating antibacterial activity in supernatants from spleen cells. Thus,

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

5.3(9) NK cell depletion.

NK cells were depleted systemically in mice (n = 5 per group) by the injection of anti-asialo GM1 antibody (Wako Chemicals, Richmond, VA), as described previously (22, 29-30). Briefly, mice were injected i.p. with 50 μg of anti-asialo GM1 antibody 24 hours prior to CLDC treatment. This treatment was found to decrease the number of splenic and lung NK cells by more than 75% (data not shown). The mice then were infected i.n. with *B. mallei* 24 hours after that (48 hours after the injection of asialo-GM1 antibody). Controls included mice injected i.p. with an equivalent amount of rabbit immunoglobulin G antibody (Jackson ImmunoResearch, West Grove, PA).

5.3(10) Inhibition of nitric oxide production *in vivo*.

Inducible nitric oxide synthase (iNOS) activity was inhibited by the treatment of mice with aminoguanidine (AG) (Sigma-Aldrich). Mice were injected i.p twice daily with 0.2 ml of a 50 mg/ml solution of AG prepared in PBS, starting 5 days prior to infection and continuing until 7 days post infection, using a previously described protocol (31-32). Control mice were injected with an equal volume of sterile PBS.

5.3(11) Statistical analysis.

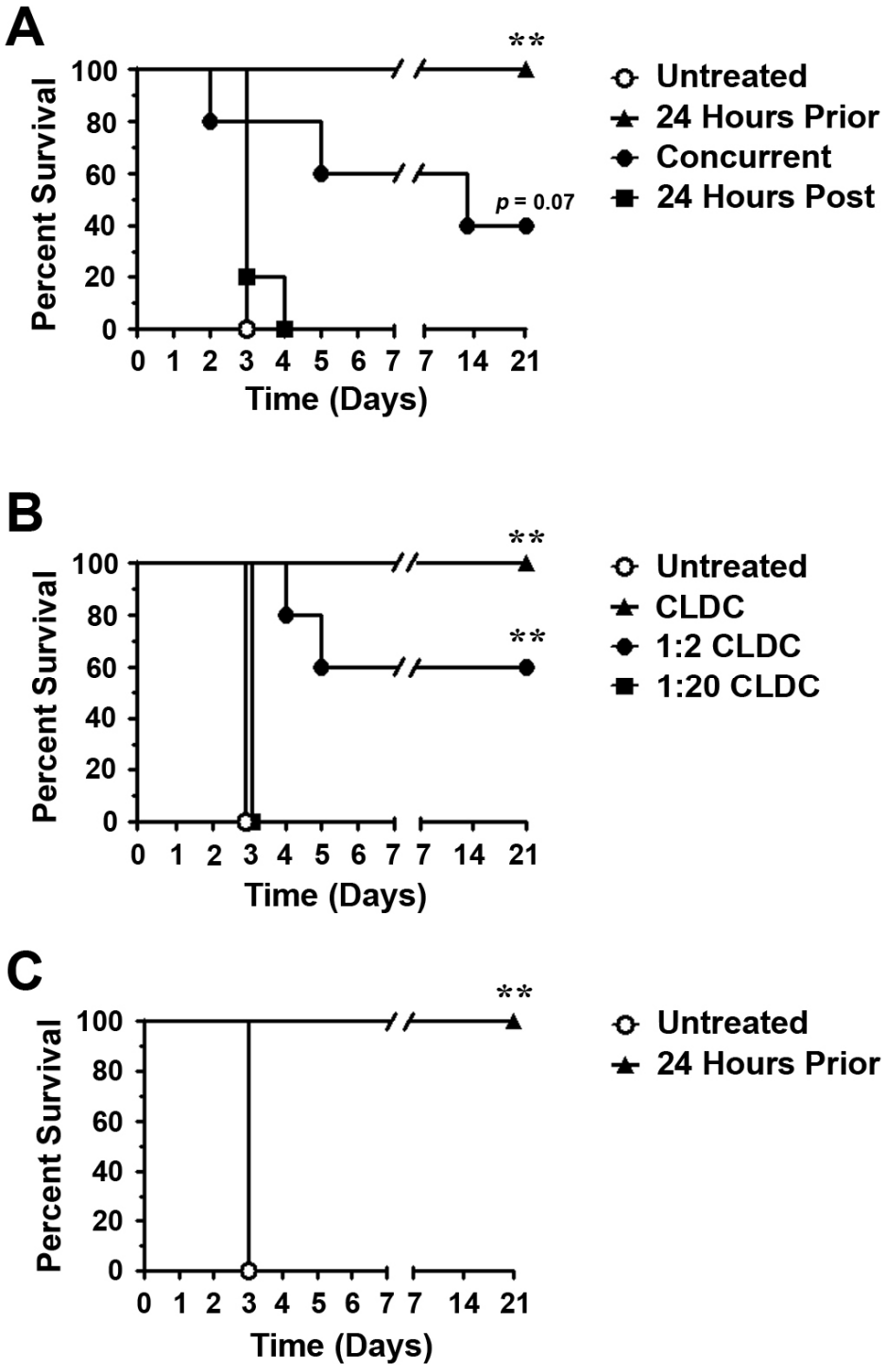
Statistical analysis was performed using Prism 5.0 software (GraphPad, La Jolla, CA). For the comparison of two groups, two-tailed Student's t-tests were performed. For the comparison of more than two groups, one-way analysis of variance (ANOVA) was done, followed by Tukey's multiple means comparison test. Survival times were compared using Kaplan-Meier curves and the log-rank test. Data were considered statistically significant for $p < 0.05$. For survival comparisons between more than two groups the Bonferroni correction for multiple comparisons was applied. *p*-values used to identify statistical significance were determined by dividing the *p*-value used to determine statistical significance ($p = 0.05$) by the number of comparisons being made.

5.4 Results.

5.4(1) Mucosal administration of CLCD immunotherapy protects mice from acute *B. mallei* pneumonic infection.

Previous studies demonstrated that systemic immunotherapy using CpG oligonucleotides could provide protection from chronic infection following low-dose *B. mallei* pneumonia (20). However, we wished to determine whether mucosal immunotherapy with a liposome-based immunotherapeutic was effective in an acutely lethal *B. mallei* pneumonia model. Therefore, we conducted protection studies in mice challenged with a high dose ($10\times LD_{50}$) of *B. mallei* by the i.n. route.

In the first studies, we assessed the effects of the timing of CLDC administration on the induction of protective immunity. BALB/c mice (n = 5 per group) were administered 20 µl of CLDC by the i.n. route, either 24 hours prior to infection, concurrently with infection, or 24 hours after infection. Mice then were infected i.n. with $10\times LD_{50}$ (approximately 10^4 CFU) of *B. mallei* and monitored for effects on survival. We observed that the administration of CLDC 24 hours prior to infection generated complete protection from lethal *B. mallei* infection **(Figure 5.1)**. Concurrent i.n. administration of CLDC immunotherapy provided partial protection from lethal infection, whereas treatment 24 hours after infection did not generate significant protection (Fig. 5.1). In addition, the administration of CLDC 6 hours after infection also failed to elicit significant protection (data not shown). We also noted that protection elicited by mucosal CLDC immunotherapy prevented lethal acute infection but failed to completely protect from chronic infection, as approximately 50% of treated mice developed chronic infection by day 60 after challenge (data not shown). CLDC treatment 24 hours prior to infection also provided significant protection against i.n. *B. mallei* infection of C57BL/6 mice, although protection was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Untreated
24 Hours Prior

Figure 5.1. Protective effects of CLDC against pneumonic *Burkholderia* infection are dependent on the timing and dose of CLDC administered. (A) BALB/c mice (n = 5 animals per group) were challenged i.n. with 10^4 CFU *B. mallei*, and the effects of the timing of the administration of CLDC immunotherapy on survival were assessed. Mice were untreated or were treated with CLDC 24 hours prior to infection, at the time of infection, or 24 hours after infection, and survival times were determined as described in Materials and Methods. (B) The effects of the CLDC dose on the induction of protective immunity were assessed. Mice (n = 5 animals per group) were treated by the i.n. administration of standard CLDC or CLDC diluted 1:2 or 1:20 24 hours before i.n. challenge with 10^4 CFU *B. mallei*, and survival times were determined. (C) BALB/c mice (n = 5 per group) were untreated or were treated with CLDC 24 hours prior to i.n. infection with $10\times LD_{50}$ (10^4 CFU) of *B. pseudomallei*. Statistical differences for the data shown were determined by Kaplan-Meier analysis followed by a log-rank test. The Bonferroni correction for multiple comparisons was applied for (A) and (B), and *p*-values ≤ 0.017 were considered significant (** $p < 0.01$). Data shown in all three panels are representative of two independent experiments each.

not complete with 40% of CLDC mice succumbing to infection by day 7 following infection with 6.1×10^3 CFU ($p < 0.01$; log-rank analysis, data not shown, pooled from 2 experiments). Thus, pretreatment by the i.n. administration of liposome-based immunotherapy was sufficient to generate significant protection from high-dose inhalational challenge with *B. mallei*.

Experiments then were conducted to assess the dose responsiveness of CLDC protection from *B. mallei* challenge by using the 24 hour pretreatment model. Mice (n = 5 per group) were pretreated with the standard concentration of CLDC or with CLDC diluted 1:2 or 1:20 in diluent (Fig. 5.1). We found that the administration of CLDC diluted 1:2 still provided significant ($p < 0.01$) protection, whereas CLDC diluted 1:20 failed to elicit protection.

We next assessed the ability of CLDC immunotherapy to protect from a higher *B. mallei* challenge dose. Mice were subjected to challenge with $50\times LD_{50}$ (5×10^4 CFU), and the effects on survival time were assessed. We found that CLDC immunotherapy still provided significant protection in this higher-dose challenge model. Though 80% of the mice succumbed to this high-dose challenge, the median survival time for CLDC-treated mice was 4 days, whereas it was 2 days for untreated mice ($p < 0.003$; log-rank analysis, data not shown). We also assessed

whether increasing the CLDC dose increased protection. Increasing the dose of CLDC administered by doubling the concentration of liposomes and plasmid DNA (and still administering the same i.n. volume of CLDC) did not, however, increase survival significantly over that elicited by the administration of the standard amount of CLDC (data not shown).

Burkholderia mallei and *B. pseudomallei* are related pathogens, but they also possess some key differences with respect to genomic complexity and the numbers of potential virulence factors (33-37). Therefore, we conducted challenge studies with *B. pseudomallei* to determine whether CLDC immunotherapy also was capable of eliciting protective immunity against pneumonic infection with this organism. BALB/c mice (n = 5 per group) were treated with CLDC i.n. 24 hours prior to infection and then were subject to i.n. infection with $10\times LD_{50}$ (10^4 CFU) of *B. pseudomallei*, and the effects on survival times were assessed (Fig. 5.1). We found that CLDC immunotherapy was effective in completely protecting mice from lethal pneumonic infection with *B. pseudomallei*. Thus, CLDC immunotherapy was capable of eliciting broadly protective immunity against two species of *Burkholderia*.

5.4(2) Reduced bacterial burden and organ pathology in mice treated with CLDC immunotherapy.

The preceding studies demonstrated that CLDC immunotherapy could protect against lethal respiratory *Burkholderia* infection in a dose-dependent fashion. Therefore, studies were conducted next to elucidate the mechanisms of protection. First, the effects of CLDC treatment on bacterial burdens in the lungs, spleen, and liver were assessed. BALB/c mice (n = 5 per group) were sham treated or were treated with CLDC i.n. 24 hours prior to infection and then were subjected to infection with approximately 10^4 CFU *B. mallei* i.n. The mice were euthanized

3 days after infection and bacterial were burdens determined, as described in Materials and Methods. We found that there were significant ($p < 0.0001$) reductions in the bacterial burdens in lung, liver, and spleen in CLDC-treated mice compared to that in untreated mice (**Figure 5.2**).

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

Pretreatment with CLDC immunotherapy also substantially reduced acute organ pathology in *B. mallei*-infected mice. In untreated mice (n = 4 per group) euthanized 3 days after B. *mallei* challenge, there was marked neutrophil infiltration in the lungs, along with hemorrhage

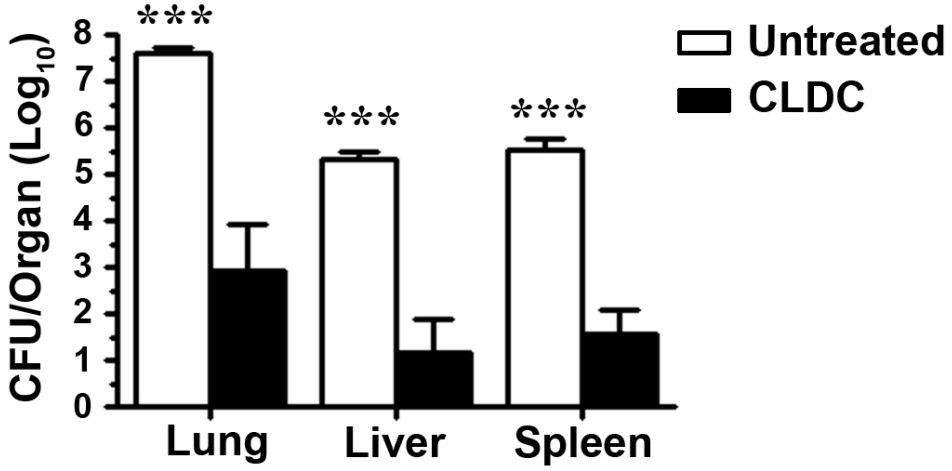

Figure 5.2. Effects of CLDC immunotherapy on bacterial burden in the lung, liver, and spleen of mice following inhalational challenge with *B. mallei*. BALB/c mice (n = 5 per group) were treated with CLDC by i.n. administration 24 hours prior to challenge and then challenge by the inhalation of 10^4 CFU *B. mallei*. Bacterial burdens (in $\log_{10}$ CFU) were determined in each organ 72 hours after *B. mallei* challenge, as described in Materials and Methods. Significant reductions in bacterial burdens were seen in mice treated with CLDC in all three organs analyzed, as assessed by a two-tailed Student's t-test (*** $p < 0.001$). Data are representative of two independent experiments.

from pulmonary vessels, the necrosis of respiratory epithelium, and the apoptosis of leukocytes and lung parenchyma (**Figure 5.3**). In contrast, in CLDC-treated mice the lung neutrophil infiltrate was reduced and hemorrhage was not observed in pulmonary vessels.

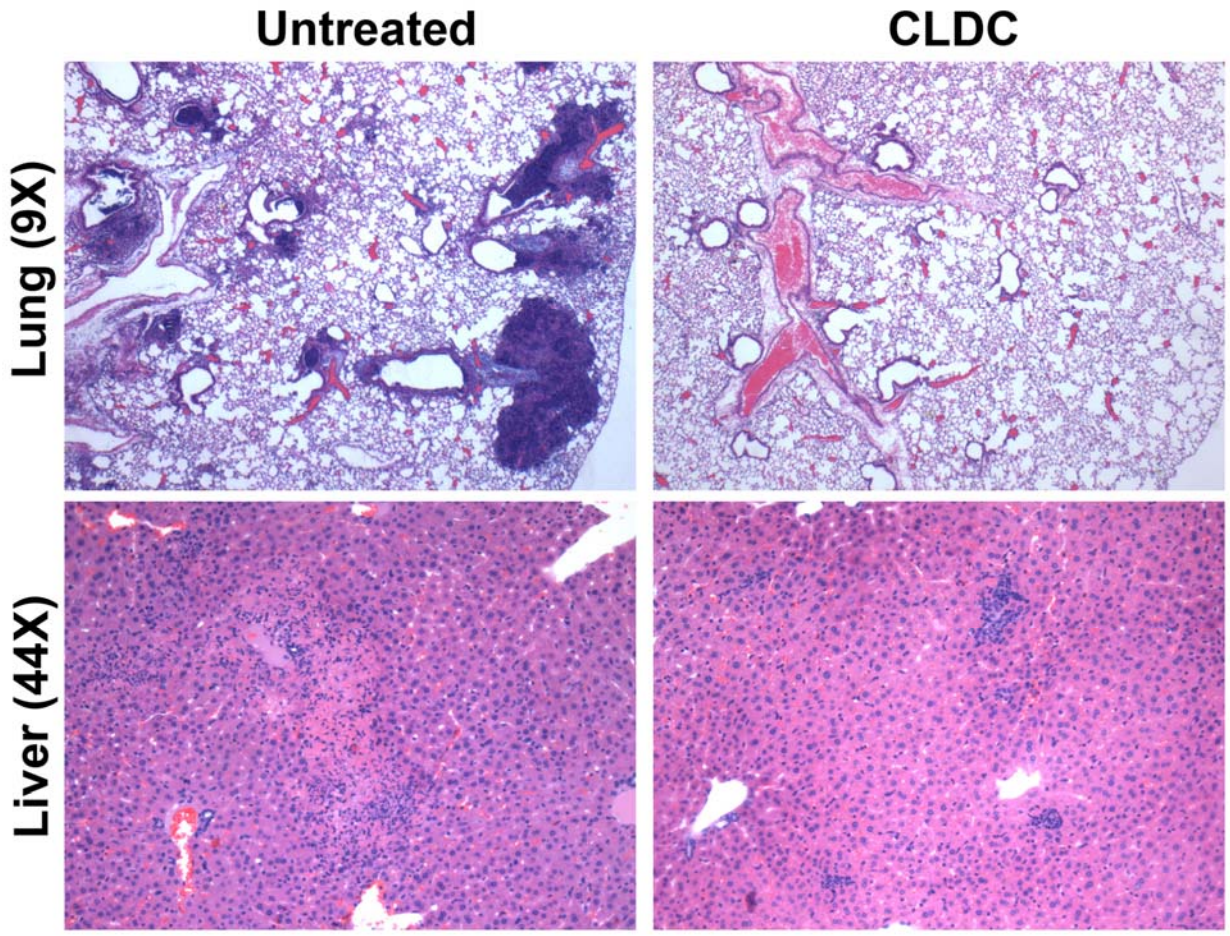

Figure 5.3. Comparison of lung and liver pathology in untreated and CLDC-treated mice following lethal *B. mallei* challenge. BALB/c mice (n = 5 per group) were untreated or were treated with CLDC 24 hours prior to i.n. infection with 10^4 CFU *B. mallei*. On day 3 after infection, mice were euthanized and lung and liver tissues were collected and processed for histopathological evaluation, as described in Materials and Methods. Representative sections from lung (top row, 2X magnification) and liver (bottom row, 20X magnification) from untreated and CLDC-treated mice were photographed. Images are representative of those obtained in two independent experiments.

In untreated *B. mallei*-infected mice, hepatic lesions consisted of infiltrates of neutrophils and monocytes. In the livers of untreated mice there were large areas of necrosis, and in some mice hepatic lesions coalesced, whereas hepatic lesions in CLDC treated mice showed no evidence of necrosis and generally were much smaller (Fig. 5.3). Important changes in spleen pathology were not observed between CLDC-treated and untreated mice. Thus, these studies demonstrated that CLDC immunotherapy significantly reduced bacterial replication and organ pathology in mice infected with *B. mallei* compared to those for infected mice not receiving immunotherapy.

5.4(3) CLDC-elicited cytokines block *B. mallei* infection of alveolar macrophages *in vitro*.

The fact that the i.n. administration of CLDC elicited a significant decrease in bacterial replication in the lungs suggested that treatment triggered the release of cytokines that were capable of eliciting antibacterial activity in macrophages. For example, it is known that the delivery of liposome-DNA complexes to the lungs can elicit the local production of IFN-γ and TNF-α (38). To investigate this mechanism further and identify possible protective cytokines, we used an *in vitro* infection system consisting of AMJ2 cells (an alveolar macrophage line) and supernatants from spleens of mice that had been injected i.v. with CLDC. In a previous study, we found that spleen cells were a major source of cytokine production in CLDC-treated mice (22). AMJ2 cells were treated with CLDC supernatants (or supernatants from untreated control mice) for 24 h. The cells then were infected with *B. mallei* at an MOI of 2, and 24 hours later the numbers of intracellular bacteria were determined.

The preincubation of macrophages with supernatants from CLDC-treated mice, but not supernatants from untreated control mice, resulted in a significant reduction in the numbers of viable intracellular *B. mallei* **(Figure 5.4)**. We also observed the inhibition of *B. mallei* infection in macrophage cultures with supernatants from CLDC-stimulated lungs, but the effect was much less potent than that with spleen supernatants (data not shown). Significant antibacterial activity was observed when supernatants were diluted 1:1 (not shown) or 1:10, but it was lost when supernatants were diluted 1:100. In previous studies, CLDC were shown to elicit large amounts of IFN-γ and smaller amounts of TNF-α, both of which have antibacterial activity against *Burkholderia* (16-19). Therefore, we used neutralization experiments to assess the relative contribution of each cytokine to antibacterial activity. The neutralization of IFN-γ activity resulted in the significant abrogation of the macrophage antibacterial activity of CLDC supernatants (Fig. 5.4). In contrast, the neutralization of TNF-α activity had a much smaller effect. However, the neutralization of both cytokines resulted in the greater inhibition of antibacterial activity, suggesting that TNF-α production contributed to the effectiveness of IFN-γ in suppressing *B. mallei* replication in infected macrophages. Finally, the pretreatment of AMJ2 cells with 10, 1, or 0.1 ng/ml recombinant IFN-γ resulted in 100, 98, and 98% inhibition of bacterial growth, respectively (data not shown). These results indicated that the CLDC-induced production of IFN-γ mediated many of the protective effects of CLDC immunotherapy noted *in vivo*, particularly since we have observed that alveolar macrophages are one of the primary early target cells for *Burkholderia* infection in the lungs (data not shown).

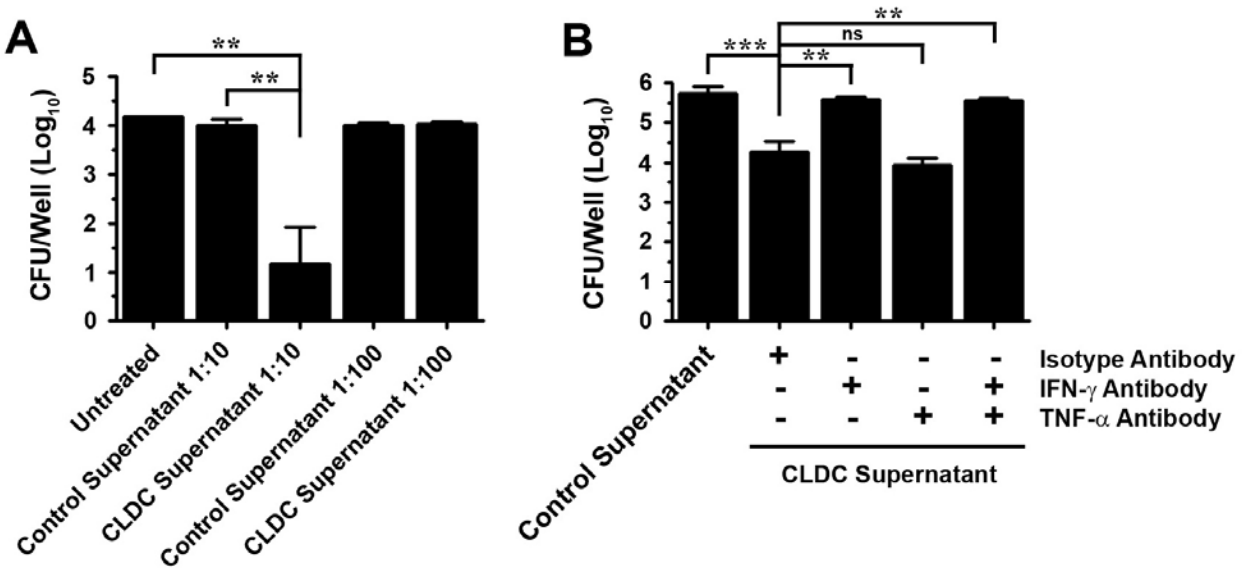

Figure 5.4. IFN-γ elicited by CLDC treatment inhibits the *B. mallei* infection of alveolar macrophages *in vitro*. (A) AMJ2 (an alveolar macrophage cell line) cells in triplicate wells of 24-well plates were treated 24 hours prior to infection with supernatants generated from CLDC-treated or untreated mouse spleen cells, as described in Materials and Methods. The cells then were infected with *B. mallei*, and 24 hours later the numbers of intracellular *B. mallei* organisms were determined. The treatment of AMJ2 cells with CLDC supernatant resulted in a significant reduction (** $p < 0.01$) in the numbers of intracellular *B. mallei* organisms. (B) Neutralizing antibodies to IFN-γ and TNF-α were utilized to determine the key cytokines present in CLDC-stimulated spleen supernatants that were responsible for the inhibition of *B. mallei* infection in macrophages, as described in Materials and Methods. The neutralization of IFN-γ resulted in the significant (** $p < 0.01$, *** $p < 0.001$, ns = not significant) inhibition of the antibacterial activity of CLDC supernatants against the *B. mallei* infection of AMJ2 cells. In contrast, the inhibition of TNF-α alone did not significantly inhibit CLDC activity. Statistical significance was assessed using a one-way ANOVA, followed by Tukey's multiple means comparison test. These results are representative of those obtained in three independent experiments.

5.4(4) Intranasal administration of CLDC elicits production of IL-12 and IFN-γ in the lungs.

Both IL-12 and IFN-γ are known to play critical roles in protective immunity to *Burkholderia* infection (15-16, 18-19, 39-41). In addition, the preceding *in vitro* studies indicated that IFN-γ could control *Burkholderia* replication in alveolar macrophages, a likely target cell for the inhalational route of infection. Therefore, we assessed the ability of the inhalational delivery of CLDC to elicit IL-12 and IFN-γ production in the lungs. Six and 24

hours after the i.n. administration of CLDC, IL-12 and IFN-γ concentrations in lung tissues were determined by ELISA **(Figure 5.5)**. Significant increases in the concentrations of both IL-12 and IFN-γ were observed in lung tissues 24 hours following the i.n. delivery of CLDC, with smaller increases noted at 6 hours after administration. These results indicated that the inhalational delivery of relatively low doses of CLDC to the lung could trigger the significant production of a key cytokine (IFN-γ) responsible for suppressing the intracellular replication of *B. mallei*. The kinetics of cytokine induction in the lung in response to CLDC immunotherapy also may explain why pretreatment with CLDC 24 hours before challenge did elicit protective immunity, whereas treatment 6 hours after challenge did not.

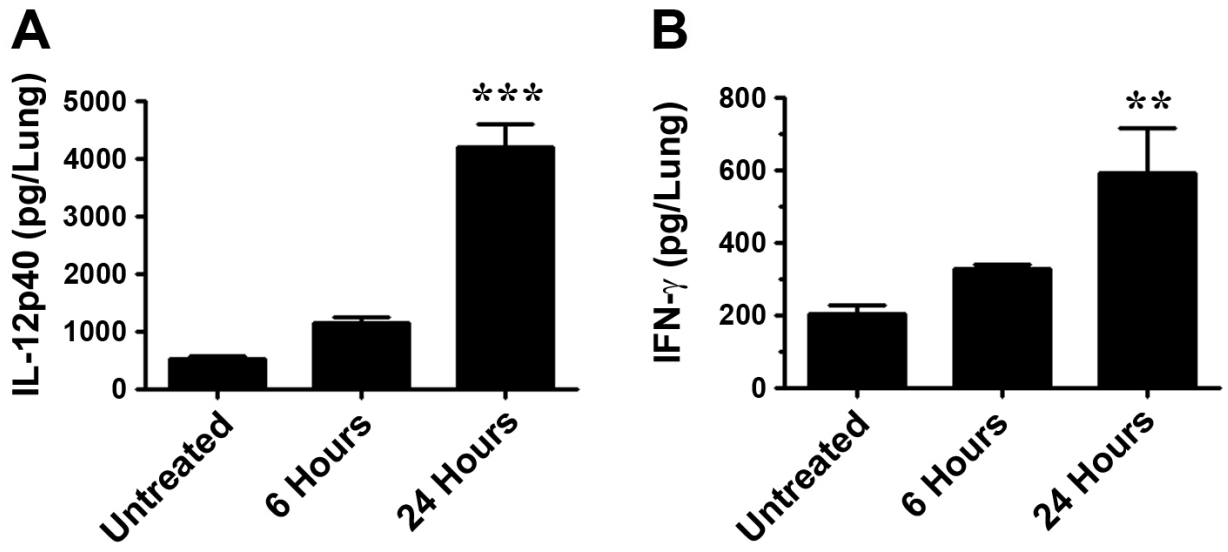

Figure 5.5. Induction of IL-12p40 and IFN-γ production in the lungs following the i.n. administration of CLDC. BALB/c mice (n = 5 per group) were treated with CLDC i.n. and then were euthanized 6 or 24 hours after treatment. Concentrations of IL-12p40 and IFN-γ were determined in homogenized lung tissues by ELISA, as described in Materials and Methods. A one-way ANOVA followed by Tukey's multiple means comparison test was used to determine significant differences between the three treatment groups (** $p < 0.01$, *** $p < 0.001$; compared to results for untreated samples). Significant increases in both IL-12 and IFN-γ were observed 24 hours after the administration of CLDC. Data are representative of two independent experiments.

5.4(5) CLDC protection is dependent on MyD88 signaling, but independent of monocyte recruitment.

Previous studies demonstrated the inability of MyD88 and chemokine receptor 2 (CCR2) knockout mice to produce IFN-γ was responsible for the increased susceptibility of these mice to *B. mallei* infection (Fig. 3.13 and Fig. 4.10). Because CLDC is known to induce IFN-γ production in the lung (Fig 5.5), the role of MyD88 signaling and monocyte recruitment was investigated. To investigate the necessity of MyD88 signaling in CLDC protection, the susceptibility of wild type, MyD88$^{-/-}$, and MyD88$^{-/-}$ mice treated with CLDC i.n. 24 hours prior to infection (MyD88$^{-/-}$ + CLDC) was investigated. In addition, a much lower challenge dose of *B. mallei* was administered (0.5×LD_{50}, ~5×10^2 CFU), since MyD88$^{-/-}$ mice were shown to be exquisitely sensitive to *Burkholderia* infection (Fig 4.1). MyD88$^{-/-}$ mice pre-treated with CLDC were not protected, and succumb to infection at the same time as untreated MyD88$^{-/-}$ mice **(Figure 5.6).** In addition, determination of bacterial burdens in wild type, MyD88$^{-/-}$, and MyD88$^{-/-}$ + CLDC mice demonstrated that both MyD88$^{-/-}$ mice and MyD88$^{-/-}$ + CLDC mice had increased bacterial burdens compared to wild type mice; although no differences were observed between MyD88$^{-/-}$ and MyD88$^{-/-}$ + CLDC mice (Fig 5.6). Therefore MyD88 signaling is necessary for the protective effects of CLDC.

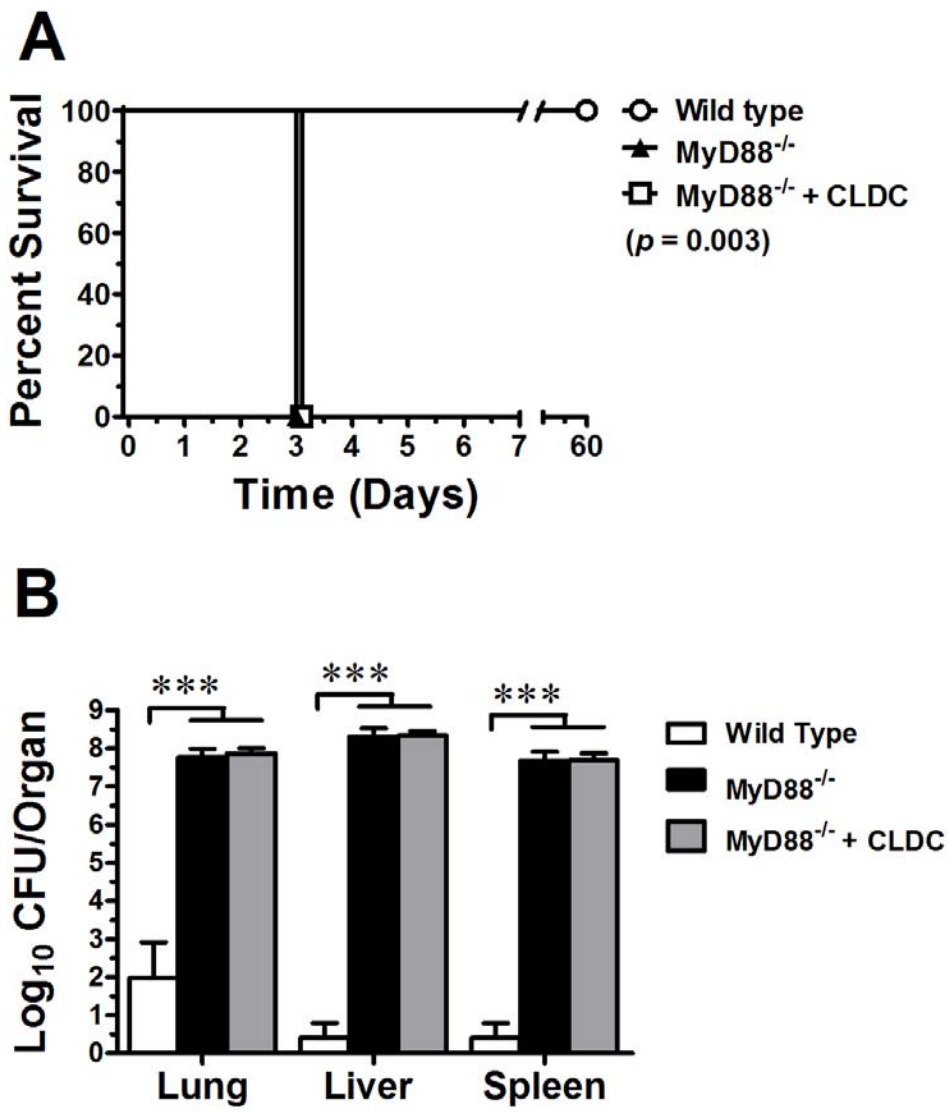

Figure 5.6. MyD88 signaling is necessary for CLDC protection following *B. mallei* challenge. (A) Survival of wild type, MyD88$^{-/-}$, and MyD88$^{-/-}$ mice treated with CLDC 24 hours prior to infection (MyD88$^{-/-}$ + CLDC) (n = 5 per group). Mice were infected with 5×10^2 CFU *B. mallei* and mice were euthanized upon reaching a pre-determined endpoint. Data are representative of 2 independent experiments. Statistical differences were determined by Kaplan-Meier analysis followed by a log rank analysis. The Bonferroni correction for multiple comparisons was applied, and *p*-values ≤ 0.017 were considered significant. (B) Wild type, MyD88$^{-/-}$ and MyD88$^{-/-}$ + CLDC mice (n = 5 per group) were infected with 5×10^2 CFU i.n. *B. mallei*. Bacterial burdens were determined 3 days after i.n. infection as described in Materials and Methods. Data are graphed as the mean ± SEM $\log_{10}$ CFU/organ titer. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple means comparison test (*** $p < 0.001$). Data are representative of 2 independent experiments.

The role of monocytes was also investigated, and in contrast to MyD88, monocyte recruitment was not necessary for CLDC protection. In preliminary studies monocyte chemoattractant protein-1 (MCP-1) knockout mice treated with CLDC 24 hours prior to infection were protected against an i.n. *B. mallei* challenge with 5×10^2 CFU *B. mallei* (data not shown). Therefore, although monocyte recruitment was necessary for protection against *B. mallei* infection (Fig 3.3), activation of local pulmonary immune responses by CLDC was sufficient to protect $MCP^{-/-}$ mice. These results are in agreement with studies from *B. pseudomallei* demonstrating that only a small amount of IFN-γ is necessary for protection, and that resident macrophages can produce sufficient IFN-γ to provide protection (19). Therefore, CLDC likely activates local pulmonary immune responses in a MyD88 dependent fashion, resulting in protection against *B. mallei* challenge.

5.4(6) IFN-γ is necessary for CLDC-mediated protection *in vivo*.

Experiments next were conducted to directly elucidate the *in vivo* role of IFN-γ in CLDC-mediated protection from lethal challenge with *B. mallei*. For these experiments, we used $IFN\text{-}\gamma^{-/-}$ mice on the BALB/c and C57BL/6 background. Untreated $IFN\text{-}\gamma^{-/-}$ mice, $IFN\text{-}\gamma^{-/-}$ mice treated with CLDC 24 hours prior to infection ($IFN\text{-}\gamma^{-/-}$ + CLDC), and wild-type mice were infected with $0.5\times LD_{50}$ (~5×10^2 CFU) of *B. mallei* i.n. **(Figure 5.7)**. We observed that the CLDC pretreatment of $IFN\text{-}\gamma^{-/-}$ mice failed to elicit protective immunity, indicating that IFN-γ was a key component of the protective immunity elicited by CLDC immunotherapy.

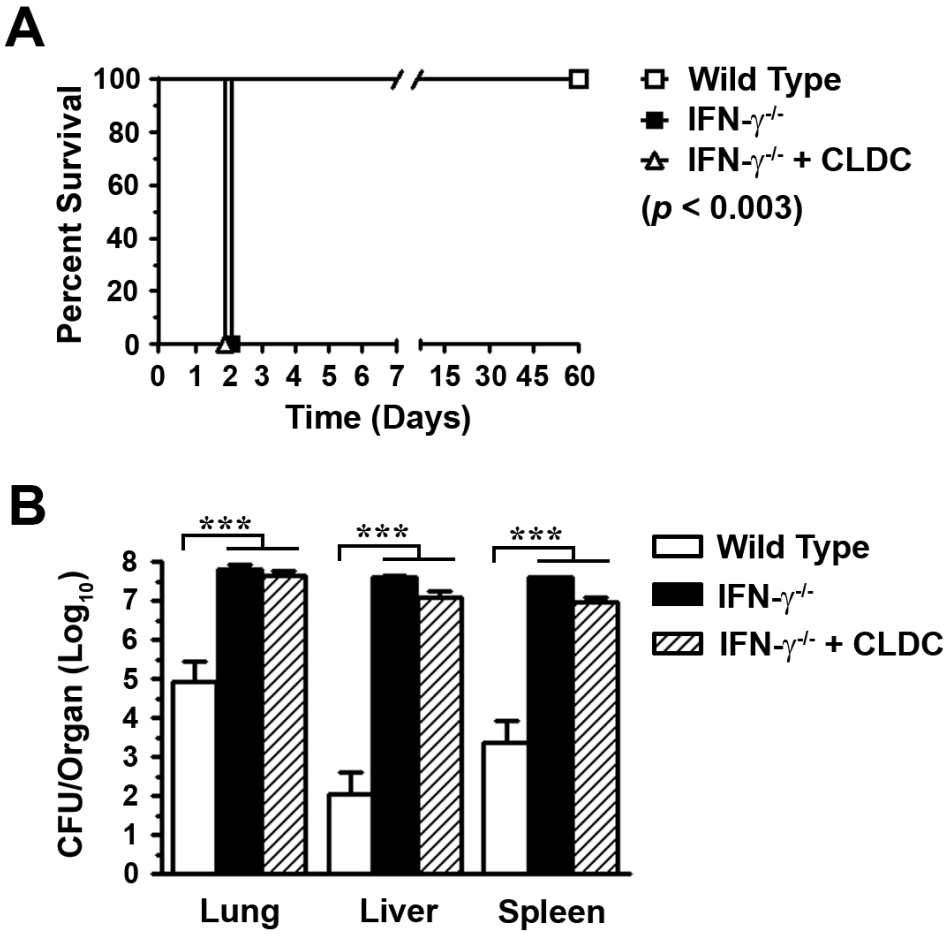

Figure 5.7. IFN-γ is necessary for the *in vivo* protective of CLDC immunotherapy. (A) BALB/c wild-type mice (n = 5 per group) or IFN-$\gamma^{-/-}$ mice (n = 5) were treated with CLDC 24 hours prior to infection or were left untreated. Mice were infected by low-dose challenge using the i.n. administration of 5×10^2 CFU *B. mallei*, and survival was assessed. Survival times in the IFN-$\gamma^{-/-}$ mice treated with i.n. administered CLDC were not significantly different in terms of infection than untreated IFN-$\gamma^{-/-}$ mice. Statistical differences were determined by Kaplan-Meier analysis using a log-rank test. The Bonferroni correction for multiple comparisons was applied, and p-values ≤ 0.017 were considered significant. Similar results were obtained in a second experiment done in IFN-$\gamma^{-/-}$ mice on a C57BL/6 background. (B) Wild-type BALB/c mice (n = 5) or IFN-$\gamma^{-/-}$ mice (n = 5) were treated with i.n. administered CLDC 24 hours prior to infection or were left untreated. Mice were infected with 5×10^2 CFU *B. mallei*, and on day 2 after infection mice were euthanized and bacterial burdens in the lungs, liver, and spleen were determined, as described in Materials and Methods. The bacterial burdens in all three organs of IFN-$\gamma^{-/-}$ mice treated with CLDC were not significantly different from those of untreated IFN-$\gamma^{-/-}$ mice. Similar results were obtained in a second experiment done in IFN-$\gamma^{-/-}$ mice on a C57BL/6 background. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple means comparison test (*** $p < 0.001$).

The role of IFN-γ in suppressing *B. mallei* replication *in vivo* also was assessed. Bacterial burdens in the lung, liver, and spleen of IFN-$\gamma^{-/-}$ mice treated with CLDC were compared to those of untreated IFN-$\gamma^{-/-}$ mice and wild-type mice on day 2 after low-dose i.n. challenge with *B. mallei*. As expected, the numbers of bacteria in all three organs from IFN-$\gamma^{-/-}$ mice were significantly higher than those in organs from wild type mice (Fig. 5.7). However, significant differences were not observed when the numbers of bacteria in untreated IFN-$\gamma^{-/-}$ mice and CLDC-treated IFN-$\gamma^{-/-}$ mice were compared (Fig. 5.7). Thus, these data are consistent with the conclusion that IFN-γ was necessary for CLDC immunotherapy to effectively suppress *B. mallei* replication and to increase survival following pulmonary challenge.

5.4(7) Role of NK cells in CLDC-induced protection from *B. mallei* challenge.

Previous studies have indicated that NK cells were the major source of IFN-γ production following systemic (intravenous) treatment with CLDC (23, 42). However, the role of NK cells in mediating CLDC-induced pulmonary immunity to bacterial challenge has not been explored previously. Prior studies have revealed that multiple cell types, including macrophages, $CD8^+$ T cells, and NK cells, produce IFN-γ in response to *Burkholderia infection* (16, 19, 41, 43). To elucidate the role of NK cells in CLDC protection, NK cells were depleted systemically using the asialo GM1 antibody (22, 29-30, 44). The antibody was administered 48 hours prior to infection, and then the mice were treated 24 hours prior to infection by the i.n. administration of CLDC. Mice then were infected with $10\times LD_{50}$, $\sim 10^4$ CFU of *B. mallei* and monitored for survival.

There was a significant reduction in survival of CLDC-treated mice depleted of NK cells compared to that of CLDC-treated mice receiving the isotype antibody ($p = 0.016$) **(Figure 5.8)**. However, CLDC-treated and NK-depleted mice still had a significant improvement in survival

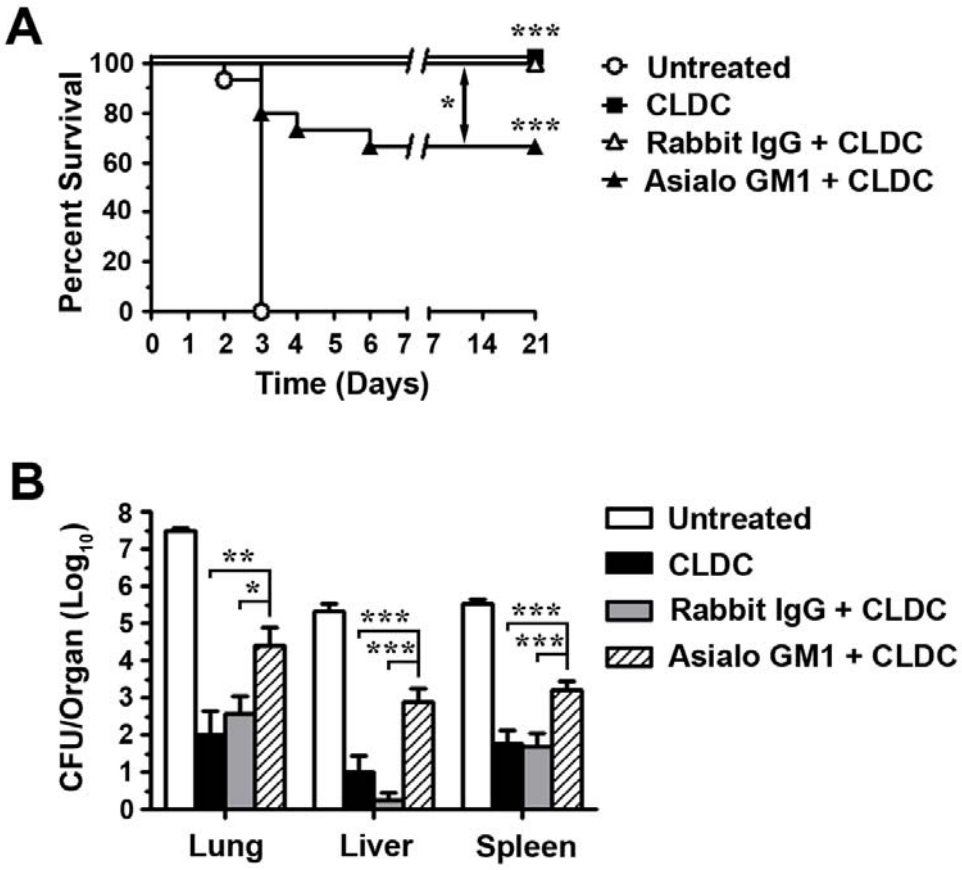

Figure 5.8. Natural killer cells play an important role in the protective effects of CLDC immunotherapy. (A) BALB/c mice (n = 10 per group) were depleted of NK cells by the i.p. administration of an NK cell-depleting antibody 48 hours prior to infection (Asialo GM1), as described in Materials and Methods. Control mice were treated with an irrelevant control rabbit antibody (Rabbit IgG). Twenty-four hours after NK cell depletion mice were treated with CLDC or were left untreated, and 24 hours later the mice were subjected to infection with 10^4 CFU *B. mallei* and survival times were assessed. Statistical differences were determined by Kaplan-Meier analysis using a log-rank test. The Bonferroni correction for multiple comparisons was applied, and p-values ≤ 0.017 were considered significant. Differences between Rabbit IgG + CLDC and Asialo GM1 + CLDC are indicated by an arrow, and all other differences are compared to untreated mice (* $p < 0.017$, *** $p < 0.001$). (B) The effects of NK cell depletion and CLDC treatment on bacterial burdens in lung, liver, and spleen tissues were assessed 72 hours after i.n. infection with *B. mallei*. Treatment groups are the same as those noted for panel A. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple means comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data were pooled from two independent experiments.

compared to that of untreated mice ($p < 0.001$), and CLDC still protected mice treated with the isotype antibody as compared to untreated mice ($p < 0.001$) (Fig. 5.8). Thus, these results indicated that NK cells were necessary for full CLDC-mediated protection. However, partial protection still was observed in NK cell-depleted mice, suggesting either that other cell types besides NK cells also contributed to the protective effects of CLDC therapy or that the residual NK cells not depleted by the administration of anti-asialo GM1 antibody were sufficient to elicit partial protection.

The role of NK cells in regulating the bacterial burden also was assessed. Bacterial burdens in the lung, liver, and spleen of mice depleted of NK cells and treated with CLDC were determined 3 days after infection. Bacterial burdens in NK cell-depleted and CLDC-treated mice were significantly increased in all three organs compared to those of mice treated with CLDC immunotherapy alone (Fig. 5.8). In addition, significant reductions in bacterial burdens also were observed in NK cell-depleted and CLDC-treated mice compared to those of untreated mice. These results are consistent with the idea that NK cells activated by CLDC immunotherapy contribute to antibacterial activity, most likely by the secretion of IFN-γ.

5.4(8) Nitric oxide production is not necessary for CLDC-mediated protection.

The preceding *in vitro* and *in vivo* experiments demonstrated that IFN-γ was critical to the therapeutic effectiveness of CLDC immunotherapy. IFN-γ also is known to be a potent inducer of NOS2 (iNOS) activity, with the subsequent production of nitric oxide (45). Several *in vitro* studies utilizing both *B. pseudomallei* and *B. mallei* have demonstrated that the preactivation of cells with IFN-γ results in nitric oxide production, which in turn mediates bacterial killing. Other *in vitro* studies have shown that *Burkholderia* species may down

modulate nitric oxide production in order to enhance its survival (46-50). In contrast to the results obtained in these *in vitro* studies, *in vivo B. pseudomallei* challenge studies using $NOS2^{-/-}$ mice have failed to demonstrate a role for nitric oxide in controlling infection (51).

Given the critical role of IFN-γ in mediating the protective effects of CLDC immunotherapy, we wished to determine whether the induction of iNOS activity and nitric oxide production was necessary for CLDC activity. To address this question, we used the iNOS inhibitor AG to block the production of nitric oxide *in vivo*. Mice were treated with AG beginning 5 days prior to infection. Mice then were treated with CLDC i.n. 24 hours prior to infection and then were infected i.n. with $15\times LD_{50}$ (1.5×10^4 CFU) of *B. mallei*. We observed that pretreatment with AG had no effect on CLDC protection **(Figure 5.9)**. To ensure that the AG treatment protocol we used effectively inhibited nitric oxide production, in a separate experiment we injected AG-treated mice with lipopolysaccharide and assessed nitric oxide production in serum 6 hours later, and we found that AG treatment completely suppressed lipopolysaccharide-induced nitric oxide production (data not shown). Moreover, in naive mice not treated with AG, we also noted that CLDC treatment failed to induce detectable nitric oxide production in lung tissues or alveolar lavage fluid (data not shown). By real-time PCR analysis, we also failed to observe increased iNOS expression in lung tissues of mice treated with CLDC (data not shown). Thus, several lines of evidence indicated that the induction of nitric oxide production was not critical for CLDC-mediated protection from pneumonic infection with *B. mallei*, although we cannot completely rule out a minor role for NO in protection.

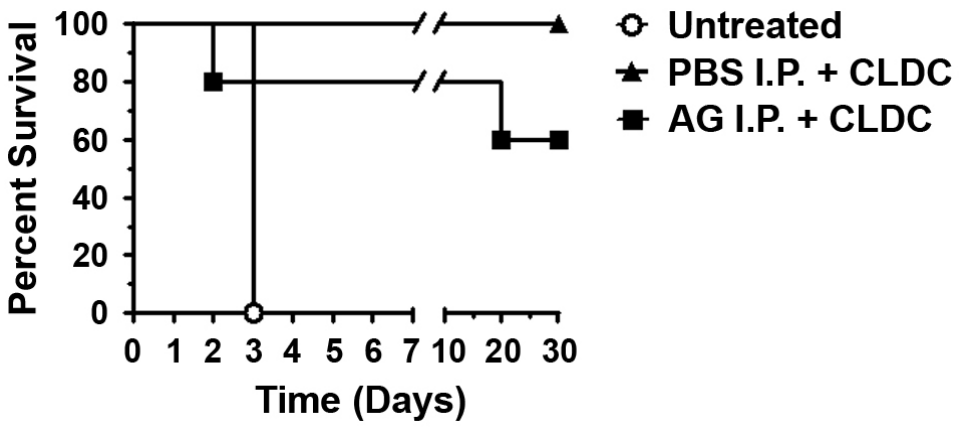

Figure 5.9. Nitric oxide production is not necessary for the protective effects of CLDC immunotherapy. BALB/c mice (n = 5 per group) were treated i.p. twice daily with AG (or PBS) starting 5 days prior to infection and continuing until 7 days post infection, as described in Materials and Methods. Mice were treated i.n. with CLDC 24 hours prior to infection with $15\times LD_{50}$ (1.5×10^4 CFU) of *B. mallei* i.n., and survival times were determined. Statistical differences were deter-mined by Kaplan-Meier analysis and a log-rank test. Data are representative of two independent experiments.

5.5 Discussion.

The results presented here indicated that the mucosal delivery of a potent activator of innate immunity could provide significant protection from lethal pneumonic infection with both *B. mallei* and *B. pseudomallei*. The most effective protection was achieved when mice were pretreated with CLDC 24 hours prior to challenge, which suggested that critical immune protective mechanisms had to be active at the time of initial contact with the inhaled bacteria to be fully effective. The local, but not systemic, MyD88 dependent induction of IFN-γ release was required for protection, and protection appeared to be independent of nitric oxide production. Although previous studies found that the systemic administration of CpG-based immunotherapeutics could protect mice from the development of chronic pneumonia following low-dose *Burkholderia* challenge, these are the first studies to our knowledge to demonstrate effective protection from acutely lethal respiratory *Burkholderia* infection following the inhalational delivery of an immunotherapeutic (15, 20-21).

IFN-γ played a critical role in the protective effects elicited by CLDC immunotherapy. In support of this, we observed that IFN-$\gamma^{-/-}$ mice were completely unprotected by CLDC immunotherapy. It is likely that the primary source of IFN-γ production elicited by CLDC was from activated NK cells. The potent activation of NK cells and IFN-γ release from the lungs has been reported previously following CLDC immunotherapy (22). In the current study, we also demonstrated that NK cells played a role in the protective effects mediated by CLDC. However, the requirement for NK cells to mediate the protective effects of CLDC was more apparent at lower challenge doses. For example, NK-depleted, CLDC-treated mice challenged with $8\times LD_{50}$ (8×10^3 CFU) had a 60 to 80% survival rate, whereas mice infected with $12\times LD_{50}$ (1.2×10^4 CFU)

has only a 20% survival rate. Nevertheless, both of these challenge doses produced 100% mortality in untreated mice.

Despite a series of *in vitro* studies that have shown that nitric oxide is important for the bactericidal effects of IFN-γ against both *B. pseudomallei* and *B. mallei*, in our studies we failed to find a role for nitric oxide in CLDC-mediated protective activity (46-50). Our findings thus are in agreement with a previous *in vivo* study wherein mice lacking the iNOS gene ($NOS2^{-/-}$) did not exhibit increased susceptibility to *B. pseudomallei* infection (51). Thus, we concluded that CLDC-mediated protection from lethal *B. mallei* pneumonia was largely independent of nitric oxide production. In summary, these studies indicate that appropriately timed mucosally administered immunotherapy may induce effective nonspecific protection against pneumonic intracellular bacterial pathogens such as *B. mallei* and *B. pseudomallei*.

More recent reports published after completion of these studies have shown that CLDC combined with membrane fractions from *F. tularensis* results in reduced colonization of human macrophages by *B. pseudomallei* (52), and that i.n. delivery of CpG DNA can also protect against a low dose aerosol *B. pseudomallei* challenge (53). To the best of our knowledge, CLDC remains the only therapeutic to protect against an acute *Burkholderia* challenge. Although CLDC was not effective in these studies when administered after *B. mallei* infection, CLDC has been used therapeutically in combination with ceftazidime to treat mice following *B. pseudomallei* infection. In these studies CLDC therapy was found to synergize with ceftazidime, and treatment with CLDC and ceftazidime provided enhanced protection compared to either treatment alone (54).

Although the mucosal administration of CLDC in this study elicited complete protection against acute *B. mallei* infection, the infection was not completely eliminated. For example,

when mice surviving the acute challenge were monitored out to 60 days, we observed the recrudescence of infection in the spleens and livers of 80 to 90% of mice. Similar results were also observed in studies investigating CLDC and ceftazidime combination therapy where 60% of mice developed chronic disease (54). Repeated treatment with CLDC with or without antibiotics may be necessary to clear chronic infection.

In contrast to acute disease, relatively little is known about chronic infection with *B. mallei* or *B. pseudomallei*. Although naturally occurring glanders is rare, melioidosis is endemic in southeast Asia and northern Australia, and occurs in most tropical areas of the world (55). Chronic melioidosis has developed in patients up to 62 years after exposure, although the site of bacterial persistence is not known (56). Therefore, the next set of studies was performed to better understand chronic melioidosis, with a focus on possible reservoirs of bacterial persistence during periods of asymptomatic carriage.

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CHAPTER 6.

BURKHOLDERIA PSEUDOMALLEI PERSISTENTLY COLONIZES AND DISSEMINATES FROM THE GASTROINTESTINAL TRACT FOLLOWING ORAL OR INTRANASAL INOCULATION

The studies in this chapter describe persistent colonization of gastrointestinal (GI) tissues, and dissemination to systemic organs following oral infection with *B. pseudomallei*. I acknowledge Dr. Sharon Peacock and Dr. Herbert Schweizer for providing *B. pseudomallei* strains, Dr. Helle Bielefeldt-Ohmann for analysis of histopathology and for capturing micro photos, and Dr. Drew Rholl for assistance plating *B. pseudomallei* strains in experiments testing the selective media used for enteric isolation of *B. pseudomallei*.

6.1 Summary.

Burkholderia pseudomallei is a soil and water bacterium found in tropical and subtropical regions of the world that causes human disease (melioidosis), which may manifest as acute, overwhelming sepsis or as chronic subclinical or recurrent infections. At present, it is unclear where the *B. pseudomallei* organism resides in infected patients during chronic, subclinical infection. We hypothesized that the gastrointestinal (GI) tract was a potential site of persistent infection following oral inoculation with *B. pseudomallei*. Therefore mouse models were used to investigate the ability of *B. pseudomallei* to establish chronic enteric infection following mucosal

or parenteral inoculation. Selective media allowed for quantitative culture from various regions of the GI tract and in other organs following oral inoculation. The relationship between intestinal colonization with *B. pseudomalle*i and dissemination to extra-intestinal sites was investigated. These studies demonstrated that *B. pseudomallei* readily and persistently infected the GI tract following oral or intranasal inoculation, followed by sustained low-level fecal shedding. Using fluorescent *in situ* hybridization (FISH) *B. pseudomallei* was localized to the stomach following oral infection, with low numbers of bacteria in the ingesta of the small intestine and cecum and feces in the colon. Moreover, there was an absence of lesions throughout the GI tract during either acute or chronic infection with *B. pseudomallei*. Notably, oral or intranasal inoculation was also much more likely to lead to disseminated infection in the liver and spleen than was subcutaneous inoculation. Thus, we conclude that *B. pseudomallei* can readily establish low-level persistent colonization of the GI tract, and that the chronically colonized GI tract may serve as a reservoir for dissemination of infection to extra-intestinal sites.

6.2 Introduction.

Burkholderia pseudomallei is a soil bacterium that also infects humans and other mammals and causes the disease melioidosis in humans in southeast Asia (S.E. Asia), northern Australia (N. Australia), Brazil, and other parts of the world (1-3). Infection with *B. pseudomallei* can produce either acute, septicemic infections or chronic disseminated infections with long latency periods (4-9). Melioidosis is a particularly dangerous disease in humans because of the rapidity with which *B. pseudomallei* can cause disseminated infection and sepsis and because the organism displays high levels of intrinsic antibiotic resistance (7, 10-11). These features, plus the fact that the organism can be easily disseminated, have caused *B. pseudomallei* to be classified as a category B select agent by the United States Centers for Disease Control and Prevention (CDC) (12).

Infection with *B. pseudomallei* typically develops following exposure to bacteria in soil or water, though in 20 to 76% of cases the initial source of exposure remains unknown (7, 13-17). Patients infected with *B. pseudomallei* may remain asymptomatic for extended periods of time, with cases of melioidosis developing up to six decades following the original exposure to the organism (4-5, 8-9, 13). While acute melioidosis has been relatively well-studied, much less is known about how chronic disease develops or where the bacterium persists during long periods of asymptomatic infection.

B. pseudomallei is well-suited for survival in the soil and moist environments. For example, *B. pseudomallei* has been reported to survive without the addition of nutrients in distilled water for up to 2 decades (18-21). Environmental surveys have shown that *B. pseudomallei* can be isolated from soil over a wide pH range (from 2.8 to 7.4), and from water with a pH range of 2 to 9 (22-23). Studies have also shown that chlorine-treated water does not

effectively kill the *B. pseudomallei* organism (24-26). Moreover, *B. pseudomallei* can survive in feces for up to 27 days, and in urine for up to 17 days (27).

Early studies of the pathogenesis of *B. pseudomallei* infection done nearly a century ago using animal challenge models provided convincing evidence of susceptibility to oral infection (27-30). For example, melioidosis could be induced by feeding *B. pseudomallei*-contaminated vegetables to monkeys, guinea pigs, rabbits, and black rats (27, 30). Melioidosis was also reported in a dog and a pig that ate meat contaminated with *B. pseudomallei*, and contaminated drinking water was responsible for two outbreaks on pig farms (31-34).

In humans, *B. pseudomallei* has been isolated from gastric fluids, intestinal contents and from feces of melioidosis patients (30, 35-37). Human infection has been attributed to ingestion of contaminated lake or pond water, and contaminated drinking water was blamed for two melioidosis outbreaks in Australia (38-40). Ulcers have been observed in the stomach, small intestine and colon of human melioidosis patients, and infants have developed melioidosis following consumption of culture positive breast milk (6, 9, 41-44). Moreover, *B. pseudomallei* has been isolated from 26% of drinking water sources in Thailand and Australia (45-48). Therefore, there is mounting evidence that *B. pseudomallei* infection may be contracted orally in humans and that enteric infection may be more common than previously realized.

The goal of the current study was to determine whether *B. pseudomallei* could establish persistent intestinal infection in mice and to investigate the impact of route of inoculation on enteric infection and dissemination. To address these questions, we investigated and compared the relative susceptibility of different mouse strains to oral inoculation with several different strains of *B. pseudomallei*. We also explored the ability of *B. pseudomallei* to colonize the gut and cause pathology and the ability of the organism to cause persistent gastrointestinal (GI)

infection, fecal shedding, and systemic dissemination. Here we present evidence that *B. pseudomallei* can readily establish persistent GI infection in mice following oral or intranasal inoculation, even with very low inocula. This new animal model of *B. pseudomallei* infection should prove useful in investigating host and pathogen factors that regulate GI colonization and shedding. In addition, these findings suggest a possible explanation for maintenance of asymptomatic infections with *B. pseudomallei* in humans, followed by later development of disseminated disease.

6.3 Materials and methods.

6.3(1) Mice.

BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME),129S6/SvEvTac mice were purchased from Taconic Laboratories (Germantown, NY), and ICR mice were purchased from Harlan (Indianapolis, IN). All mice used in experiments were housed under pathogen-free conditions in micro-isolator cages and mice were 6-10 weeks of age at the time of infection. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University. Mice were euthanized upon reaching one of the following pre-determined euthanasia endpoints: (1) hunched posture with decreased movement or response to stimuli; (2) development of respiratory distress (tachypnea, open-mouthed respirations); or (3) loss of > 15% body weight.

6.3(2) Bacteria.

B. pseudomallei strain 1026b (Bp1026b) is a clinical isolate recovered from a human case of septicemic melioidosis in Thailand (49). Three low-passage clinical *B. pseudomallei* isolates recovered from melioidosis patients in Thailand were chosen at random from Dr. Schweizer's collection of *B. pseudomallei* strains were also used in this study. Strain 2671a (Bp2671a) was isolated from blood culture, while strain 2685a (Bp2685a) was isolated from a pus sample, and strain 2719a (Bp2719a) was isolated from the lungs. These three strains were determined to be unique from each other and from strain 1026b based on multilocus sequence typing (MLST) analysis (data not shown). All strains, including Bp1026b, were from the S.E. Asian clade of *B. pseudomallei*. In BSL-2 experiments the purM$^-$ mutant *B. pseudomallei* strain 82 (Bp82) derived from Bp1026b was used (49). For *B. thailandensis* experiments, strain E264, an environmental

isolate from Thailand was used (50). Additional *Burkholderia* species used include, *B. oklahomensis* strain C6786, *B. dolosa* strain AU0158, *B. gladioli* subspecies cocovenenans, *B. ubonensis* strain A20, and *B. vietnamiensis* strain H4102. All bacterial strains were kindly provided by Herbert Schweizer, Colorado State University. All strains were grown in Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA), and stationary phase cultures were frozen at -80°C in LB broth + 20% glycerol (Fisher Scientific, Pittsburgh, PA). All experiments were performed with strains from a single freezing event. All procedures involving *B. pseudomallei* were performed in a Biosafety Level 3 (BSL3) facility, in accordance with approved BSL3 and Select Agent protocols in place at Colorado State University.

6.3(3) Animal infections.

Immediately prior to animal inoculation bacterial stocks that had been frozen in LB broth with 20% glycerol were thawed and diluted in sterile phosphate buffered saline (PBS). Infectious doses were determined by plating serial dilutions of each inoculum on LB agar (BD Biosciences). Oral (p.o.) inoculations were done using a stainless steel 22 gauge gavage needle and mice were inoculated using a total volume of 100 µl. For intranasal (i.n.) inoculation, mice were anesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg) (Pfizer, New York, NY), and xylazine (10 mg/kg) (Lloyd Laboratories, Shenandoah, IA). Intranasal inoculations were done using a volume of 20 µl (10 µl per nostril). Subcutaneous (s.c) inoculations were done in the right groin and mice were inoculated with a total volume of 100 µl. Intraperitoneal inoculation was done by i.p. injection in a total volume of 200 µl.

LD_{50} values for acute disease (i.e., euthanasia required on or before day 7) were calculated using the Reed-Muench method (51). LD_{50} values in BALB/c used in this study

include 3.8×10^7 CFU for s.c. inoculation, 1.7×10^6 CFU for i.p. inoculation, and as described previously, 9×10^2 CFU for i.n. inoculation (49).

Preliminary experiments were performed to determine if pulmonary infection occurs following oral infection due to inadvertent aspiration of bacteria during oral gavage with *B. pseudomallei*. Following p.o. inoculation with 5×10^5 CFU *B. pseudomallei*, BALB/c mice were euthanized two hours after infection and bacterial burdens were determined in the lungs as described below (Limit of detection = 4 CFU/organ). On average *B. pseudomallei* was cultured from the lungs of 3/14 (21%) mice (data pooled from 3 independent experiments). These results are similar to previous studies, and due to the potential for aspiration into the lungs following p.o. inoculation, any mouse succumbing to acute disease with higher bacterial burdens in the lung than in GI tissues was excluded from the analysis (52).

Previous studies demonstrated that following i.n. inoculation, ~40% of the inoculum reaches the lungs (Chapter 3). Briefly, BALB/c mice (n = 9) were inoculated i.n. as described above, and pulmonary bacterial burdens were determined 3 hours after infection.

6.3(4) Selective medium for isolation of *B. pseudomallei* from gastrointestinal tissues.

The selective medium used most often for isolation of *B. pseudomallei* from clinical samples is Ashdown's medium (ASH) (53-55). In preliminary studies we found that ASH failed to prevent the growth of normal gut commensal bacteria and in many cases *B. pseudomallei* was overgrown by enteric bacteria. Therefore, to suppress the growth of commensal bacteria we added norfloxacin, ampicillin, and polymyxin B to ASH media (NAP-A) for selective isolation of *B. pseudomallei* from intestinal contents and feces, based on previously reported media and antibiotic susceptibility profiles of *B. pseudomallei* (54-57). To prepare NAP-A medium, we

used ASH medium as the basal medium (53). Briefly, 4% glycerol (Fisher Scientific), 5 μg/ml crystal violet (EMD Science, Gibbstown, NJ), 50 μg/ml neutral red (Sigma-Aldrich, St. Louis, MO), and 4 μg/ml gentamicin (Sigma-Aldrich) were added to trypticase soy agar (BD Biosciences). After ASH was autoclaved and cooled to 50-60°, norfloxacin (4 μg/ml) (Sigma-Aldrich), ampicillin (10 μg/ml) (Sigma-Aldrich) and polymyxin B (300 units/ml) (Sigma-Aldrich) were added to prepare NAP-A medium.

6.3(5) Determination of sepsis and organ bacterial burden.

For quantitative blood culture, serial 10-fold dilutions of heparinized blood were diluted in sterile PBS and dilutions were plated on LB agar plates. To determine bacterial burdens in lung, liver, spleen, brain and kidney tissues of infected mice, the mice were humanely euthanized, and organs from each mouse were harvested and placed in 4 ml sterile PBS. Organs were homogenized using a Stomacher 80 Biomaster (Seward, Bohemia, NY). Serial 10-fold dilutions of supernatants then were prepared in sterile saline and plated on LB agar plates (BD Biosciences). For GI organs, the stomach and cecum were cut into ~1-2 cm^2 sections, while small intestine and colon tissues were cut open longitudinally, and then cut into 2-3 cm lengths prior to homogenization. Stomach, small intestine (SI), cecum and colon homogenates were plated on NAP-A agar (described above). All agar plates were incubated at 37°C and colonies were counted at 48 hours. The limit of detection in blood was 10 CFU/ml, while the limit of detection in organ homogenates ranged from 1-20 CFU/organ.

6.3(6) Isolation of *B. pseudomallei* from fecal pellets.

Fecal pellets were collected by transferring mice from their cage into a plastic container, where the pellets were collected and placed in sterile PBS at a concentration of 0.1 gram feces per ml PBS. Multiple fecal pellets from each mouse (typically 5-6 pellets per mouse) were homogenized using a Stomacher 80 Biomaster. Serial dilutions of fecal homogenates were prepared in sterile PBS and plated on NAP-A agar plates. The limit of bacterial detection in feces was 10-60 CFU/gram of feces.

6.3(7) Fluorescent *in situ* hybridization.

Fluorescent *in situ* hybridization (FISH) was performed using antisense ssDNA probes targeting the 16S rRNA. *B. pseudomallei* specific probes used in this study were designed in our laboratory. Two probes designated Bpm427 (5'-CCACTCCGGGTATTAGCCAGA-3') (positions 427 to 447) and Bpm975 (5'-CGCCCAACTCTCATCGGG-3') (positions 975 to 992) were identified based on binding regions on the 16S rRNA gene of *B. pseudomallei* strain 1026b. Probe specificity was confirmed in preliminary experiments performed on pure bacterial cultures which demonstrated that both Bpm427 and Bpm975 bound to Bp82 and *B. mallei* ATCC23344 but not to *B. thailandensis* E264 or fecal bacteria (data not shown). The previously described Eub338 probe (5'- GCTGCCTCCCGTAGGAGT-3') which recognizes a conserved sequence present in the 16S rRNA of all bacteria, and the irrelevant Non338 probe (5'-ACTCCTACGGGAGGCAGC-3') containing a sequence complementary to the Eub338 probe were also used (58-59). All probes were purchased from Integrated DNA Technologies (San Diego, CA). Bpm427 and Bpm975 probes were 5' labeled with Cy3, and Eub338 and Non338 probes were 5' labeled with 6-FAM.

Tissue fixation was performed by placing tissues in 10% neutral buffered formalin (NBF) (Sigma-Aldrich) for 48 hours. The entire small intestine was collected as a "Swiss roll" and fixed in 10% NBF for 48 hours. After 48 hours in 10% NBF, all organs were transferred into a solution of 70% ethanol for 7 days. Tissues were then embedded in paraffin, and sectioned.

FISH was performed as described previously (60). Prior to performing the FISH assay tissue sections were baked for 1 hour at 60°C. Sections were then deparaffinized with histoclear (National Diagnostics, Atlanta, GA) and re-hydrated in solutions with decreasing ethanol concentration. Sections were post fixed in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) in PBS for 15 minutes at room temperature and washed in PBS. Tissue sections were then permeabilized using one of two proteinase K (PK) digestion protocols. PCR grade PK was purchased from Roche (Indianapolis, IN) and was diluted in 10 mM Tris pH 7.5, 5 mM $CaCl_2$, and 0.2% Triton X-100 (All reagents from Fisher Scientific). Preliminary experiments were performed to determine the optimal digestion conditions resulting in maximal signal strength from enteric bacteria, or the maximal digestion procedure which did not alter tissue morphology. Maximal signal from enteric bacteria was obtained following digestion in 20 µg/ml PK for 30 minutes at 37°C, as assessed by signal intensity following hybridization with the Eub338 probe. In contrast, the digestion which did not alter tissue morphology was determined to be 5 µg/ml PK for 8 minutes at 37°C, as assessed by changes in nuclear morphology following DAPI staining (data not shown). Therefore, for identification of *B. pseudomallei* in ingesta of the stomach and cecum, and fecal material in the colon sections were digested in 20 µg/ml PK for 30 minutes at 37°C; and for localization of *B. pseudomallei* in tissues a separate set of sections was digested in 5 µg/ml PK for 8 minutes at 37°C. All small intestine sections were digested in 5µg/ml PK for 8 minutes at 37°C, as preliminary experiments demonstrated that no

increase in signal from enteric bacteria in the ingesta of the small intestine was observed regardless of the PK digestion protocol used (data not shown). Following PK digestion sections were washed in 30 mM glycine (Fisher Scientific) to stop proteolysis, followed by a PBS wash. Next, tissue sections were hybridized with ssDNA probes. Probes were diluted in hybridization buffer consisting of 4X saline sodium citrate (SSC) (Fisher Scientific), 200 mg/ml dextran sulfate (Sigma-Aldrich), 20% formamide (Sigma-Aldrich), 0.25 mg/ml PolyA (Sigma-Aldrich), 0.25 mg/ml salmon sperm DNA (Invitrogen, Carlsbad, CA), 0.25 mg/ml tRNA (Invitrogen), and 0.5X Dendhart's solution (Sigma-Aldrich). Slides were hybridized with either a cocktail of the Bpm427, Bpm975 and Eub338 probes each used at a final concentration of 1 μg/ml, or the Non338 probe at a final concentration of 3 μg/ml. Probes were hybridized with tissue sections in a humidified chamber at 37°C for 24 hours. Following hybridization sections were washed to remove non-specific probe binding. Washes included, one 15 minute wash in 1X SSC at 37°C, two 15 minute washes in 1X SSC at 55°C, two 15 minute washes in 0.5X SSC at 55°C, and one 10 minute wash in 0.5X SSC at room temperature. Slides were washed in dH_2O at room temperature for two minutes, air dried, and mounted with Pro-Long gold containing DAPI (Invitrogen).

6.3(8) Fluorescent microscopy.

Following hybridization with FISH probes tissue sections were observed at 1000X final magnification using an Olympus BX51 fluorescent microscope (Olympus, Center Valley, PA) with DAPI (Ex. 377/50 nm; Dichroic 409 nm; Em. 477/60 nm), FITC (Ex. 482/35 nm; Dichroic 506 nm; Em. 536/40 nm) and Cy3 (Ex. 531/40 nm; Dichroic 562 nm; Em. 593/40 nm) filter sets (Semrock, Lake Forest, IL). Photomicrographs were captured with a DP71 camera using

CellSens Entry software version 1.5 (Olympus). Fluorescent overlays were created by combining individual fluorescent images in Photoshop CS3 software (Adobe, San Jose, CA). When necessary multiple images were obtained at different focal planes and combined using layer masks in Photoshop software. All other manipulations were applied to the images globally. Settings used on images obtained from tissues hybridized with the Bpm427, Bpm975 and Eub338 probes were determined from images captured from tissues hybridized with the Non338 probe.

6.3(9) Histological analysis.

Tissues were fixed as described above for FISH analysis, and lungs were inflated with NBF (Sigma-Aldrich) via the trachea for 5 minutes prior to removal, and then placed in NBF for 48 hours. After fixation tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissues were examined by a veterinary pathologist experienced in mouse pathology. Photomicrographs were optimized using Photoshop CS3 software (Adobe) and all changes were applied globally.

6.3(10) Statistical analysis.

Statistical analyses were done using Prism 5.0 software (Graph Pad, San Diego, CA). Analyses comparing two groups were done using a two-tailed Student's t-test, and analyses comparing more than two groups were performed using a one-way ANOVA followed by a Tukey's multiple means comparison test. Differences in percentages of positive samples were compared using a two-tailed Fisher's exact test. Differences were considered statistically significant for $p < 0.05$, and statistical trends were considered for $p < 0.1$.

6.4 Results.

6.4(1) Development of selective medium for culture of *B. pseudomallei* from GI tissues and feces.

Ashdown's medium (ASH) is currently considered to be the most selective medium for isolation of *B. pseudomallei* from complex microbial samples (54-55). However, studies attempting to isolate *B. pseudomallei* from feces have reported that enteric bacteria can grow on ASH (36). Due to the high levels of antibiotic resistance in *B. pseudomallei*, an attempt to improve the specificity of ASH was made. Based on previous reports describing antibiotic resistance profiles of *B. pseudomallei*, as well as antibiotics which have been shown to kill enteric bacteria; additional antibiotics were screened for their ability to kill enteric bacteria without affecting growth of *B. pseudomallei* (56, 61-62).

Modified Trypticase Soy Agar medium (M-TSA), consisting of TSA containing 5 μg/ml crystal violet, 4% glycerol and 50 μg/ml neutral red, was prepared as described previously (53). Fecal pellet plating was used to screen M-TSA containing gentamicin (4 μg/ml) (ASH), ampicillin (10 μg/ml), norfloxacin (4 μg/ml) or polymyxin B (300 U/ml). Feces plating from BALB/c, C57BL/6 and ICR mice demonstrated that all four antibiotics significantly reduced enteric bacterial titers compared to M-TSA alone ($p < 0.001$) **(Figure 6.1)**. Norfloxacin was the most effective antibiotic and was significantly better than gentamicin, which is currently used in ASH ($p < 0.05$). In addition, both ampicillin and norfloxacin were found to be more selective than polymyxin B ($p < 0.05$) (Fig. 6.1). Preliminary studies were also performed with vancomycin and rifampicin, although these antibiotics were less effective against enteric bacteria (data not shown). Therefore, this screen identified multiple antibiotics which could potentially improve the specificity of ASH.

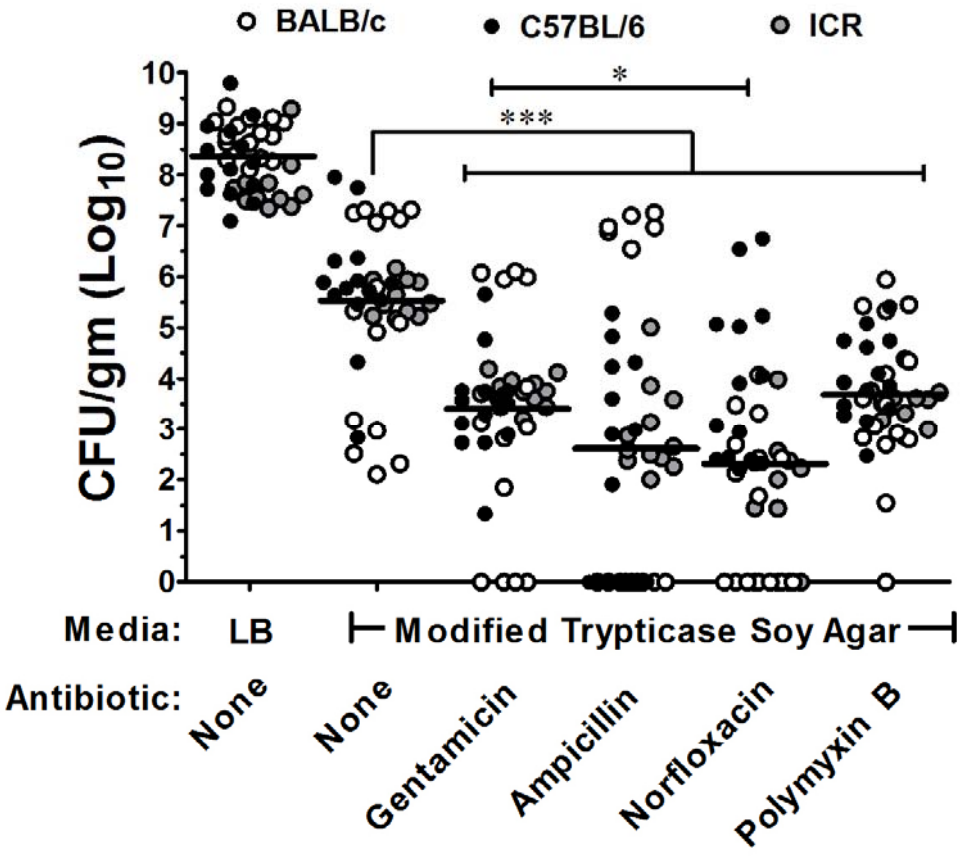

Figure 6.1. Ability of various antibiotics to prevent growth of enteric bacteria. Fecal pellets were homogenized in PBS and serial dilutions were plated on various agar mediums. Modified Trypticase Soy Agar was supplemented with Gentamicin (4 μg/ml), Ampicillin (10 μg/ml), Norfloxacin (4 μg/ml) or Polymyxin B (300 U/ml). Data from BALB/c (n = 15), C57BL/6 (n = 14) and ICR (n = 12) were pooled (n = 41). Data are presented as individual $\log_{10}$ CFU/gram of feces with bars representing the mean titer for each group. Statistical differences between antibiotics were determined by a one-way ANOVA followed by a Tukey's multiple mean's test (* $p < 0.05$, *** $p < 0.001$). Data were pooled from 7 individual experiments.

Next the effect of combining all four antibiotics was investigated. Organ homogenate from GI tissues and fecal pellets from BALB/c, C57BL/6 and ICR mice plated on ASH medium containing norfloxacin, ampicillin and polymyxin B (NAP-A) resulted in a significant decrease in enteric bacterial growth as compared to growth on ASH **(Figure 6.2)**. In addition, qualitative differences could also be observed. Growth of *B. pseudomallei* colonies on ASH results in a red/purple/ruffled morphology. In Fig 6.2(B) a number of *B. pseudomallei* colonies can be

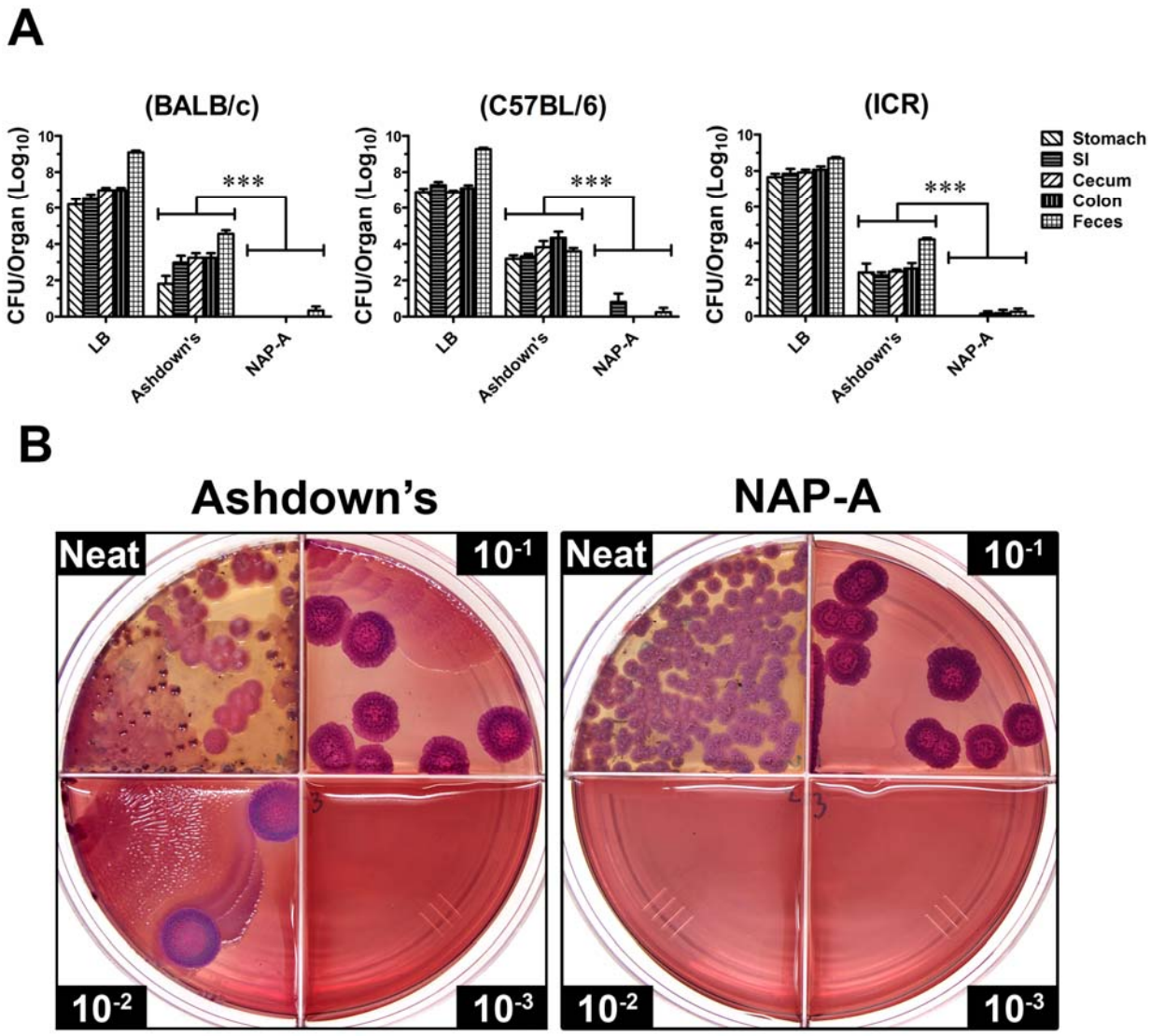

Figure 6.2. Increased specificity of NAP-A medium compared to ASH. (A) Stomach, SI, cecum, LI and feces tissues from BALB/c (n = 10), C57BL/6 (n = 11) and ICR (n = 11) mice were homogenized and serial dilutions were plated on LB, ASH or NAP-A medium agar plates. Data are graphed as $\log_{10}$ CFU/organ ± SEM for stomach, SI, cecum and LI, and as $\log_{10}$ CFU/gram ± SEM for feces. Differences in bacterial titers between different mediums in all organs were determined by a two way-ANOVA followed by a Bonferroni post test (*** $p < 0.001$). For each mouse strain data were pooled from 2 individual experiments. (B) Organ homogenate from the small intestine of a BALB/c mouse infected with *B. pseudomallei* 1026b. Serial dilutions were plated on both ASH and NAP-A medium. Agar plates were incubated at 37°C for 5 days. Quadrants where undiluted (neat) homogenate and serial dilutions were plated are labeled accordingly.

identified on ASH media, although all colonies in the neat quadrant appear to be enteric bacteria. In contrast, all colonies in the neat quadrant of NAP-A medium have a morphology characteristic of *B. pseudomallei*. These results suggested that NAP-A medium may provide increased sensitivity compared to ASH, allowing for improved detection of low level colonization.

To test if the additional antibiotics added to ASH medium affected growth of *B. pseudomallei*, overnight broth cultures of *B. pseudomallei* were serially diluted and plated on LB, ASH and NAP-A agar medium. These studies demonstrated that growth of 25 different strains was nearly identical on all 3 mediums ($p = 0.99$) **(Figure 6.3)**. In addition, 3 strains of *B. thailandensis* also grew equivalently on LB, ASH and NAP-A medium (data not shown). Furthermore equivalent growth of *B. oklahomensis, B. dolosa, B. gladioli, B. ubonensis*, and *B. vietnamiensis* was observed on ASH and NAP-A (data not shown).

To determine if NAP-A medium provided increased sensitivity organ homogenates from GI tissues from *B. pseudomallei* infected mice were plated on both ASH and NAP-A mediums. When all organ titers from GI organs plated on both ASH and NAP-A medium were compared no differences were observed ($p = 0.35$; two-tailed Student's t-test). However, the major effect of NAP-A medium compared to ASH was observed when neat organ homogenate was plated (Fig. 6.3B and 6.3C). To determine if NAP-A medium improved detection of low level GI infection, bacterial burdens from GI organs where *B. pseudomallei* could only be identified in the neat homogenate were compared (titer $\leq$ 1000 CFU/organ). This analysis revealed that NAP-A was more sensitive than ASH for detection of low level colonization (Fig 6.3B). Therefore the addition of norfloxacin, ampicillin and polymyxin B to ASH medium resulted in a more specific and more sensitive medium for the isolation of *B. pseudomallei* from intestinal tissues.

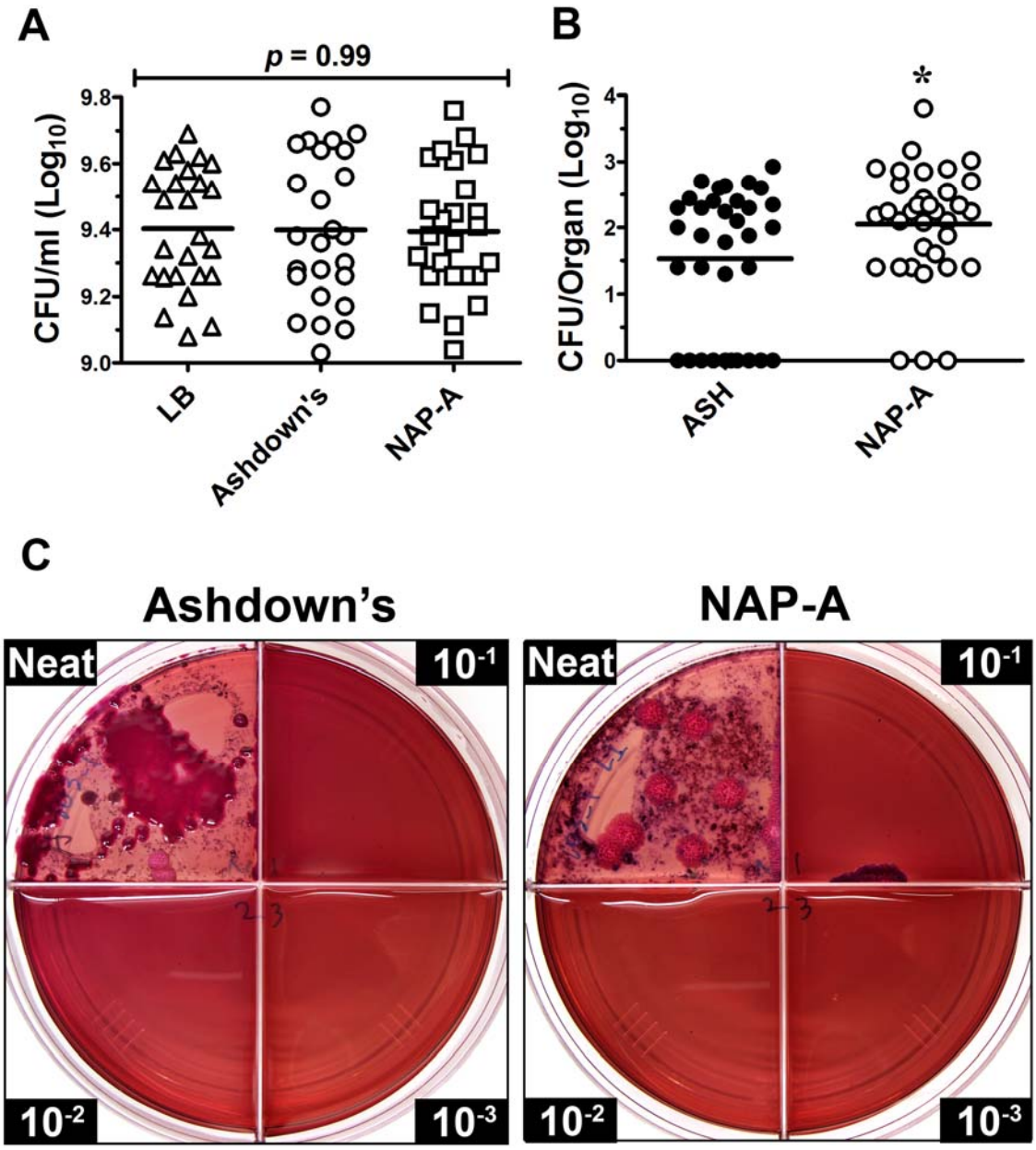

Figure 6.3. Increased sensitivity of NAP-A compared to ASH medium. (A) Overnight cultures of 25 *B. pseudomallei* isolates (12 clinical, 10 environmental and 3 laboratory strains) were grown in LB broth. Serial dilutions were plated on LB, ASH or NAP-A medium agar plates. Data are presented as individual $\log_{10}$ CFU/ml titers with bars representing the mean titer. Bacterial titers were compared using a one-way ANOVA. Data are representative of 2 independent experiments. (B) NAP-A medium improves isolation of *B. pseudomallei* from tissues with low bacterial burden (titers ≤ 1000 CFU/organ). Data from GI organs plated on ASH and NAP-A medium are graphed as individual $\log_{10}$ CFU/organ values with bars representing mean values (n = 34). Statistical differences were determined by a two-tailed Student's t-test (* $p < 0.05$). (C) Large intestine organ homogenate from a BALB/c mouse infected with *B. pseudomallei* strain 2685a, plated on both ASH and NAP-A medium. Agar plates were incubated at 37°C for 5 days.

6.4(2) Persistent GI colonization develops following low-dose oral inoculation with *B. pseudomallei*, but not *B. thailandensis*.

Previous studies have demonstrated infection with *B. pseudomallei* following acute, high-dose oral inoculation, but effects of oral inoculation on development of chronic infection have not been previously assessed (27-29, 52, 63). To investigate the ability of *B. pseudomallei* to establish chronic enteric infection, we first determined the minimal oral challenge dose of *B. pseudomallei* strain 1026b required to cause persistent GI colonization. To ensure that environmental persistence traits of *B. pseudomallei* were not responsible for enteric persistence, oral challenge studies were also performed with *B. thailandensis* E264, a closely related but avirulent environmental bacterium (64-66). BALB/c mice (n = 9 - 10 per group) were inoculated with *B. pseudomallei* or *B. thailandensis* orally and quantitative cultures were done on GI organs 8 weeks after inoculation. Any animal where *B. pseudomallei* or *B. thailandensis* could be recovered from the stomach, SI, cecum or colon on day 60 after inoculation was considered to be persistently infected. Oral inoculation with 4×10^5 and 4×10^4 CFU *B. pseudomallei* yielded similar results, with 89% and 78% of mice becoming chronically infected, respectively. Challenge with 4×10^3 CFU *B. pseudomallei* resulted in a 44% rate of chronic infection, while inoculation with 2.5×10^2 CFU *B. pseudomallei* resulted in a 10% chronic infection rate **(Table 6.1)**. The infectious dose needed to persistently colonize 50% of mice (ID_{50}) following oral *B. pseudomallei* infection was determined to be 1.5×10^4 CFU according to the Reed-Muench method (Table 6.1). Therefore, for most of the experiments done in this report, we selected an oral challenge dose of 5×10^5 CFU *B. pseudomallei* strain 1026b, as this dose reliably caused persistent GI infection without triggering acutely fatal disease.

Table 6.1. Colonization rates following oral infection.

B. pseudomallei		*B. thailandensis*	
Dose (CFU)	**% GI Infection**	**Dose (CFU)**	**% GI Infection**
3.9×10^5	89% (8/9)	2.8×10^{10}	50% (5/10)
4.2×10^4	78% (7/9)	2.6×10^9	44% (4/9)
3.8×10^3	44% (4/9)	2.2×10^8	20% (2/10)
2.5×10^2	10% (1/10)		
ID_{50}[a] = 1.5×10^4 CFU		ID_{50}[a] = 1.3×10^{10} CFU	

[a] ID_{50} values calculated by the Reed-Muench method (51).

In contrast to oral *B. pseudomallei* infection, very high doses of *B. thailandensis* were required to cause persistent GI colonization. Organ plating performed at day 60 following oral infection with *B. thailandensis* revealed that 50% of mice infected with 2.8×10^{10} CFU, 44% of mice infected with 2.6×10^9, and 20% of mice infected with 2.2×10^8 CFU were persistently infected (Table 6.1). The ID_{50} following oral *B. thailandensis* infection determined by the Reed Muench method was 1.3×10^{10} CFU. This is about a 6 $\log_{10}$ increase from the ID_{50} observed for *B. pseudomallei* (Table 6.1). Additionally, fecal shedding titers determined at earlier time points were also reduced following oral infection of mice with 2.8×10^{10} CFU *B. thailandensis* as compared to oral infection with 5×10^5 CFU *B. pseudomallei*. For example, fecal shedding titers were significantly reduced in *B. thailandensis* mice at day 14 ($p < 0.05$), 35 ($p < 0.01$) and 56 ($p < 0.01$) (data not shown; two-tailed Student's t-test). These studies demonstrate that environmental survival traits are not sufficient to explain GI persistence observed following *B. pseudomallei* infection.

In addition, we investigated the ability of oral challenge with *B. pseudomallei* to elicit acutely lethal infection by determining LD_{50} values according to the Reed-Muench method. LD_{50} values for acute disease following oral challenge (i.e., mice euthanized on or before day 7) in BALB/c, C57BL/6 and 129S6/SvEv mice (n = 6-10 per dose), were 1.04×10^7 CFU, 7.1×10^6 CFU, and 1.9×10^3 CFU respectively. From these studies, we observed mouse strain-specific differences in susceptibility to acute, lethal infection following oral inoculation. These mouse strain differences in susceptibility were similar to those we and others have noted for inhalation, and oral inoculation (49, 52). Following oral infection of BALB/c mice (n = 30) with *B. thailandensis* strain E264 only one death was observed 25 days after infection with 2.6×10^9 CFU, while no deaths were observed following oral infection with 2.2×10^8 or 2.2×10^{10} CFU. Thus, oral inoculation with *B. pseudomallei* strain 1026b, but not *B. thailandensis*, reliably produced either chronic or acute infection in mice, depending on the challenge dose delivered.

6.4(3) *B. pseudomallei* is present in all GI organs following oral inoculation.

Using the low-dose oral challenge model, we next investigated whether there were differences in the location of persistent *B. pseudomallei* infection amongst GI organs. BALB/c mice (n = 8-9 per group) were inoculated orally with 5×10^5 CFU *B. pseudomallei* and bacterial burdens in various locations in the GI tract were determined on days 3, 14 and 56 post-challenge. We observed that persistent infection could be detected in most mice at all levels of the GI tract examined, from the stomach through the colon (**Figure 6.4**). Bacteria could be recovered more frequently from the small intestine, cecum, and colon than from the stomach.

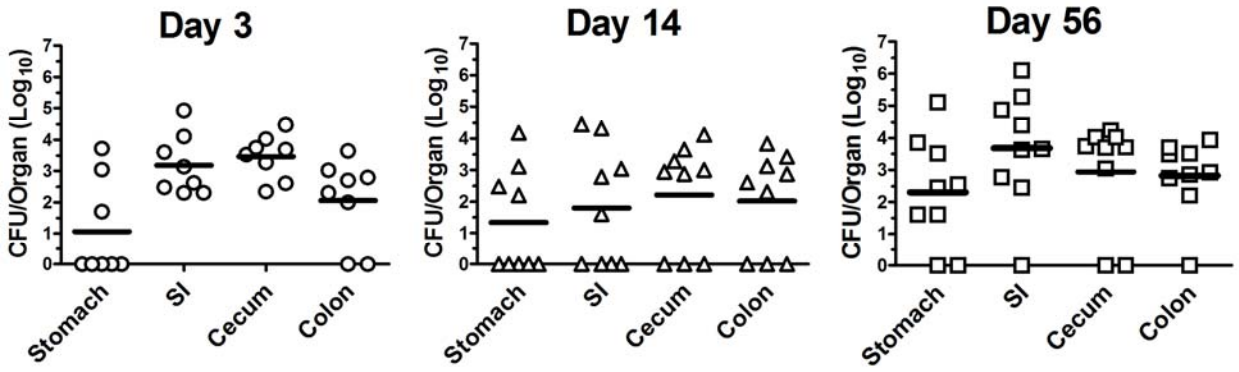

Figure 6.4. Gastrointestinal bacterial burden following oral *B. pseudomallei* challenge. BALB/c mice (n = 8-9) were inoculated orally with 5×10^5 CFU *B. pseudomallei* strain 1026b. On day 3, 14 and 56 after inoculation, mice were euthanized and organs were processed for determination of bacterial burden as described in Materials and Methods. Data are presented as individual $\log_{10}$ CFU/organ values with bars representing the mean titer for each organ. The limit of detection was 20 CFU/organ. Data were pooled from two independent experiments.

In mice inoculated orally with *Salmonella*, *Yersinia enterocolitica,* or *Shigella dysenteriae*, the gallbladder and mesenteric lymph nodes are often infected (67-72). However, in mice challenged orally with *B. pseudomallei*, we found that the mesenteric lymph node or gall bladder were rarely infected, or were infected at a very low level. For example, mesenteric lymph node infection was found in only 8 of 36 mice, with a mean titer of 9 CFU/organ (data not shown). In addition, the gall bladder was infected in only 2 of 26 mice, with a mean titer of 3 CFU/organ (data not shown).

Given the ability of *B. pseudomallei* to persistently colonize the GI tract, we next asked whether infected mice also shed the organism in their feces. Therefore, we examined fecal shedding of *B. pseudomallei* from orally-inoculated mice (5×10^5 CFU) over a 60 day period (**Figure 6.5**). We found that fecal shedding of *B. pseudomallei* could be detected beginning as early as 24 hours following oral inoculation (data not shown). Remarkably, the level of fecal shedding remained relatively constant over the next 60 days of observation. The concentration of *B. pseudomallei* in feces of persistently infected mice (range = $10^2 - 10^5$ CFU/gm feces,

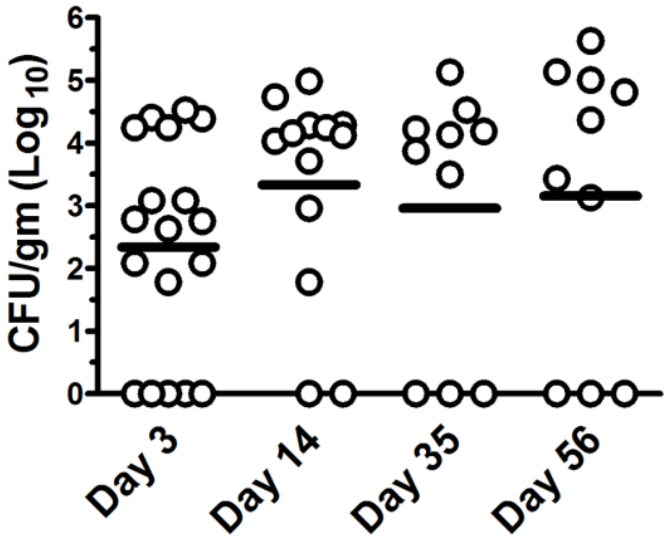

Figure 6.5. *B. pseudomallei* is persistently shed in the feces following oral inoculation. BALB/c mice (n = 10-18 animals per group) were inoculated orally with 5×10^5 CFU. On days 3, 14, 35 and 56 after inoculation, feces were collected and processed for determination of bacterial burden as described in Materials and Methods. Data are presented as individual $\log_{10}$ CFU/gram values with bars representing the mean titer at each time point. The limit of detection was 10-60 CFU/gram, depending on the number of fecal pellets collected from each mouse. Data was pooled from two independent experiments.

average = 10^3 CFU/gm feces). It should also be noted that persistently infected mice showed no outward signs of GI infection, maintaining normal body weight and fecal pellet consistency.

6.4(4) GI infection develops following oral inoculation with multiple different *B. pseudomallei* strains.

The preceding experiments done with *B. pseudomallei* strain 1026b demonstrated a marked propensity to colonize the GI tract and establish persistent infection following oral inoculation. To determine whether the ability to establish GI infection was a general property of *B. pseudomallei*, or instead reflected a strain-specific phenomenon, we also subjected mice to oral challenge with 3 additional strains (Bp2671a, Bp2685a, Bp2791a) of *B. pseudomallei*. These 3 strains were randomly selected from a panel of 22 clinical *B. pseudomallei* isolates

kindly provided by Dr. S. Peacock (now at the University of Cambridge). In initial experiments, we observed that oral challenge with a challenge dose of ~5×10^5 CFU with the 3 new strains of *B. pseudomallei* produced a high percentage of acutely lethal infections, suggesting that these 3 strains were each more virulent than the 1026b strain (data not shown). Due to the increased acute lethality of these 3 new strains, colonization of GI organs at day 3 post infection was used to compare infection with 1026b (Fig. 6.4) and the 3 new strains. BALB/c mice (n= 8-10 per strain) were challenged orally with each of the 3 additional *B. pseudomallei* strains (Bp2671a = 3.6×10^5 CFU; Bp2685a = 2.9×10^5 CFU; Bp2719a = 3.5×10^5 CFU), and the organ bacterial burden was determined on day 3. We did not observe statistically significant differences in GI bacterial burdens in mice infected with strain 1026b compared to the 3 new clinical isolates **(Figure 6.6)**.

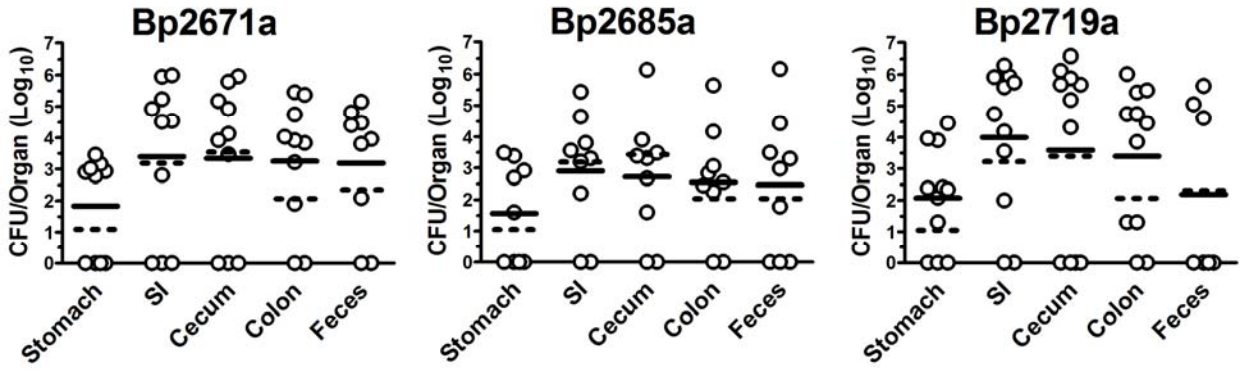

Figure 6.6. GI colonization occurs following oral inoculation with multiple *B. pseudomallei* strains. BALB/c mice (n = 9-11 animals evaluated per bacterial strain) were inoculated orally with Bp2671a (3.6×10^5 CFU); Bp2685a (2.9×10^5 CFU); or Bp2719a (3.5×10^5 CFU). At day 3 after infection, organs and feces were processed for determination of bacterial burden. Data are presented as individual values with solid bars representing the mean $\log_{10}$ titer. Organ bacterial burdens are expressed as $\log_{10}$ CFU/organ, and feces titers are graphed as $\log_{10}$ CFU/gram of feces. Dashed bars represent the mean $\log_{10}$ titers from day 3 Bp1026b bacterial burden determination (Reproduced from Figure 6.4 for reference). Data were pooled from 2 independent experiments. The limit of detection was 20 CFU/organ, and 10-60 CFU/gram of feces, depending on the number of fecal pellets collected from each mouse.

To determine whether lower oral challenge doses could elicit chronic GI infection with the 3 clinical *B. pseudomallei* strains, BALB/c mice (n = 3 – 5 per group) were inoculated with approximately 5×10^4 CFU of each of the 3 new *B. pseudomallei* strains (Bp2671a = 2.0×10^4 CFU; Bp2685a = 4.8×10^4 CFU; Bp2719a = 2.8×10^4 CFU). Even a one log reduction in challenge dose still resulted in rapid lethality with a 58% mortality rate by day 14 post-challenge (data not shown). Determination of bacterial burden in feces of surviving mice that were challenged orally with the 3 new clinical strains of *B. pseudomallei* revealed that 6 of 8 mice had GI colonization on day 7, while 3 of 5 mice were colonized on day 14, although the two surviving mice had cleared the GI infection by day 56 (data not shown). Strain Bp2719a was found to be especially virulent and p.o. infection with as few as 1.8×10^3 CFU resulted in 100% lethality 6 days after infection (data not shown). Thus, acute challenge studies revealed that all 4 strains of *B. pseudomallei* were similar in their ability to colonize the GI tract following oral inoculation. However, the three low-passage clinical isolates also appeared to be more virulent *in vivo* than *B. pseudomallei* 1026b, as reflected by more rapid spread and dissemination following oral challenge.

6.4(5) Dissemination to systemic organs following oral *B. pseudomallei* inoculation.

The liver and the spleen are two of the most frequently affected visceral organs in humans with melioidosis (3, 13, 73-75). We and others have also observed that in mice that survive high-dose intranasal challenge with *B. pseudomallei*, death due to disseminated infection to the liver and spleen often develops over a 30-90 day period (3, 76-78). Therefore, we next investigated whether disseminated infection to the liver and spleen could develop following oral inoculation with *B. pseudomallei*. To address this question, bacterial burdens in the blood, lung,

liver, spleen were determined 3, 14 and 56 days after low-dose oral challenge of BALB/c mice (n = 8 - 10) with 5×10^5 CFU *B. pseudomallei* 1026b. We observed that by day 60 post-challenge, nearly all orally-inoculated mice had *B. pseudomallei* lesions in the spleen and liver, with especially high bacterial burdens in the spleen **(Figure 6.7)**. Thus, it appeared that *B. pseudomallei* could readily disseminate from the GI tract to the liver and spleen.

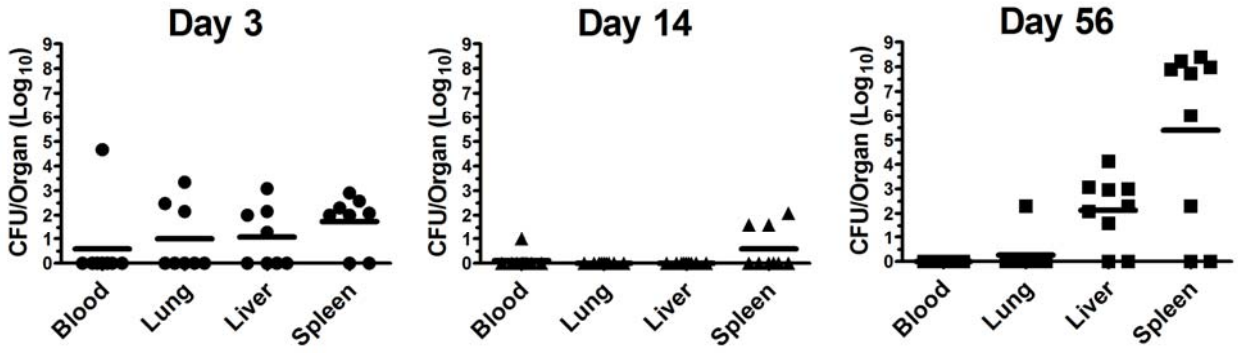

Figure 6.7. Bacterial dissemination to systemic organs following oral inoculation with *B. pseudomallei.* BALB/c mice (n = 8-10 animals per time point) were inoculated orally with 5×10^5 CFU Bp1026b. On day 3, 14 and 56 after inoculation mice were euthanized and organs were processed for determination of bacterial burden. Data are graphed as individual values with bars representing the mean titer for each group. Lung, liver and spleen titers are graphed as $\log_{10}$ CFU/organ, and blood is graphed as $\log_{10}$ CFU/ml. The limit of detection was 20 CFU/organ, and 10 CFU/ml for blood. Data were pooled from two independent experiments.

We next assessed whether the 3 low-passage *B. pseudomallei* strains could also disseminate to the liver and spleen following low-dose oral inoculation. Mice (n = 9 - 11 per challenge strain) were inoculated orally (~5×10^5 CFU) with each of the three strains and bacterial burdens were determined in blood, lung, liver, spleen three days after inoculation. Each of these three strains was able to cause infection in the blood, lung, liver, and spleen, with bacterial burdens statistically equivalent to those generated by oral inoculation with *B. pseudomallei* strain 1026b, although a trend towards increased burdens in the liver ($p = 0.08$) and spleen ($p = 0.07$) were observed following infection with strain Bp2719a as compared to Bp1026b, when analyzed by a two-tailed Student's t-test **(Figure 6.8)**.

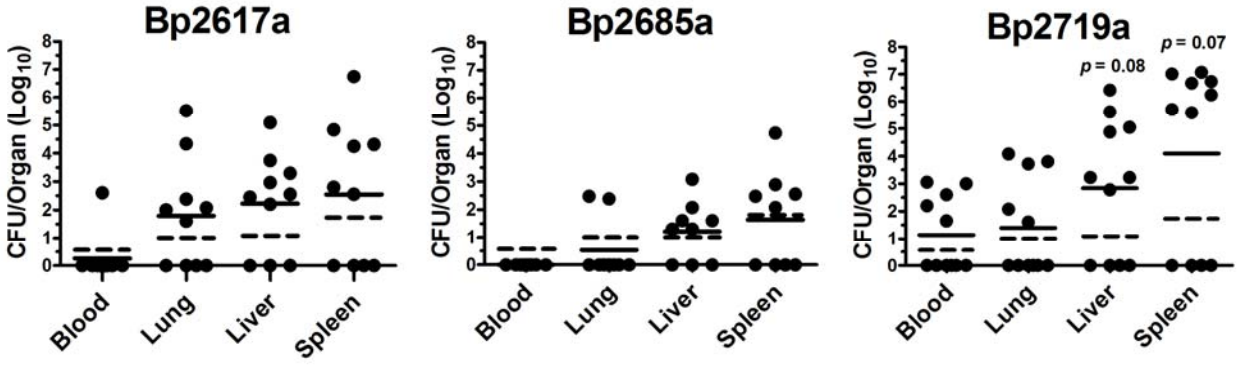

Figure 6.8. Bacterial dissemination to systemic organs following oral inoculation with multiple *B. pseudomallei* strains. BALB/c mice (n = 9-11 animals evaluated per bacterial strain) were inoculated orally with Bp2671a (3.6×10^5 CFU); Bp2685a (2.9×10^5 CFU); or Bp2719a (3.5×10^5 CFU). At day 3 after infection, organs were processed for determination of bacterial burden as described in Materials and Methods. Data are presented as individual values with solid bars representing the mean $\log_{10}$ titer. Organ bacterial burdens are expressed as $\log_{10}$ CFU/organ, and blood titers are graphed as $\log_{10}$ CFU/ml. Dashed bars represent the mean $\log_{10}$ titers from day 3 Bp1026b bacterial burden determination (Reproduced from Figure 6.7 for reference). Data were pooled from 2 independent experiments. The limit of detection was 20 CFU/organ, and 10 CFU/ml for blood. Statistical differences were determined between Bp1026b and each clinical strain using a two-tailed Student's t-test.

After receiving a low dose oral challenge (~5×10^4 CFU) with strain Bp2671a, Bp2685a or Bp2719a, 6 of 11 mice had grossly visible splenic lesions, while only 2 of 9 mice inoculated orally with Bp1026b (5×10^5 CFU) had spleen lesions at day 60 post-challenge. Thus, it was apparent that different *B. pseudomallei* strains were capable of disseminating from the GI tract following oral inoculation.

6.4(6) *B. pseudomallei* colonize the stomach following oral infection.

The above experiments demonstrated that *B. pseudomallei* could persistently colonize all organs of the GI tract. Fluorescent *in situ* hybridization (FISH) was performed to further localize *B. pseudomallei* within GI tissues. Preliminary FISH experiments performed with bacterial cultures, and purified fecal bacteria, demonstrated that the Bpm427 and Bpm975 probes bound to *B. pseudomallei* strain 82 (Bp82) but not to *B. thailandensis* or fecal bacteria (data not shown).

Additionally, when Bp82 was spiked into fecal bacteria, the Eub338 signal from Bp82 was found to be dim as compared to the signal from fecal bacteria. Therefore, when the 6-FAM signal from the Eub338 probe was combined with the Cy3 signal from the Bpm427 and Bpm975 probes *B. pseudomallei* typically appeared red in image overlays rather than yellow (data not shown).

For localization studies BALB/c mice were infected with ~5×10^5 CFU *B. pseudomallei* strain 1026b, and mice were euthanized 56 days after infection. Tissues were fixed and hybridized with FISH probes as described in materials and methods. To ensure bacterial staining could be discriminated from intestinal debris tissues were observed at 1000X final magnification. Surprisingly, despite the harsh conditions present in the stomach, FISH staining revealed that the major focus of *B. pseudomallei* infection following oral infection was localized in the stomach **(Figure 6.9)**. *B. pseudomallei* was also identified in the ingesta of the small intestine, cecum and fecal material of the colon, although at much lower levels than the stomach (Fig. 6.9B-D). *B. pseudomallei* identified in the small intestine were localized in the distal jejunum and ileum.

To ensure non-specific binding of *B. pseudomallei* probes to enteric bacteria was not occurring tissues from uninfected BALB/c mice were hybridized with the Bpm427, Bpm975 and Eub338 probes. These experiments demonstrated that the *B. pseudomallei* probes did not cross react with enteric bacteria, while the eubacterial probe did bind to enteric bacteria (Fig. 6.9E-H). Tissues from uninfected mice were also hybridized with the irrelevant Non338 probe to ensure that binding of FISH probes was sequence specific. While bacterial DAPI signal was observed in all GI sections no signal was observed with the Non338 probe, demonstrating that the FISH procedure resulted in sequence specific probe binding (Figure 6.9I-L).

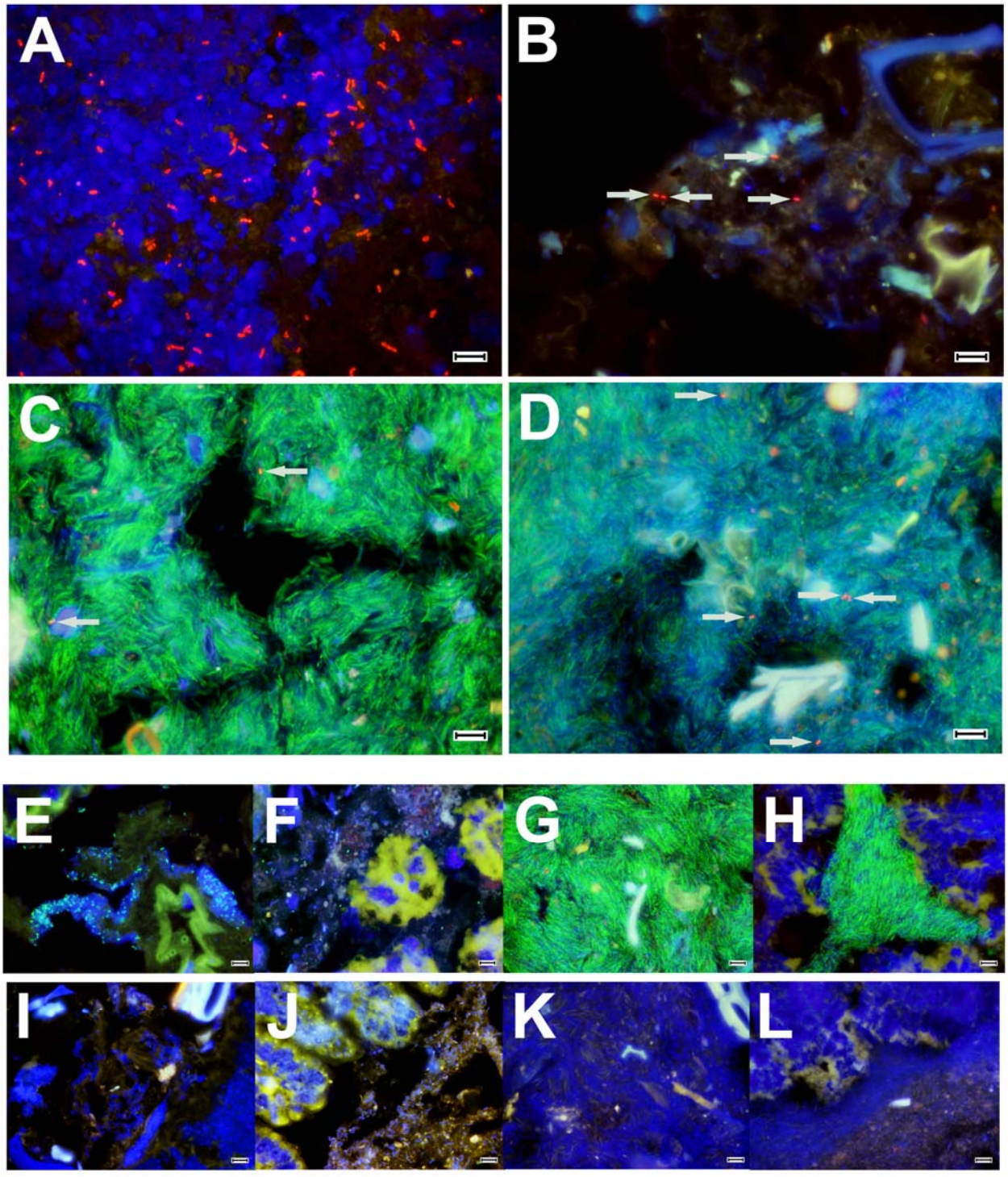
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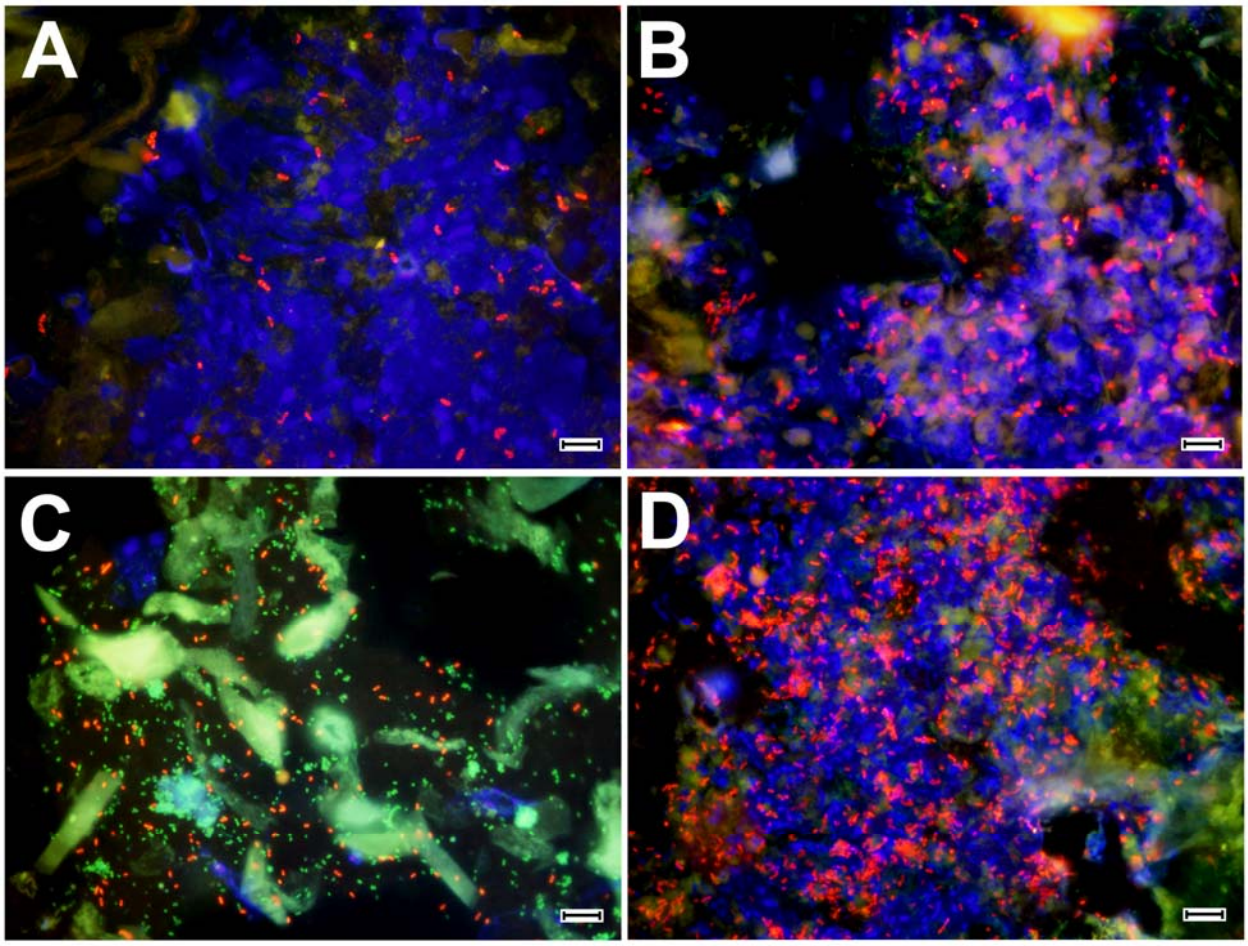
Figure 6.9. Localization of *B. pseudomallei* 1026b in gastrointestinal organs following oral infection. (A-D) Stomach (A), small intestine (B), cecum (C) and colon (D) tissues from mice infected orally with 5×10^5 CFU *B. pseudomallei* strain 1026b were collected 56 days after infection. Organs were fixed in 10% NBF and embedded in paraffin prior to sectioning. FISH was performed on tissue sections as described in materials and methods. Tissue sections were counterstained with DAPI (blue) and observed at 1000X final magnification. Tissue sections were hybridized with a eubacterial probe (green), and two *B. pseudomallei* specific probes (red). (E-L) Control tissues from uninfected BALB/c mice were processed as described for *B. pseudomallei* infected tissues. To ensure *B. pseudomallei* probes did not cross react with enteric bacteria, stomach (E) small intestine (F), cecum (G), and colon (H) tissues from uninfected BALB/c mice were hybridized with a eubacterial probe (green), and both *B. pseudomallei* specific probes (red). To ensure the FISH procedure resulted in specific probe hybridization, stomach (I), small intestine (J) cecum (K) and colon (L) tissues from uninfected BALB/c mice were hybridized with an irrelevant probe (green). Arrows in B-D indicate the location of *B. pseudomallei*. In all images the scale bar represents 10 microns.

In addition to studies with Bp1026b, BALB/c mice were also infected orally with three additional isolates (Bp2671a = 2.0×10^4 CFU; Bp2685a = 4.8×10^4 CFU; Bp2719a = 2.8×10^4 CFU). Organs were harvested from Bp2671a on day 21, from Bp2685a on day 3, and Bp2719a on day 4 post infection. Regardless of the *B. pseudomallei* strain used, or the time point organs were harvested, the stomach was the most heavily colonized organ. *B. pseudomallei* was also observed at low levels in the ingesta of the small intestine and cecum as well as the fecal material in the colon (Additional FISH images are presented in Appendix II, Figures A2.1-3).

Further investigation of stomach tissue revealed that infection with all four *B. pseudomallei* isolates resulted in colonization of the stomach **(Figure 6.10)**. Following infection with Bp1026b, Bp2671a or Bp2719a, *B. pseudomallei* was almost exclusively co-localized with tissue DAPI staining. In contrast, following infection with strain Bp2685a, the majority of *B. pseudomallei* were identified amongst the ingesta in the lumen of the stomach. While infection with Bp1026b, Bp2671a or Bp2719a resulted in a few concentrated foci of infection within the stomach, strain Bp2685a colonization was evenly distributed over larger areas of the stomach. To identify what regions of the stomach were colonized following oral infection the location of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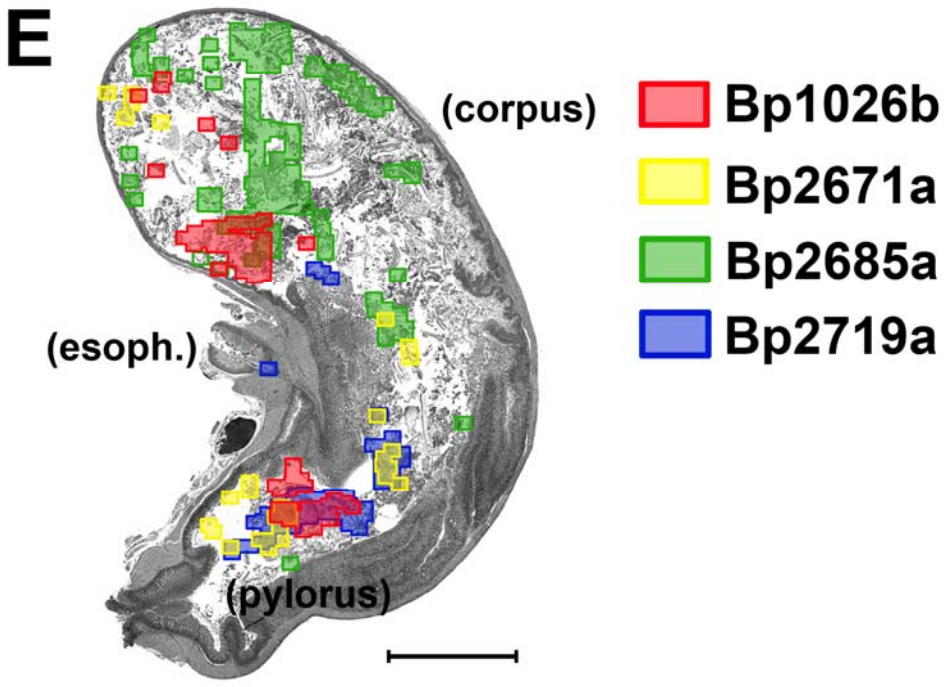
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(corpus)
(esoph.)
(pylorus)
Bp1026b
Bp2671a
Bp2685a
Bp2719a

Figure 6.10. Stomach colonization following oral infection with different *B. pseudomallei* isolates. BALB/c mice were infected orally with *B. pseudomallei* strain Bp1026b (5×10^5 CFU), Bp2671a (2.0×10^4 CFU), Bp2685a (4.8×10^4 CFU) or Bp2719a (2.8×10^4 CFU). Stomach tissues were collected from Bp1026b mice 56 days after infection, Bp2671a mice 21 days after infection, Bp2685a mice 3 days after infection, and Bp2719a mice 4 days after infection. All tissues were fixed in 10% NBF and embedded in paraffin before sectioning. FISH was performed on stomach tissue sections from mice infected with Bp1026b (A), Bp2671a (B), Bp2685a (C) and Bp2719a (D) as described in materials and methods. Tissue sections were hybridized with a eubacterial probe (green) and two *B. pseudomallei* specific probes (red), counterstained with DAPI (blue), and observed at 1000X final magnification. (E) The location of 1000X fields positive for *B. pseudomallei* was determined for each *B. pseudomallei* isolate. Positive 1000X fields are indicated by red (Bp1026b), yellow (Bp2671a), green (Bp2685a) or blue (Bp2719a) shaded outlines. Outlines were overlaid onto a representative stomach image created by combining images from a hematoxylin and eosin stained section. The esophagus (esoph.), body (corpus) and pylorus of the stomach are labeled for reference. In (A-D) the scale bar represents 10 microns, and in (E) the scale bar represents 2 mm.

positive 1000X fields from each stomach were determined. Positive 1000X fields were overlaid over a representative stomach image (Fig. 6.10E). This analysis revealed that *B. pseudomallei* was located in both the pylorus and body (corpus) of the stomach, and was even identified in the esophagus in one animal (Fig 6.10E). These results are in contrast to *Helicobacter pylori* infection where the bacteria are located mainly in the pylorus (79). Individual localization overlays generated using DAPI composite images from each stomach are shown in Appendix II, Figure A2.4.

While no foci of infection were identified outside of the stomach, individual bacteria could be identified in small intestine tissue (data not shown). Although rare, localization of *B. pseudomallei* within small intestine tissue consistently occurred following infection with any of the four *B. pseudomallei* strains tested. In contrast, no cecum or colon *B. pseudomallei* tissue staining of any kind was observed with any *B. pseudomallei* strain (data not shown). In conclusion, results from FISH experiments suggest that following oral infection *B. pseudomallei* establishes a persistent infection of the stomach from which bacteria are shed into the distal GI

tract resulting in identification of low numbers of *B. pseudomallei* in the intestinal contents of these organs and shedding in the feces.

6.4(7) Mice lack gastrointestinal pathology following oral *B. pseudomallei* infection.

To investigate if oral infection and colonization of the stomach induces pathologic changes in the GI tract tissue sections from the same mice used for FISH analysis were analyzed for pathologic changes. BALB/c mice (n = 32) were infected orally with one of four *B. pseudomallei* strains (Bp1026b = 5×10^5 CFU; Bp2671a = 2.0×10^4 CFU; Bp2685a = 4.8×10^4 CFU; Bp2719a = 2.8×10^4 CFU). Tissues were analyzed from *B. pseudomallei* infected mice euthanized at multiple time points following infection (Bp1026b days 2, 3, 14, and 56; Bp2671a days 4, 8, and 21; Bp2685a days 2 and 3; Bp2719a days 4, 8 and 11). Fecal shedding titers determined prior to euthanasia, demonstrated that the GI tracts of mice were heavily colonized (up to 10^6 CFU/gm, data not shown). Despite the large number of bacteria identified in the stomach by FISH, no gastric histological changes were observed compared to uninfected BALB/c mice **(Figure 6.11)** (Additional stomach images in Appendix II, Figure A2.5). Additionally, while no pathology was observed in the cecum or colon, mild neutrophil (PMN) and macrophage influx was observed in the ileum in 2 of 32 mice. In one of these mice there was noticeable necrosis in the serosa associated with PMN and macrophage infiltration, while necrosis was not observed in the other mouse (data not shown).

The lack of inflammation in the stomach was unexpected due to the high number of bacteria observed following FISH analysis. The lack of inflammation in the stomach suggests that *B. pseudomallei* is likely persisting between Paneth cells in gastric pits. Survival in the mucosa would also allow *B. pseudomallei* to avoid the low pH in the lumen of the stomach.

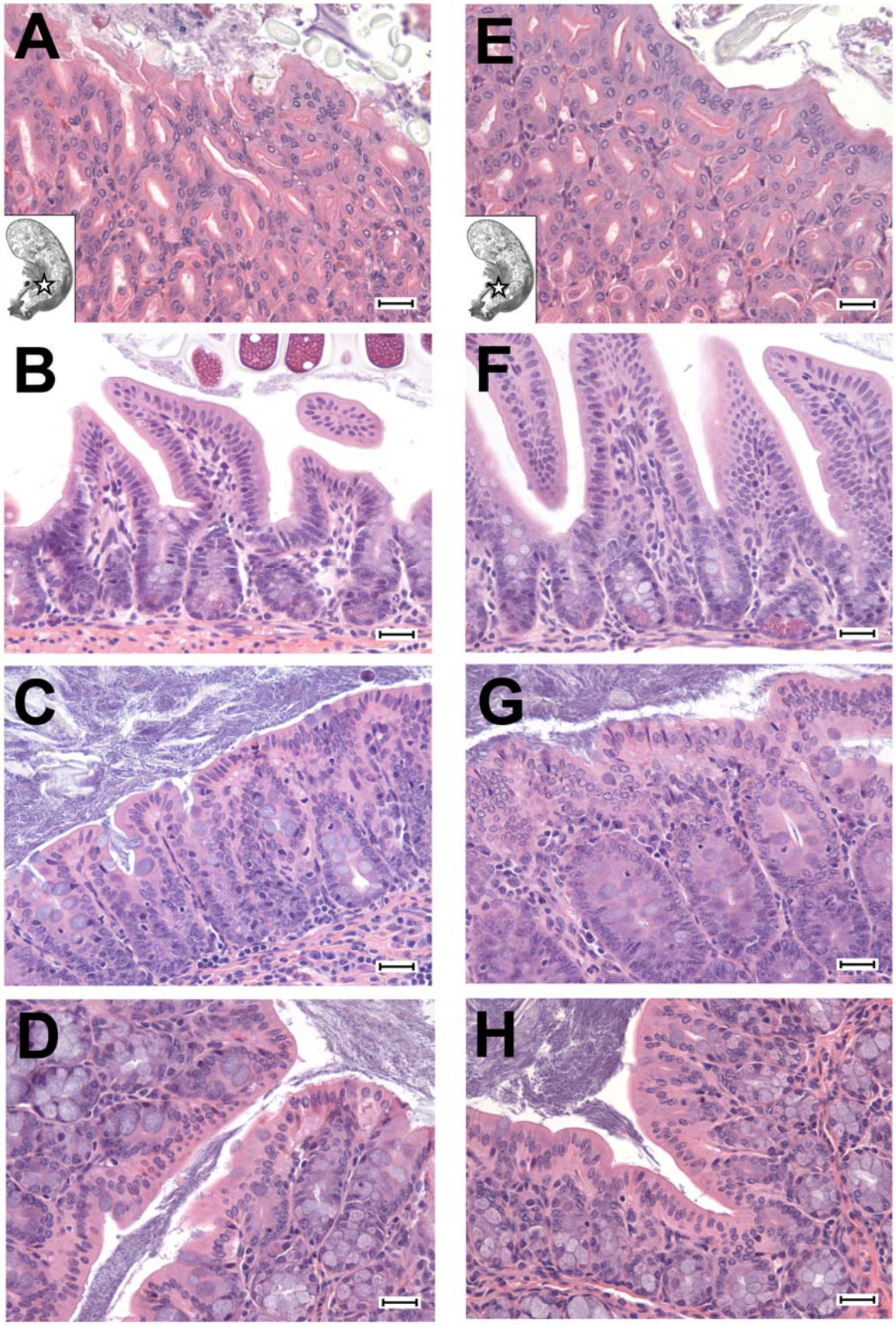
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Figure 6.11. Histology in gastrointestinal organs following oral *B. pseudomallei* infection. BALB/c mice were in infected with 5×10^5 CFU *B. pseudomallei* 1026b. Mice were euthanized 56 days after infection and tissues were processed and stained with hematoxylin and eosin as described in methods. Tissues were also collected from uninfected BALB/c mice as control tissues. Stomach (A), small intestine (B), cecum (C) and colon (D) images from uninfected BALB/c mice are shown in the left column. Stomach (E), small intestine (F), cecum (G) and colon (H) images from *B. pseudomallei* infected mice are shown in the right hand column. For stomach images (A, E) the location of each H+E image within the stomach is indicated by the star on the stomach outline shown in the bottom left corner of each image. All small intestine images are from the ileum, and the colon images are from the proximal colon. Images were captured at 400X final magnification, and the scale bar on all images represents 25 microns.

For instance, compared to the body of the stomach (pH = 3), the pH in the mucosa would be much higher (pH 4-5.5), a range where *B. pseudomallei* is known to grow (80-82). Additionally, *H. pylori* also survives in the gastric mucosa, avoiding the low pH of the stomach, and the immune response of the host (79).

6.4(8) Infection of the GI tract after challenge by non-oral routes of inoculation.

The preceding studies indicated that *B. pseudomallei* readily colonized the GI tract following oral inoculation. We therefore asked whether other routes of inoculation could also produce chronic enteric infection. Fecal shedding following infection with *B. pseudomallei* strain 1026b was used to survey mice for evidence of GI colonization following intranasal (i.n.), intraperitoneal (i.p.), or subcutaneous (s.c.) inoculation.

Intranasal inoculation resulted in high levels of GI infection, whereas inoculation by the s.c. or i.p. routes produced very different results **(Figure 6.12)**. For example, following i.n. inoculation of BALB/c mice with a low bacterial challenge dose (approximately 5×10^2 CFU per mouse), 13 of 15 mice (87%) developed persistent GI infection. Interestingly, i.n. inoculation also resulted in a higher percentage of mice with fecal shedding compared to mice subjected to

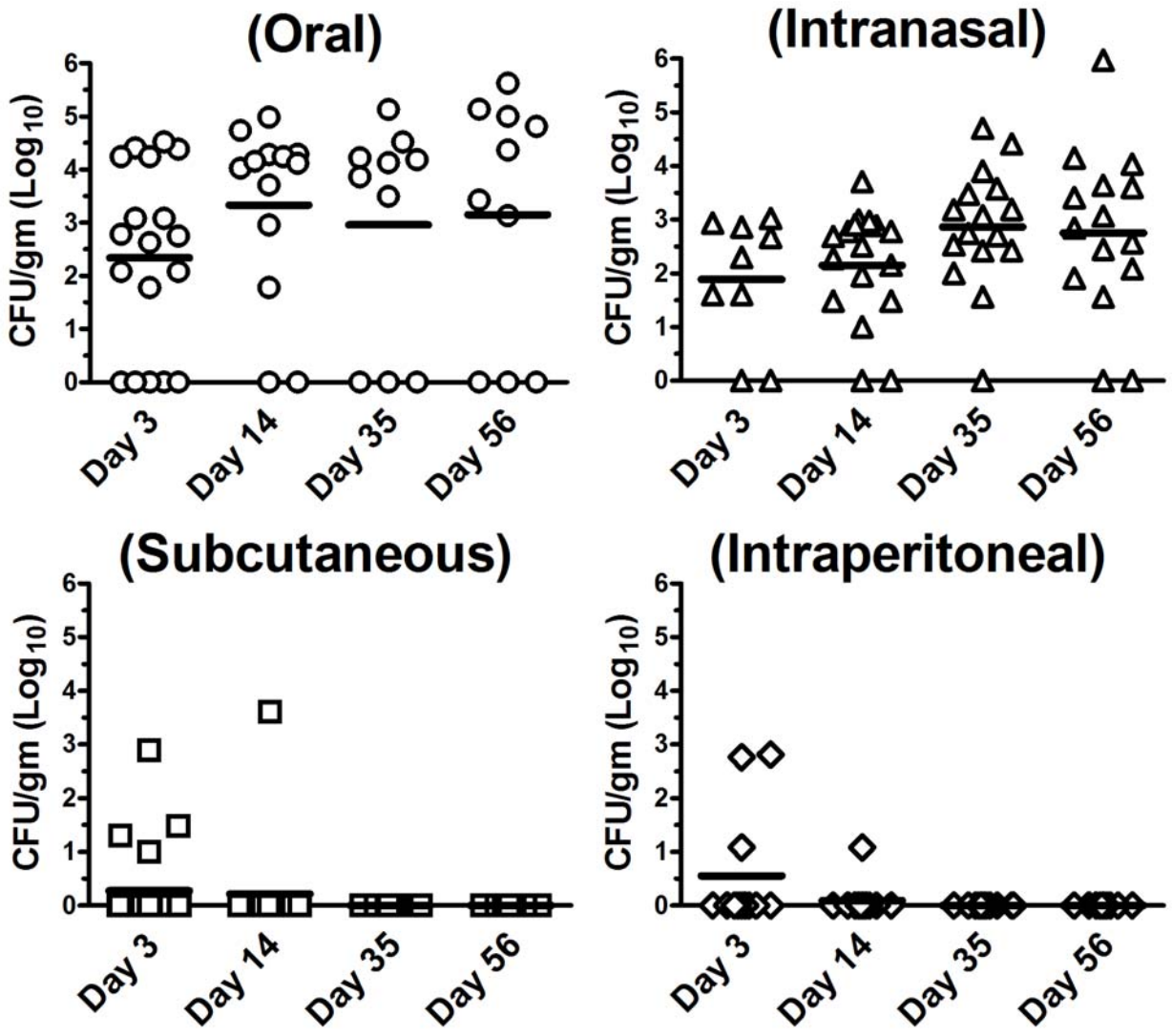

Figure 6.12. *B. pseudomallei* is persistently shed in feces following i.n., but not s.c. or i.p. inoculation. BALB/c mice were inoculated with *B. pseudomallei* using the following route and dose combinations: Oral inoculation (5×10^5 CFU) (n = 10-18 animals), i.n. inoculation (5×10^2 CFU) (n = 9-17 animals), s.c. inoculation (5×10^4 CFU - 5×10^7 CFU) (n = 11-24 animals), or i.p. inoculation (10^6 CFU – 10^8 CFU) (n = 11-12 animals). On day 3, 14, 35 and 56 after infection, fecal pellets were collected and processed for determination of bacterial burden. Data are graphed as individual $\log_{10}$ CFU/gram values with bars representing the mean value for each time point. Oral fecal shedding data from figure 6.5 are reproduced in this figure for reference. Data were pooled from 2-6 experiments per infection route.

oral inoculation (87% versus 70%). At present, the route by which *B. pseudomallei* colonizes the GI tract following i.n. inoculation has not been conclusively determined.

The preceding experiments led us to hypothesize that the GI tract was the primary site of bacterial persistence in mice inoculated by the oral or i.n. routes. To test this hypothesis, we inoculated BALB/c mice (n = 11) with a low dose (5×10^2 CFU) i.n. challenge with *B. pseudomallei* 1026b. Mice were euthanized on day 21, when all mice were clinically asymptomatic. Bacterial burdens were determined in blood, lung, liver, spleen, kidney, brain, stomach, small intestine, cecum, colon, and feces. To increase the sensitivity of bacterial detection, the entire organ homogenate from each organ was plated, using large agar plates (limit of detection of 1 CFU/organ). When bacterial burdens in all organs were compared, significant differences in bacterial burden were observed between the GI tract and extra-intestinal organs (lung, liver, spleen, brain and kidney) **(Figure 6.13)**. For example, the small intestine, cecum, and colon each had significantly higher bacterial counts than the lung, liver, brain, kidney, or spleen ($p < 0.001$). Differences were also observed between the stomach and systemic organs, although to a lesser degree ($p < 0.05$). However, bacterial counts in the lung, liver, spleen, kidney or brain were not significantly different from one another, nor were significant differences observed between bacterial counts in various regions of the GI tract, including the stomach, small intestine, cecum, and colon. The GI tract was also much more likely to be infected compared to extra-intestinal organs. For example, the GI tract was colonized significantly more often than the lung, brain ($p < 0.01$), spleen ($p < 0.05$), or kidney ($p < 0.001$) (Fig. 6.13). Only 1 of 11 mice with GI colonization had no evidence of extra-intestinal infection.

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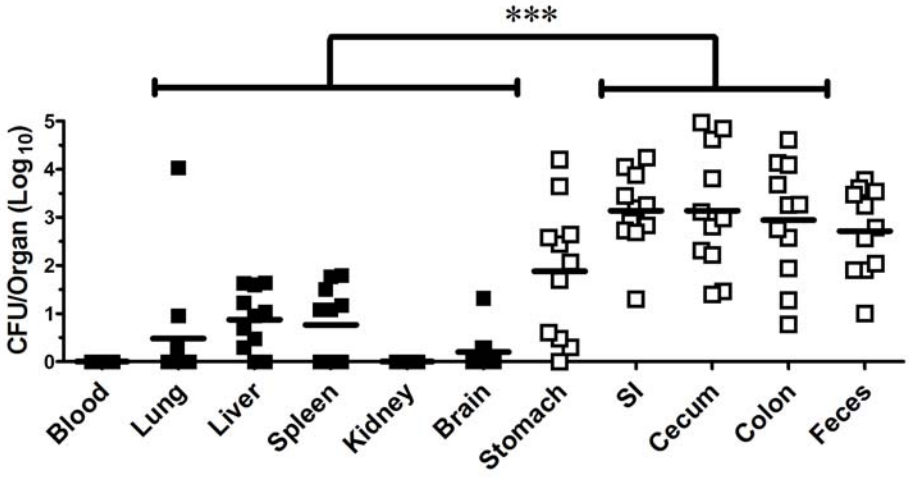

B

Tissue	Percent Infected (Day 21)	*p* value[a]
Blood	0% (0/11)	$p < 0.0001$
Lung	27% (3/11)	$p = 0.001$
Liver	82% (9/11)	ns
Spleen	55% (6/11)	$p = 0.04$
Kidney	0% (0/11)	$p < 0.0001$
Brain	36% (4/11)	$p = 0.004$
Stomach	91% (10/11)	ns
SI	100% (11/11)	—
Cecum	100% (11/11)	ns
Colon	100% (11/11)	ns
Feces	100% (11/11)	ns

[a] Statistical differences compared to SI were determined by Fisher's exact test.

Figure 6.13. GI organs are colonized more heavily and more frequently than systemic organs after i.n. challenge. BALB/c mice (n = 11 animals per group) were infected via the i.n. route with 5×10^2 CFU Bp1026b. After 21 days, blood, organs and feces were processed for determination of bacterial burden. (A) Bacterial burden in each organ following low-dose i.n. infection. Data are graphed as individual values with bars representing the mean $\log_{10}$ titer. Organ titers are plotted as $\log_{10}$ CFU/organ, blood as $\log_{10}$ CFU/ml, and feces as $\log_{10}$ CFU/gram. Statistical differences between organs were determined using a one-way ANOVA followed by a Tukey's multiple means test (*** $p < 0.001$). (B) The percentage of mice with positive *B. pseudomallei* cultures from each organ was determined. Statistical differences between percentages in the intestine versus other organs were determined using a two-tailed Fisher's exact test. The limit of detection was 1 CFU/organ, 10 CFU/ml for blood, and 10-60 CFU/gram for feces, depending on the number of fecal pellets collected from each mouse. Both figures were generated by pooling data from 3 independent experiments. ns = not significant.

6.4(9) Bacterial dissemination occurs more rapidly following oral inoculation than s.c. inoculation.

Most humans are thought to acquire *B. pseudomallei* infection via the inadvertent cutaneous inoculation with the organism (83). However, it is also possible that other routes of infection, including oral exposure, may also lead to the development of disseminated infection and melioidosis. To compare the relative frequency with which chronic disease developed following cutaneous versus oral inoculation with *B. pseudomallei* strain 1026b mice were infected with an equivalent number of bacteria. Mice were inoculated s.c. or orally with an infectious dose of 5×10^4 CFU, then euthanized on day 56 post infection and bacterial concentrations were determined in blood, lung, liver, spleen, kidney, brain, stomach, small intestine, cecum, colon and feces. None of the mice inoculated s.c. had detectable bacteria in any organ cultured **(Table 6.2)**. In contrast, 67% of mice inoculated orally had evidence of systemic infection. For example, *B. pseudomallei* was isolated from the liver of 6 of 9 mice ($p < 0.01$) and from the spleen of 5 of 9 mice ($p < 0.05$) inoculated orally. Similar results were also obtained at day 56 following low dose i.n. inoculation, which also produced persistent GI colonization (Figure 6.13). Compared to s.c. inoculation with 5×10^4 CFU *B. pseudomallei*, i.n. inoculation with $\sim5\times10^2$ CFU *B. pseudomallei* resulted in liver infection in 6 of 7 mice ($p < 0.001$) and splenic infection in 4 of 7 mice ($p < 0.05$) (data not shown; Fisher's exact test).

Mice inoculated orally were also much more likely to die from chronic *B. pseudomallei* infection than mice inoculated subcutaneously. While the day 56 LD_{50} for s.c. inoculation was 4.9×10^6 CFU, the day 56 LD_{50} for oral inoculation was only 5.9×10^4 CFU. These results were consistent therefore with the idea that bacterial dissemination and chronic disease developed

Table 6.2. Systemic and gastrointestinal colonization following oral or subcutaneous infection.

	Challenge Dose = 5×10^4 CFU		
Tissue	**Oral**	**Subcutaneous**	***p* value[a]**
Blood	44% (4/9)	0% (0/10)	$p = 0.03$
Lung	56% (5/9)	0% (0/10)	$p = 0.01$
Liver	67% (6/9)	0% (0/10)	$p = 0.003$
Spleen	56% (5/9)	0% (0/10)	$p = 0.01$
Stomach	56% (5/9)	0% (0/10)	$p = 0.01$
SI	78% (7/9)	0% (0/10)	$p = 0.0007$
Cecum	78% (7/9)	0% (0/10)	$p = 0.0007$
Colon	78% (7/9)	0% (0/10)	$p = 0.0007$
Feces	60% (3/5)	0% (0/10)	$p = 0.02$

[a] Statistical differences between oral and subcutaneous infection were determined by Fisher's exact test.

much more readily following oral inoculation and the establishment of persistent GI colonization with *B. pseudomallei* strain 1026b.

As noted above, systemic infection was not observed in any of 10 mice subjected to a s.c. challenge dose with 5×10^4 *B. pseudomallei* strain 1026b. However, in 6 mice surviving much higher challenge doses of *B. pseudomallei* by the s.c. route (dose range: $6.6\times10^6 - 6.1\times10^7$ CFU), we did observe systemic infection developing in some of the animals. Notably, all of these mice developed cutaneous lesions at the s.c. injection site. We subjected each of these injection site reactions to bacterial culture and found that these lesions contained very high bacterial titers, with an average titer of 3×10^7 CFU *B. pseudomallei* per lesion. A similar phenomenon was noted in mice inoculated by the i.p. route, where only mice with skin lesions developed systemic infection (data not shown). Therefore, these results suggest that efficient dissemination of *B.*

pseudomallei to other organs may require either a nidus of high-level infection (e.g., cutaneous lesions) or persistent, low-level infection of a large organ such as the gastrointestinal tract.

6.5 Discussion.

Most reports indicate that melioidosis in humans results primarily from either inhalation or cutaneous inoculation of *B. pseudomallei* from the environment (83). However, melioidosis also develops in 20 to 76% of patients with no known exposure to the organism (7, 13-17). Thus, it is possible that an alternative route of infection, such as oral inoculation, may be responsible for a number of melioidosis cases. In fact, compelling data from studies done at the turn of the century indicated that *B. pseudomallei* was in fact quite infectious in a variety of animal species following ingestion of the organism (27-30). Moreover, recent epidemiological studies also indicate that oral infection with *B. pseudomallei* may be possible (36, 41). Recent studies in mice have demonstrated that oral infection with *B. pseudomallei* can cause acute disease, results in antibody production and systemic infection (52, 63). The current study demonstrates that low dose oral infection results in infection of the stomach, persistent colonization of all GI organs, and low level fecal shedding. The present study also demonstrated that melioidosis develops following oral inoculation with multiple different *B. pseudomallei* strains, and that *B. pseudomallei* colonizes the GI tract following i.n. inoculation, but not following s.c. or i.p. infection.

Though our studies indicated that *B. pseudomallei* was infectious orally in mice, the mouse model of oral infection with *B. pseudomallei* also has a number of unique features when compared with infection with other more well-known enteric bacterial pathogens. For example, the doses of *B. pseudomallei* required to infect mice orally (5×10^3 to 5×10^4 CFU) are relatively low compared to other enteric pathogens. The infectious doses reported for most *Escherichia coli* and *Salmonella* strains in mice are in the range of 10^4 to 10^9 CFU (84-85). In contrast, the level of enteric colonization and fecal shedding with *B. pseudomallei* was relatively low

compared to other enteric pathogens. *Salmonella* and *E. coli* infection of mice results in fecal shedding titers ranging from 10^3–10^8 CFU/gram of feces (84-86), whereas, fecal titers following *B. pseudomallei* infection of mice ranged from 10^2-10^5 CFU/gm feces, with average titer of 10^3 CFU/gm feces (Fig. 6.5).

Equally important may be the fact that following oral infection with *B. pseudomallei* in mice, low-level fecal shedding remained relatively stable for months in infected animals, with only minor fluctuations over time. In contrast, *E. coli*, *Salmonella* or *S. flexneri* fecal titers initially increase after inoculation and then decline to very low levels or disappear as the infection is controlled by the immune system (84-85, 87). Thus, in the *B. pseudomallei* model, the stable level of infection suggests enteric colonization rather than tissue invasion. Previous studies have demonstrated that oral infection results in antibody production, suggesting an immune responses is mounted following oral infection (52). However, in the current study fecal shedding titers did not appear affected by the development of an immune response (Fig 6.5). If fecal shedding titers were the result of enteric infection, a reduction in fecal shedding titers would be expected as the infection was controlled. Moreover, the entire GI tract was infected in *B. pseudomallei* inoculated mice. This finding is quite remarkable considering the very different and inhospitable environments within the stomach and small intestine. Thus, our studies indicated that *B. pseudomallei* is an efficient and adaptable colonizer of the GI tract of mice.

In addition to stable fecal shedding titers, the lack of tissue pathology provides further evidence that *B. pseudomallei* is likely an enteric colonizer rather than a true enteric pathogen. For example, infected mice did not develop significant lesions at any site in the intestinal tract following oral or intranasal inoculation. This was true even in mice subjected to very high challenge doses of *B. pseudomallei*, or in very susceptible strains of mice (e.g., 129Sv/EvTac

mice). In contrast, infection with other enteric pathogens such as *Salmonella*, *S. dysenteriae*, and most *E. coli* strains produces intestinal pathology (88-89). Although enterotoxigenic *E. coli* infection can cause disease without inducing organ pathology, the toxins produced by this bacterium also result in diarrhea following infection (89). Notably, mice infected orally with *B. pseudomallei* did not show signs of diarrhea or evidence of weight loss.

Localization of *B. pseudomallei* to the mucosa of the stomach provided an explanation for many of these differences. The lack of tissue invasion by *B. pseudomallei*, in either the stomach or intestine, likely explains both the lack of an immune response and diarrhea in mice. The gastric mucosal lining is also where *H. pylori* colonize the stomach, and allows the bacteria to avoid the low luminal pH of the stomach (79). While the pH in the lumen of the murine stomach is 3, the pH of the mucosa is thought to range from 4-6.5, and can be as high as 6.9 at epithelial surfaces (79, 81-82). *B. pseudomallei* is known to grow in broth culture at a pH of 4.5, and can increase the pH of broth media from 4.5 to 7 (80). In humans the lumen of the human stomach typically has a pH of 2, but can vary from 2-5 (90-91). *B. pseudomallei* is known to survive in saline at a pH of 2 for one day, and has been isolated from surface water with a pH of 2 (92-93). Localization of *B. pseudomallei* to the stomach is also in agreement with previous case reports describing gastric ulcers in melioidosis patients (6, 41). While reports of gastric ulcers are rare in melioidosis patients, the results of this study, as well as original experiments performed by Whitmore in guinea pigs and oral infection of horses with *B. mallei*, suggest that colonization without ulceration may be far more common (29, 94). Similar to *H. pylori* colonization, gastric disease may occur only if *B. pseudomallei* becomes invasive (79).

Major risk factors associated with development of melioidosis include diabetes, alcoholism and chronic kidney disease (CKD) (83). Previous studies have identified that all of

these disorders result in reduced neutrophil function (83). While clinical trials investigating treatment with G-CSF to increase neutrophil function have resulted in reduced morbidity, no differences in mortality were observed (83, 95-98). This result suggests that additional factors associated with these disorders may be important for development of melioidosis. Interestingly, patients with diabetes, alcoholism and CKD also frequently develop gastrointestinal lesions, and more specifically gastric lesions.

Dyspepsia is a common symptom in diabetics, and diabetic patients are known to develop gastric ulcers more frequently than non-diabetics (99-100). In addition, diabetes patients can develop gastric ulcers, erosions and severe acute gastritis without dyspepsia symptoms (101). Excessive alcohol consumption is known to directly damage the mucosa of the esophagus, stomach and small intestine, with exposure to high concentrations resulting in gastric hemorrhaging (102-104). Excessive alcohol consumption can increase intestinal permeability and toxin release, leading to increased risk for infection (104-105). Finally, the association between GI pathology and chronic kidney disease is well described, and patients with chronic kidney disease have a higher frequency of upper GI tract lesions than the general population (106-109). Additionally, GI bleeding occurs in 19% of chronic kidney disease patients, and 61% of these lesions were localized to the duodenum (110). In summary, multiple risk factors associated with melioidosis also increase the risk for formation of GI lesions which could allow for increased dissemination of *B. pseudomallei* from the GI tract. All of the risk factors associated with melioidosis are complex diseases, and therefore their impact on melioidosis is likely to be multifactorial.

Since enteric colonization with *B. pseudomallei* does not induce signs of GI disease in infected mice, and possibly not in humans either, the GI tract could well be considered a

sanctuary for persistent subclinical infection with *B. pseudomallei*. If the organism can maintain chronic low level GI infection, then it may persist undetected for months or years. This could then account for the long lag following initial exposure to the organism and the development of overt disseminated disease to extra-intestinal sites. Alternatively, over time low numbers of the organism may spontaneously enter the bloodstream, leading to eventual organ seeding and eventual disseminated infection. In this model, the relative risk of developing disseminated *B. pseudomallei* infection would increase with the absolute duration of enteric infection. Although a carrier state of *B. pseudomallei* in humans has not been identified thus far, only throat swabs have been tested, and GI colonization may be occurring in endemic areas (11, 111).

Oral colonization may have been overlooked due to multiple technical challenges involved with processing GI samples and low level of *B. pseudomallei* colonization. For instance, previous studies have shown that enteric bacteria from fecal swabs often overwhelmed *B. pseudomallei* when grown on Ashdown's medium (36). In the current study, fecal shedding titers in mice were low, and entire bowel movements had to be processed to isolate bacteria. This would be difficult in humans, and may require culture in enrichment broth or concentration prior to analysis. Alternatively, molecular or fluorescent techniques may be necessary to identify *B. pseudomallei* amongst the enteric flora. Molecular techniques were recently used to isolate *B. pseudomallei* from the feces of grazing animals (112).

Exactly how *B. pseudomallei* maintains chronic colonization of the stomach is currently unknown. Capsular polysaccharide may be important as *B. thailandensis* lacks the capsular polysaccharide, and was unable to colonize the GI tract (Table 6.1) (113-117). Capsules are known to protect bacteria against environmental stresses, and may be involved with resistance to stomach acid (118). In addition, previous studies have shown that arginine deiminase regulates

acid resistance in *B. pseudomallei*, *Listeria monocytogenes* and *Lactobacillus* spp. (119-123). A preliminary survey of the *B. pseudomallei* K96243 genome indicates the presence of a number of genes and regulatory factors involved with acid resistance in other enteric pathogens. For example, all genes necessary for urea utilization in *H. pylori* are present in *B. pseudomallei*, including a urease enzyme, accessory proteins, a urease transporter, and a nickel transporter (79). *B. pseudomallei* contains multiple other systems including the PhoPQ, Fur, RpoS, and OmpR/EnvZ systems known to modulate gene expression in *E. coli* and *Salmonella* following acid exposure (124-125). In addition, the lysine decarboxylase important in both *Vibrio cholera* and *Salmonella*, the wca locus involved with exopolysaccharide changes in *E. coli* and *Salmonella*, and DNA methyl transferase genes in *Salmonella* (124, 126). Therefore *B. pseudomallei* contains multiple systems known to protect other enteric bacteria against the low pH of the stomach.

Further studies will be needed to identify how *B. pseudomallei* is disseminating from the GI tract. In the current study *B. pseudomallei* was not frequently isolated in the mesenteric lymph nodes, which are commonly colonized with *Salmonella* and *Y. enterocolitica* infection (67, 69-71). Furthermore, unlike infection with *Salmonella* or *Shigella*, the gall bladder was rarely colonized with *B. pseudomallei* (68-69, 71-72). Because FISH experiments localized *B. pseudomallei* to the stomach, dissemination may be occurring through the gastric lymph node. This would be in agreement with isolation of *B. pseudomallei* from the gastric lymph node of monkeys, and the gastrohepatic lymph node of pigs (33, 127). Another possibility is that *B. pseudomallei* may be disseminating from the ileum, as both *B. pseudomallei* and PMN recruitment were localized to the ileum by FISH and histopathology experiments. Although both

of these observations were rare, the identification of both exclusively in the ileum warrants future investigation.

Experiments performed with low passage clinical isolates of *B. pseudomallei* demonstrated that multiple isolates of *B. pseudomallei* could cause disseminated infection following oral inoculation (see Fig 6.8). Interestingly all three low-passage *B. pseudomallei* strains were more acutely virulent than Bp1026b. While Bp1026b was originally isolated from a patient in Thailand, passage in the laboratory may have attenuated this strain. Natural infection of humans with *B. pseudomallei* causes a wide range of disease manifestations, including chronic disease that may develop in patients up to 60 years after original exposure (4-5, 8, 13, 128-130). Despite being the least acutely virulent strain tested in this study, the 1026b strain consistently induced chronic infection following oral or i.n. inoculation.

Studies were also conducted to accurately localize bacteria immediately after oral or i.n. inoculation (see Methods). Immediately following oral inoculation with 5×10^5 CFU *B. pseudomallei*, 21% of mice had *B. pseudomallei* in the lungs (limit of detection = 4 CFU/lung). This rate of inadvertent aspiration is much lower than the 89% of mice which developed disseminated infection following oral infection with Bp1026b (5×10^5 CFU) (Fig. 6.7). Therefore observations in this study are unlikely to be due solely to aspiration during oral inoculation. Furthermore, 3 days following oral infection with Bp2671a (3.6×10^5 CFU), Bp2685a (2.9×10^5 CFU) or Bp2719a (3.5×10^5 CFU) systemic infection occurred in 70%, 78%, and 64% of mice respectively (Fig. 6.8).

Following intranasal infection, previous studies have demonstrated that infection of mice with ~5×10^2 CFU results in 40% of the challenge dose reaching the lungs (Chapter 3). Therefore, a dose of ~300 CFU (60% of 500) could potentially be ingested during i.n. infection

with 500 CFU. Following i.n. infection with 5×10^2 CFU we observed that 87% of mice were shedding *B. pseudomallei* in their feces. In contrast, low dose oral infection with 250 CFU resulted in colonization in just 10% of mice (Table 1). Additionally, fecal shedding was delayed following i.n. inoculation, as compared to direct oral inoculation. For example, following oral infection mice were shedding bacteria in their feces 24 hours after oral infection, while fecal shedding was not observed until day 3 following intranasal infection (data not shown). Therefore it is unlikely that GI colonization following i.n. infection is due to direct ingestion. However we cannot exclude the possibility that *B. pseudomallei* may be colonizing lymphoid tissue of the head following i.n. infection, and subsequent shedding of bacteria into the stomach may result in GI infection. Infection of lymphatic tissue of the head may be a more likely cause of GI colonization in melioidosis patients as either inhalation or ingestion could result in colonization of these tissues. Poratid lesions observed in Thai children are thought to occur following ingestion, and may represent such an exposure (131-133).

Some of the findings from our mouse model of chronic *B. pseudomallei* enteric infection need to be confirmed in humans infected with *B. pseudomallei*. Particularly, patients with melioidosis as well as asymptomatic patients from melioidosis endemic regions could be screened using sensitive assays or repeated cultures for detection of low-level fecal shedding of the organism. If substantial numbers of people with persistent enteric colonization with *B. pseudomallei* were identified, this finding would have significant implications for understanding human melioidosis and developing attempts to control or prevent infection.

In summary, our findings in a mouse model of oral inoculation with *B. pseudomallei* indicate clearly that this organism is an efficient colonizer of the GI tract. *B. mallei* is also infectious following oral inoculation, but does not survive in the environment as well as *B.*

pseudomallei. Therefore, genes associated with environmental survival which are shared by both *B. mallei* and *B. pseudomallei* may be important for survival in the GI tract (94, 134-136). Understanding the mechanisms that *B. pseudomallei* uses to sustain persistent GI colonization are also likely to yield important insights into how the organism disseminates from the GI tract to organs such as the spleen and liver and central nervous system.

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CHAPTER 7.

CONCLUDING REMARKS

7.1 Significance of work.

The research described in this dissertation provides valuable information related to early immune responses to infection, efficacy of immunotherapeutics, and potential reservoirs of bacterial persistence during chronic melioidosis. Chapters 3 and 4 in this dissertation report on components of the innate immune response necessary for protection against respiratory *B. mallei* infection. These studies revealed that myeloid differentiation factor 88 (MyD88) dependent monocyte recruitment results in increased numbers of dendritic cells in the lung. Dendritic cells (DC) were shown to be the major source of IL-12, which results in IFN-γ production by natural killer (NK) cells. Therefore, therapeutics which target TLRs, stimulate monocyte recruitment, or induce IFN-γ production will likely be effective treatments for *B. mallei* or *B. pseudomallei* infection. Indeed, the studies in chapter 5 demonstrated that cationic liposome DNA complexes (CLDC) provided prophylactic protection against both glanders and melioidosis; and both MyD88 dependent Toll-like receptor (TLR) signaling and IFN-γ production were required for CLDC mediated protection. Finally, studies investigating chronic disease revealed that oral infection of mice with *B. pseudomallei* results in a persistent low level colonization of the stomach and the development of spleen and liver infection 45-60 days after infection. Confirmation of these findings in human melioidosis patients, or in healthy individuals from endemic areas, could have implications for treatment of melioidosis.

7.2 Specific aims 1 and 2.

These two specific aims focused on investigation of the innate immune response of the host following *Burkholderia mallei* infection. Naturally occurring glanders is rare, and therefore protective innate immune responses are largely unknown (1). The classification of *B. mallei* as a select agent has resulted in a renewed interest in pathogenesis and therapeutic development (2). In specific aim 1 the necessity of monocytes for protection against respiratory glanders was investigated. These studies demonstrated that monocyte chemoattractant protein-1 (MCP-1) and chemokine receptor 2 (CCR2) knockout mice were highly susceptible to *B. mallei* infection. Depletion of monocytes in wild type mice with clodronate liposomes also increased susceptibility to infection. $CCR2^{-/-}$ mice infected with *B. mallei* had reduced numbers of monocytes and dendritic cells in the lungs, while a significant increase in neutrophil recruitment was observed. Cytokine analysis revealed that TNF-α and CXCL1 production remained intact, while IL-12 and IFN-γ production was reduced in $CCR2^{-/-}$ mice. The critical role of IFN-γ was confirmed by administration of recombinant IFN-γ (rIFN-γ) to $CCR2^{-/-}$ mice which conferred protection against *B. mallei* infection.

In specific aim 2 studies on innate immunity were expanded to investigate signaling pathways necessary for protection, specifically the role of MyD88 dependent TLR signaling. TLRs are located at the cell surface or in endosomal compartments, and the majority of TLRs involved with recognition of bacterial infection signal through MyD88 (3). Following respiratory *B. mallei* infection $MyD88^{-/-}$ mice were found to be highly susceptible to infection. Investigation of individual TLRs revealed that $TLR2^{-/-}$ mice were protected against infection, while no role of TLR4 was observed following *B. mallei* infection. Reduced chemokine production was observed in $MyD88^{-/-}$ mice including both MCP-1 and CXCL1. In agreement

with diminished chemokine production, reductions in neutrophil, monocyte and dendritic cell recruitment to the lung were also observed. Intracellular cytokine staining demonstrated that dendritic cells and monocytes were the major sources of IL-12, NK cells produced IFN-γ, and TNF-α was produced mainly by neutrophils. While early production of multiple cytokines was reduced in MyD88$^{-/-}$ mice, knockout mice were unable to produce IFN-γ. Similar to CCR2$^{-/-}$ mice, treatment with rIFN-γ protected MyD88$^{-/-}$ mice against acute glanders, although protection was not complete.

Taken together results from these studies demonstrate that following respiratory *B. mallei* infection, ligation of TLRs and MyD88 signaling is necessary for recruitment of monocytes to the site of infection. Monocytes likely differentiate into dendritic cells and produce IL-12, which stimulates IFN-γ production by NK cells. Therefore potential therapeutics targeting monocyte recruitment, as well as TLRs agonists may be effective treatment options for *B. mallei*.

A number of question still remain regarding innate immune responses to *B. mallei* infection. For instance, in specific aim 1 CCR2$^{-/-}$ mice produced equivalent levels of nitric oxide compared to WT mice, suggesting that activation of iNOS is not how IFN-γ stimulation is killing *B. mallei*. Therefore, although IFN-γ is critical for protection against both *B. mallei* and *B. pseudomallei* further studies will be needed to identify the bactericidal mechanisms activated by IFN-γ. Although, IFN-γ protected both CCR2$^{-/-}$ and MyD88$^{-/-}$ mice, administration at the time of infection may have prematurely activated antimicrobial responses. Studies delaying IFN-γ treatment may more accurately determine the ability of IFN-γ to protect susceptible mouse strains. In addition, the incomplete protection observed in MyD88$^{-/-}$ mice following IFN-γ treatment suggests that other cytokines reduced at early time points such as IL-6 and TNF-α may also be important. Investigation of genes uniquely or commonly induced by each of these three

cytokines may provide additional candidates for investigation. In addition, multiple bactericidal effects may be working together to kill *B. mallei*, and simultaneous inhibition of multiple pathways may be necessary. Preliminary microarray data from macrophages stimulated with IFN-γ *in vitro* suggests that induction of antimicrobial peptides may be one mechanism of IFN-γ mediated killing (S. Dow, unpublished observations).

One of the more surprising results from TLR studies was that TLR2$^{-/-}$ mice were protected against *B. mallei* infection, which was also observed following *B. pseudomallei* and *B. thailandensis* infection (4-5). Following *Yersinia pestis* infection a polarizing role of TLR2 on the immune response has been described. In these studies signaling through TLR2-TLR1 heterodimers resulted in immune activation and bacterial clearance, while singling through TLR2-TLR6 heterodimers resulted in immune suppression and uncontrolled bacterial replication (6). Investigation of TLR6 signaling following *B. mallei* or *B. pseudomallei* infection would demonstrate if a similar phenomenon is occurring following *Burkholderia* infection.

Lastly, while MyD88 was essential for protection in response to *B. mallei* infection, and is also known to be necessary in response to *B. pseudomallei* and *B. thailandensis* infection, individual TLRs are not necessary for protection (TLR2, TLR4, TLR5 and TLR9) (5, 7-8). In addition to TLR signaling, MyD88 is an adapter molecule used to signal following ligation of IL-1β and IL-18 receptors (9). IL-18 is known to be necessary for protection against *B. pseudomallei* infection, and recent studies in *Listeria monocytogenes* have demonstrated that MyD88 is necessary for IL-18 signaling in NK cells (10-13). Further studies will be needed to identify if MyD88 is necessary for IL-18 signaling following *Burkholderia* infection.

7.3 Specific aim 3.

Current antibiotic treatment for glanders and melioidosis requires an extensive course of antibiotic treatment, and treatment failure occurs in 11-17% of melioidosis cases (14-15). Therefore immune based therapeutics provide an attractive treatment option. This set of studies demonstrated that the cationic liposome DNA complex (CLDC) therapeutic protected mice when administered prophylactically, but not therapeutically. In an *in vitro* macrophage system CLDC mediated killing was dependent on IFN-γ, and to a lesser extent TNF-α. CLDC administration into the lungs of mice induced production of IL-12 and IFN-γ. Following *B. mallei* infection CLDC protection was shown to be dependent on MyD88 signaling and IFN-γ production, but independent of monocyte recruitment and nitric oxide production.

Subsequent studies from Dr. Dow's laboratory have demonstrated that CLDC is an effective adjuvant when used in a *B. pseudomallei* vaccine. Mice vaccinated with heat killed *B. pseudomallei* complexed with CLDC provided significant protection as compared to mice receiving heat killed bacteria alone (16). In addition, CLDC has been used therapeutically when delivered in combination with ceftazidime following *B. pseudomallei* infection. In this previous study delivery of CLDC in combination with ceftazidime resulted in a synergistic interaction, and provided increased protection compared to either treatment alone (17). Many vaccines and treatments have conferred protection against acute disease, although in all cases chronic disease develops in surviving animals. For instance, CLDC therapy described in chapter 5 of this dissertation, CLDC vaccination, and CLDC/ceftazidime combination therapies all protected against acute disease, however mice in all studies untimely developed chronic disease (16-17). Therefore future studies using repeat administration of CLDC with antibiotics may provide enhanced protection against the development of chronic disease.

7.4 Specific aim 4.

Chronic disease in melioidosis patients in endemic areas is well described and patients have developed melioidosis up to six decades after infection (18-21). However, the site of bacterial persistence during asymptomatic stages of disease is not known. Low dose oral infection of mice with *B. pseudomallei* resulted in persistent colonization of the stomach, and *B. pseudomallei* could be isolated from all gastrointestinal (GI) organs, as well as the feces. Similar results were obtained with 3 additional *B. pseudomallei* isolates, demonstrating that GI colonization is a common trait of multiple *B. pseudomallei* strains. In addition to GI colonization dissemination to the spleen and liver of infected mice was observed 45-60 days after infection. Investigation of GI colonization following non-oral routes of infection demonstrated that fecal shedding occurred following oral or intranasal infection, but not following intraperitoneal or subcutaneous infection. Following intranasal infection *B. pseudomallei* was present in GI organs at higher titers as compared to systemic organs, and chronic disease occurred more rapidly following oral infection as compared to subcutaneous infection.

Topics for further study include how *B. pseudomallei* is disseminating from the GI tract. Monocytes are known to be important for *Salmonella* dissemination, and studies investigating the role of monocytes in *B. pseudomallei* dissemination are currently underway (22). How *B. pseudomallei* survives the low pH of stomach is also currently unknown. *Helicobacter pylori* is known to utilize a urease enzyme resulting in ammonia production and local pH buffering (23-24). *B. pseudomallei* is known to contain a urease enzyme as well as all the necessary accessory proteins and transporters. In addition, the arginine deiminase in *B. pseudomallei* has been shown to be involved with acid resistance (25). Further studies will be needed to determine the role of these and other acid resistance mechanisms to survival of *B. pseudomallei* in the stomach.

7.5 References.

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APPENDIX I.

CYTOKINE PRODUCTION IN MYD88$^{-/-}$ MICE AND NORMALIZATION ANALYSIS.

A1.1 Summary.

This appendix contains four tables comparing cytokine production in wild type (WT) and myeloid differentiation factor 88 (MyD88) knockout mice infected intranasally (i.n.) with *B. mallei*. Data sets include: Differences in cytokine production between WT and MyD88$^{-/-}$ mice infected with 5×10^2 CFU *B. mallei* i.n. (Table A1.1); Differences in cytokine production between WT mice infected with 10^4 CFU, and MyD88$^{-/-}$ mice infected with 5×10^2 CFU *B. mallei* i.n. (Table A1.2); Results from correlation analysis investigating the association between bacterial burdens and cytokine production in the lung, plasma and spleen of WT mice infected with 5×10^2 or 10^4 CFU *B. mallei* i.n. (Table A1.3); Differences in cytokine production following normalization to bacterial burden in WT and MyD88$^{-/-}$ mice infected with 5×10^2 CFU *B. mallei* i.n. (Table A1.4).

Table A1.1. Cytokine production following i.n. infection of wild type and MyD88$^{-/-}$ mice with 5×10^2 CFU *B. mallei*[a].

Lung						
Cytokine / Chemokine	**24 Hours**		**48 Hours**		**72 Hours**	
	WT	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**
IL-6	52.4 ± 12 †	9.1 ± 0.6	54.2 ± 28.6	63.4 ± 17	15 ± 0.8	118 ± 26 †
TNF-α	38 ± 10.4 *	11.8 ± 0.6	28 ± 13.8	9.2 ± 1.5	5 ± 0.6	25 ± 3.6 §
IFN-γ	2.4 ± 0.4 †	1.0 ± 0.07	39.3 ± 6.5 †	1.7 ± 0.04	11.5 ± 4.7 *	0.82 ± 0.03
CCL2	56.2 ± 4.4 †	39.2 ± 2.1	62.7 ± 8.1	50.1 ± 8	23.8 ± 1	260 ± 39 §
CXCL1	241 ± 28 *	160 ± 16	170 ± 35.7	203 ± 20.4	147 ± 3.1	307 ± 21 §

Plasma						
Cytokine / Chemokine	**24 Hours**		**48 Hours**		**72 Hours**	
	WT	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**
IL-6	84 ± 11 *	7.5 ± 1.1	59.4 ± 20	135 ± 3.1 *	9 ± 0.6	3922 ± 777 §
TNF-α	3.4 ± 0.06	3.2 ± 0.1	8.6 ± 1 *	3.7 ± 0.2	4.5 ± 0.3	180 ± 21 §
IFN-γ	7.5 ± 1.4 †	1.3 ± 0.7	42.2 ± 4.8 §	0.8 ± 0.04	3.9 ± 0.6	1.9 ± 0.9
CCL2	234 ± 33 §	29.2 ± 3.7	86 ± 14	52.3 ± 13.6	33.6 ± 7.7	1338 ± 355 †
CXCL1	645 ± 103 †	132 ± 22.5	1369 ± 422	3314 ± 453 *	105 ± 66.6	28789 ± 5677 §

Spleen						
Cytokine / Chemokine	**24 Hours**		**48 Hours**		**72 Hours**	
	WT	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**	**WT**	**MyD88$^{-/-}$**
IL-6	14 ± 0.13	13.6 ± 0.16	5.7 ± 0.13	25.4 ± 8.1 *	5.4 ± 0.1	99 ± 42 *
TNF-α	4.3 ± 0.09	4 ± 0.14	5.5 ± 0.3	9.5 ± 1.8 *	5 ± 0.3	48 ± 13.6 †
IFN-γ	0.7 ± 0.04	1.2 ± 0.4	10.5 ± 3.8 *	2.7 ± 0.1	3 ± 0.9	2.1 ± 0.2
CCL2	22.6 ± 0.1	22.4 ± 0.1	21 ± 0.3	35 ± 6.6 *	19.7 ± 0.2	1126 ± 64 §
CXCL1	87.5 ± 5.2 §	26 ± 5.4	17 ± 6.4	82 ± 15.3 †	3.3 ± 1.4	224 ± 32 §

[a] Data are representative of two independent experiments. Data are presented as mean ± SEM pg/ml concentrations, and differences between WT and MyD88$^{-/-}$ mice were determined by a two-tailed Student's t-test (* $p < 0.05$, † $p < 0.01$, § $p < 0.001$).

Table A1.2. Cytokine production following i.n. infection of wild type mice with 10^4 CFU and MyD88$^{-/-}$ mice with 5×10^2 CFU *B. mallei*[a].

Lung						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	450 ± 138 *	9.1 ± 0.6	1057 ± 127 §	63.4 ± 17	714 ± 110 §	118 ± 26
TNF-α	48 ± 12.1 *	11.8 ± 0.6	256 ± 23 §	9.2 ± 1.5	188 ± 35 †	25 ± 3.6
IFN-γ	3.1 ± 0.5 †	1.0 ± 0.07	8 ± 2.4	1.7 ± 0.04	5 ± 0.3 §	0.82 ± 0.03
CCL2	200 ± 50.4 *	39.2 ± 2.1	1367 ± 202 §	50.1 ± 8	2626 ± 176 §	260 ± 39
CXCL1	1233 ± 320 *	160 ± 16	5757 ± 1123 †	203 ± 20.4	4203 ± 855 †	307 ± 21

Plasma						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	1169 ± 419 †	7.5 ± 1.1	621.8 ± 107 *	135 ± 3.1	3392 ± 1070	3922 ± 777
TNF-α	3.5 ± 0.5	3.2 ± 0.1	7.3 ± 0.8 *	3.7 ± 0.2	69 ± 16.7	180 ± 21 *
IFN-γ	8.7 ± 1.6 †	1.3 ± 0.7	90.4 ± 32.6 *	0.8 ± 0.04	275 ± 38 †	1.9 ± 0.9
CCL2	275 ± 107 *	29.2 ± 3.7	257 ± 54 *	52.3 ± 13.6	1153 ± 166	1338 ± 355
CXCL1	10777 ± 5176 *	132 ± 22.5	26937 ± 3603 §	3314 ± 453	18649 ± 3032	28789 ± 5677

Spleen[b]						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	14.7 ± 0.2 †	13.6 ± 0.16	27 ± 8.9	25.4 ± 8.1	59 ± 3.4	99 ± 42
TNF-α	3.9 ± 0.1	4 ± 0.14	7.5 ± 1.4	9.5 ± 1.8	23 ± 1.6	48 ± 13.6 *
IFN-γ	1.2 ± 0.1	1.2 ± 0.4	49.4 ± 0.4 *	2.7 ± 0.1	134 ± 8.8 §	2.1 ± 0.2
CCL2	22.5 ± 1.4	22.4 ± 0.1	101 ± 21	35 ± 6.6	367 ± 52	1126 ± 64 §
CXCL1	169 ± 32 †	26 ± 5.4	404 ± 41 †	82 ± 15.3	546 ± 58 †	224 ± 32

[a] Data are representative of two independent experiments. Data are presented as mean ± SEM pg/ml concentrations, and differences between WT and MyD88$^{-/-}$ mice were determined by a two-tailed Student's t-test (* $p < 0.05$, † $p < 0.01$, § $p < 0.001$).

[b] Bacterial burdens in the spleen of WT mice infected with 10^4 CFU and MyD88$^{-/-}$ mice infected with 5×10^2 CFU were similar at 24 and 48 hours, while MyD88$^{-/-}$ mice had increased bacterial burdens at 72 hours (analyzed by a two-tailed Student's t-test).

24 Hrs: Below limit of detection in both WT and MyD88$^{-/-}$.

48 Hrs: WT = 4.3 $\log_{10}$ CFU/organ; MyD88$^{-/-}$ = 4.4 $\log_{10}$ CFU/organ ($p = 0.64$).

72 Hrs: WT = 4.8 $\log_{10}$ CFU/organ; MyD88$^{-/-}$ = 6.5 $\log_{10}$ CFU/organ ($p < 0001$).

Table A1.3. Correlation of cytokine production and bacterial burden following i.n. infection of wild type mice with 5×10^2 or 10^4 CFU *B. mallei*[a].

	Lung		Plasma		Spleen	
Cytokine / Chemokine	**p-value**	**r^2**	**p-value**	**r^2**	**p-value**	**r^2**
IL-6	†	0.35	$p = 0.35$	0.15	§	0.88
TNF-α	§	0.53	$p = 0.26$	0.2	$p = 0.36$	0.11
IFN-γ	$p = 0.26$	0.06	$p = 0.7$	0.03	$p = 0.21$	0.19
CCL2	§	0.75	$p = 0.32$	0.16	$p = 0.50$	0.07
CXCL1	§	0.45	$p = 0.45$	0.08	$p = 0.93$	0.001

[a] Data were pooled from two independent experiments. Statistical results from a Person's correlation analysis are displayed, and regression values from correlation analysis are also reported (* $p < 0.05$, † $p < 0.01$, § $p < 0.001$).

Table A1.4. Comparison of cytokine production normalized to CFU in wild type and MyD88$^{-/-}$ mice infected i.n. with 5×10^2 CFU *B. mallei*[a].

Lung						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	0.38 ± 0.16	0.001 ± 0.0004	0.17 ± 0.05 †	0.001 ± 0.0004	0.30 ± 0.15	0.0001 ± 0.00003
TNF-α	0.26 ± 0.12	0.001 ± 0.0006	0.09 ± 0.02 †	0.0002 ± 0.00002	0.11 ± 0.05 *	0.00002 ± 0.000003
IFN-γ	0.02 ± 0.008 *	0.0001 ± 0.00004	0.11 ± 0.04 *	0.00001 ± 0.000005	0.17 ± 0.08 *	0.0000006 ± 0.0000001
CCL2	0.46 ± 0.18 *	0.005 ± 0.002	0.32 ± 0.1 *	0.0005 ± 0.0001	0.87 ± 0.47	0.0003 ± 0.00005
CXCL1	2 ± 0.76 *	0.02 ± 0.008	0.89 ± 0.3 *	0.002 ± 0.0004	2.5 ± 1.3	0.0002 ± 0.00004

Plasma						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	5.3 ± 0	0.4 ± 0.3	3.6 ± 2.5	0.71 ± 0.18	0.34 ± 0.06 *	0.06 ± 0.04
TNF-α	0.64 ± 0	0.25 ± 0.18	0.43 ± 0.17	0.03 ± 0.01 †	0.21 ± 0.1 †	0.0026 ± 0.0018
IFN-γ	0.38 ± 0	0.11 ± 0.1	4.6 ± 3.1	0.006 ± 0.002	0.2 ± 0.11 †	0.00006 ± 0.000002
CCL2	11.1 ± 0	2 ± 1.7	4.6 ± 1.9 *	0.53 ± 0.2	1 ± 0.37 §	0.03 ± 0.01
CXCL1	34.34 ± 0	6.5 ± 3.6	26.5 ± 5.8	24 ± 9.2	1.9 ± 1.9	0.46 ± 0.16

Spleen						
Cytokine / Chemokine	24 Hours		48 Hours		72 Hours	
	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$	WT	MyD88$^{-/-}$
IL-6	0.15 ± 0.07	0.46 ± 0	0.72 ± 0.29 †	0.005 ± 0.0007	BLD	0.0003 ± 0.00007
TNF-α	0.45 ± 0.23	1.1 ± 0	0.42 ± 0.12 §	0.002 ± 0.00004	BLD	0.00009 ± 0.00002
IFN-γ	0.05 ± 0.02	0.28 ± 0	1.2 ± 0.34 †	0.0006 ± 0.00009	BLD	0.000005 ± 0.000001
CCL2	1.6 ± 0.87	2.2 ± 0	2.6 ± 1.1 *	0.013 ± 0.003	BLD	0.001 ± 0.0002
CXCL1	6.2 ± 3.9	3.3 ± 0	0.37 ± 0.21 *	0.02 ± 0.005	BLD	0.0006 ± 0.0001

[a] Data are representative of two independent experiments. Data are presented as mean ± SEM pg/ml concentrations, and differences between WT and MyD88$^{-/-}$ mice were determined by a two-tailed Student's t-test (* $p < 0.05$, † $p < 0.01$, § $p < 0.001$).

BLD = Below limit of detection, no CFU data for normalization.

APPENDIX II.

ADDITIONAL FLUORESCENT AND HISTOPATHOLOGY IMAGES FROM MICE INFECTED ORALLY WITH *BURKHOLDERIA PSEUDOMALLEI*.

A2.1 Summary.

This appendix contains additional fluorescent and histopathology images from the oral infection model of chronic melioidosis. Figures include: Fluorescent images from fluorescent *in situ* hybridization (FISH) experiments performed on stomach, small intestine (SI), cecum and colon tissues from mice orally infected with Bp2671a (2.0×10^4 CFU; tissues collected 21 days after infection) (Fig. A2.1); Bp2685a (4.8×10^4 CFU; tissues collected 3 days after infection) (Fig. A2.2) and Bp2719a (2.8×10^4 CFU; tissues collected 4 days after infection) (Fig. A2.3). Individual localization of positive 1000X fields within the stomach of mice infected with each *B. pseudomallei* strain (Fig A2.4). Histopathology images from the stomach of mice infected with each *B. pseudomallei* strain (Fig. A2.5).

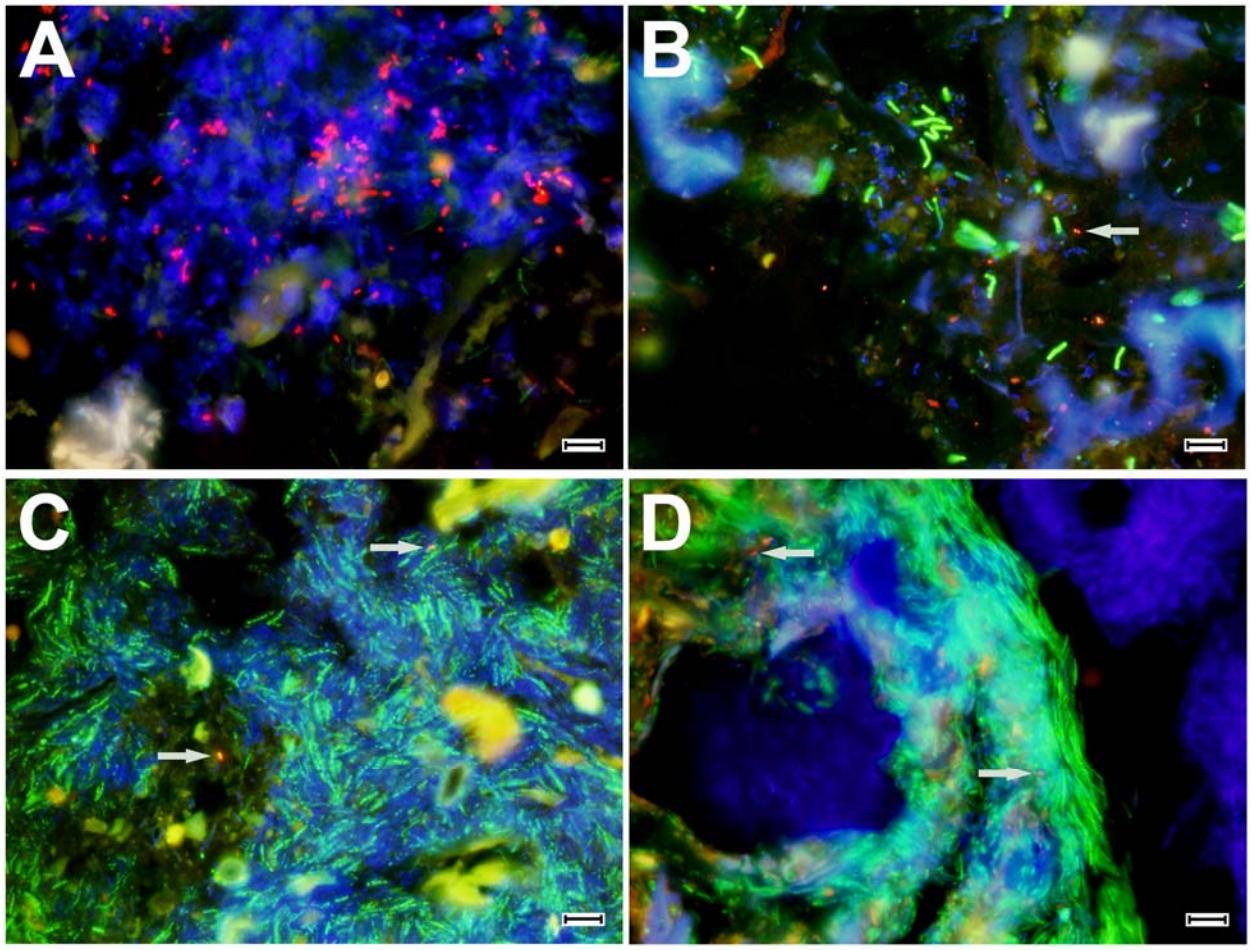

Figure A2.1. Localization of *B. pseudomallei* 2671a in gastrointestinal organs following oral infection. Stomach (A), small intestine (B), cecum (C) and colon (D) tissues from mice infected orally with 2.0×10^4 CFU *B. pseudomallei* strain 2671a were collected 21 days after infection. Organs were fixed in 10% NBF and embedded in paraffin prior to sectioning. FISH was performed on tissue sections as described in materials and methods. Tissue sections were counterstained with DAPI (blue) and observed at 1000X final magnification. Tissue sections were hybridized with a eubacterial probe (green), and two *B. pseudomallei* specific probes (red). Arrows in B-D indicate the location of *B. pseudomallei*. In all images the scale bar represents 10 microns.

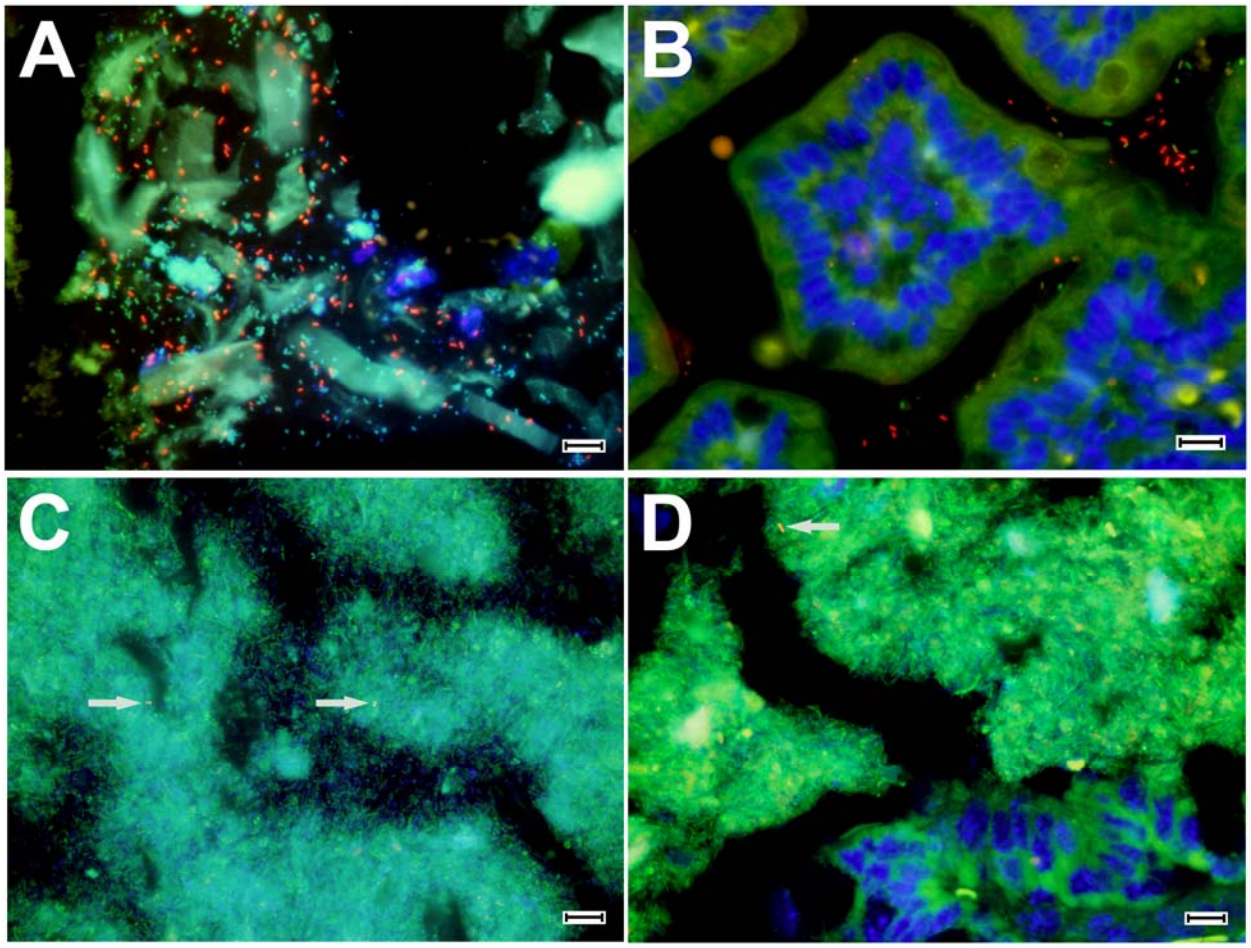

Figure A2.2. Localization of *B. pseudomallei* 2685a in gastrointestinal organs following oral infection. Stomach (A), small intestine (B), cecum (C) and colon (D) tissues from mice infected orally with 4.8×10^4 CFU *B. pseudomallei* strain 2685a were collected 3 days after infection. Organs were fixed in 10% NBF and embedded in paraffin prior to sectioning. FISH was performed on tissue sections as described in materials and methods, sections were counterstained with DAPI (blue) and observed at 1000X final magnification. Tissue sections were hybridized with a eubacterial probe (green), and two *B. pseudomallei* specific probes (red). Arrows in C and D indicate the location of *B. pseudomallei*. In all images the scale bar represents 10 microns.

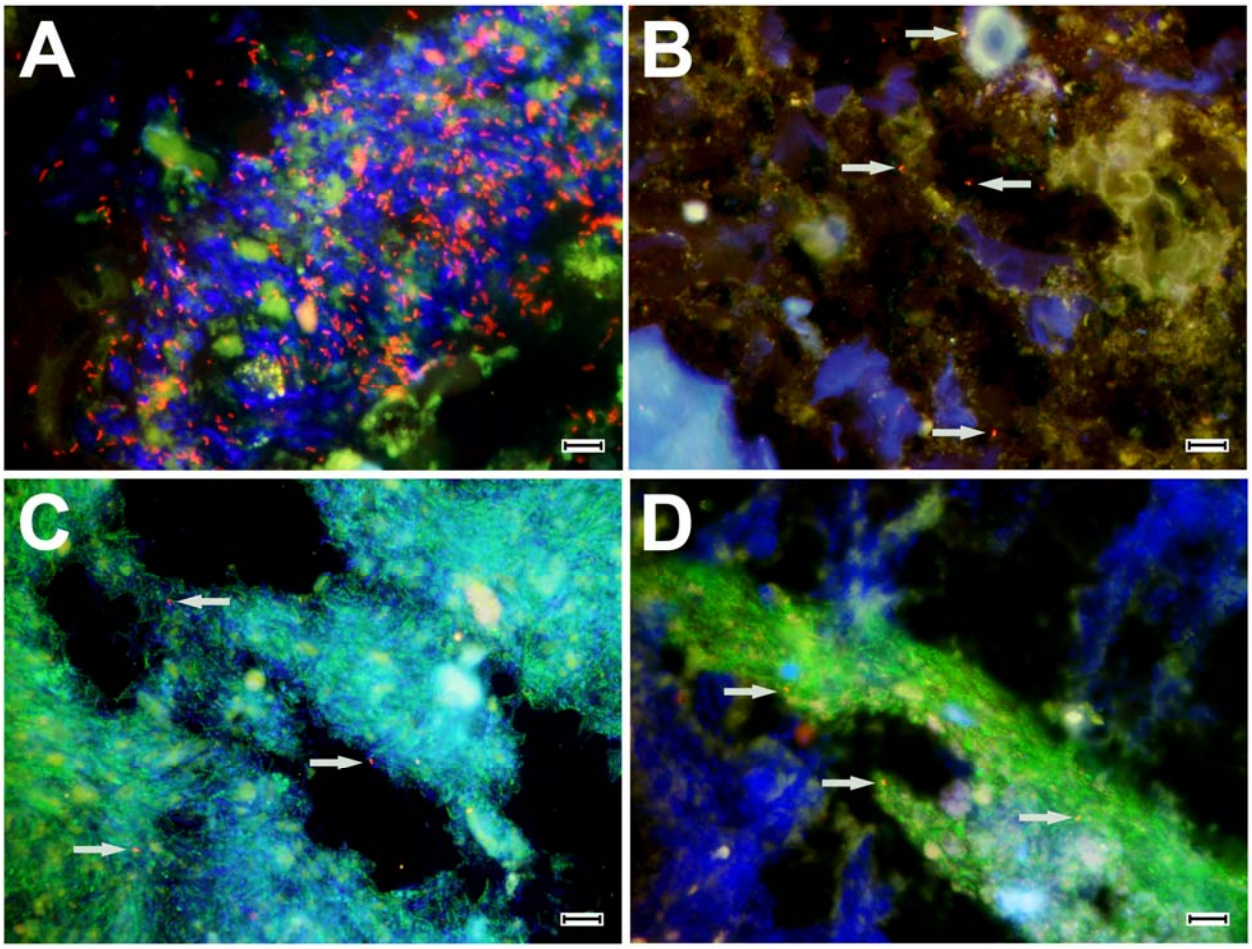

Figure A2.3. Localization of *B. pseudomallei* 2719a in gastrointestinal organs following oral infection. Stomach (A), small intestine (B), cecum (C) and colon (D) tissues from mice infected orally with 2.8×10^4 CFU *B. pseudomallei* strain 2719a were collected 4 days after infection. Organs were fixed in 10% NBF and embedded in paraffin prior to sectioning. FISH was performed on tissue sections as described in materials and methods, sections were counterstained with DAPI (blue) and observed at 1000X final magnification. Tissue sections were hybridized with a eubacterial probe (green), and two *B. pseudomallei* specific probes (red). Arrows in B-D indicate the location of *B. pseudomallei*. In all images the scale bar represents 10 microns.

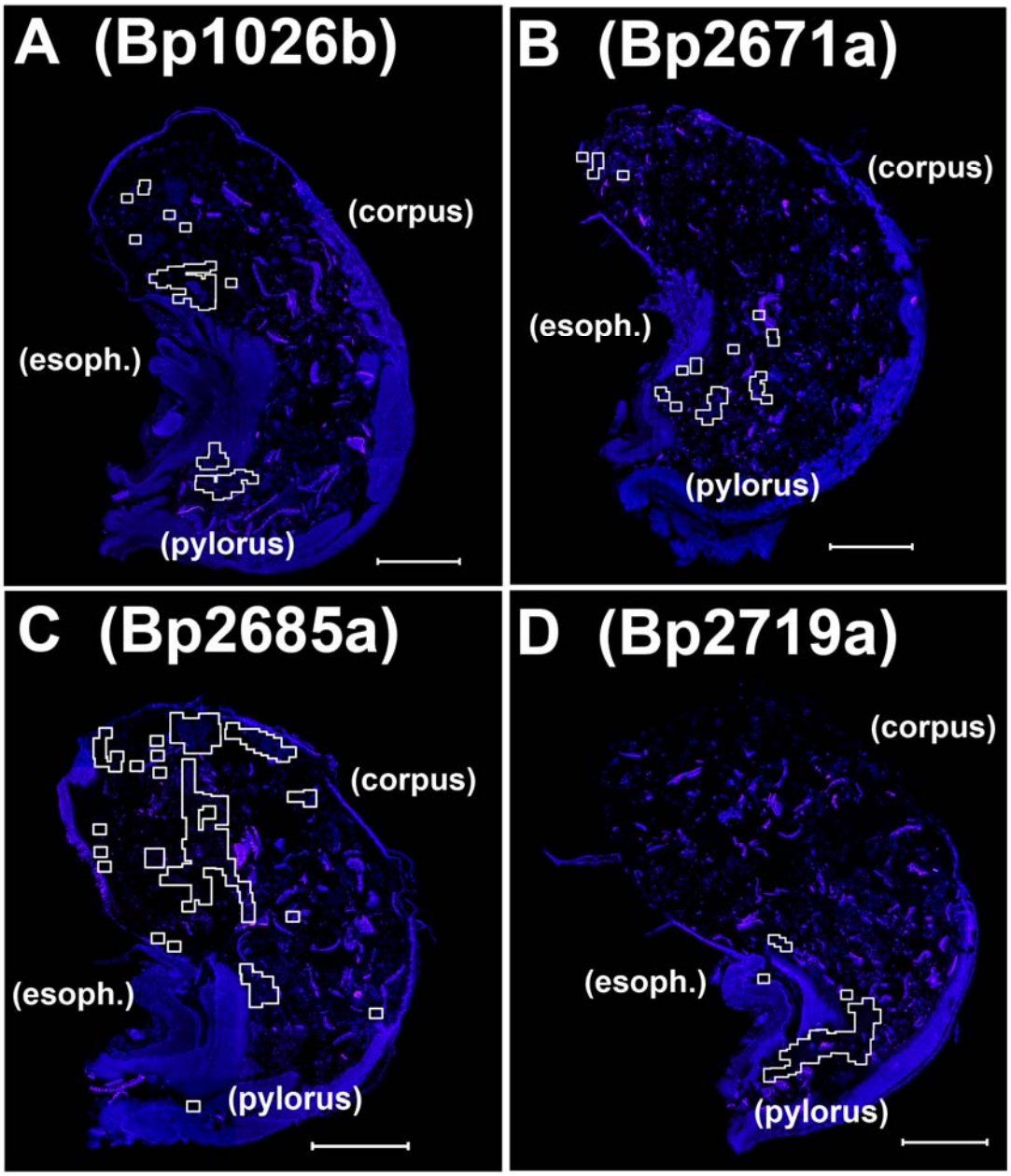

Figure A2.4. Localization of *B. pseudomallei* colonization in the stomach. BALB/c mice were infected orally with *B. pseudomallei* strain 1026b (5×10^5 CFU), Bp2671a (2.0×10^4 CFU), Bp2685a (4.8×10^4 CFU) or Bp2719a (2.8×10^4 CFU). Stomach tissues were collected from Bp1026b mice 56 days after infection, Bp2671a mice 21 days after infection, Bp2685a mice 3 days after infection, and Bp2719a mice 4 days after infection. All tissues were fixed in 10% NBF and embedded in paraffin before sectioning. FISH was performed on stomach tissue sections as described in materials and methods. Tissues were counterstained with DAPI and observed at 1000X final magnification. Positive 1000X fields containing *B. pseudomallei* from mice infected with Bp1026b (A), Bp2671a (B), Bp2685a (C) or Bp719a (D) are indicated by white outlines. Outlines are overlaid onto stomach images created by combining images of DAPI staining obtained from each stomach. The esophagus (esoph.), body (corpus) and pylorus of the stomach are labeled for reference. The scale bar in all images represents 2 mm.

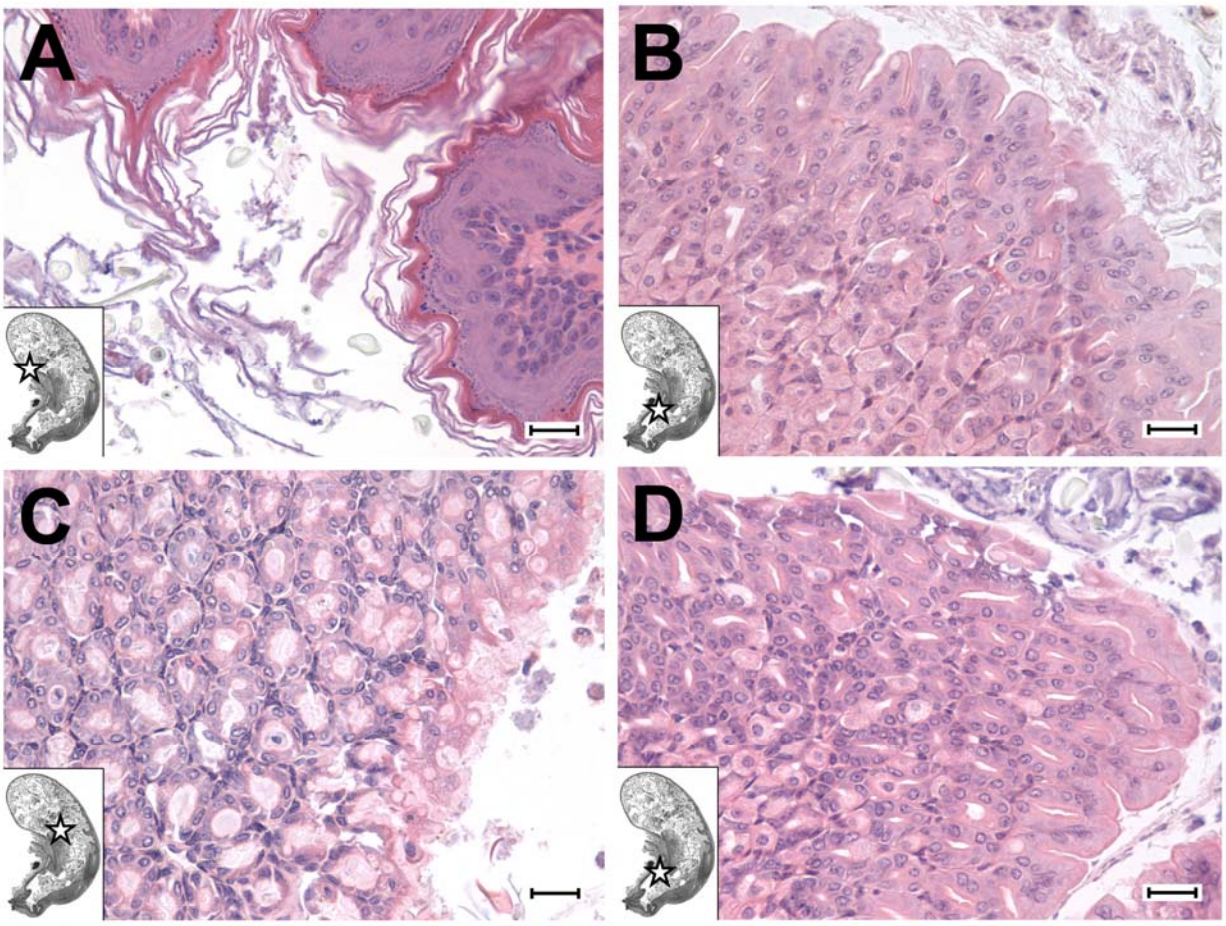

Figure A2.5. Mice lack gastric pathology following oral infection with different *B. pseudomallei* isolates. BALB/c mice were infected orally with *B. pseudomallei* strain Bp1026b (5×10^5 CFU), Bp2671a (2.0×10^4 CFU), Bp2685a (4.8×10^4 CFU) or Bp2719a (2.8×10^4 CFU). Stomach tissues were collected from Bp1026b mice 56 days after infection, Bp2671a mice 21 days after infection, Bp2685a mice 3 days after infection, and Bp2719a mice 4 days after infection. All tissues were fixed in 10% NBF, embedded in paraffin and stained with hematoxylin and eosin. Representative stomach images from Bp1026b (A), Bp2671a (B), Bp2685a (C) and Bp2719a (D) are shown. The location of each image within the stomach is indicated by a star on the representative stomach image in the bottom left corner of each image. The scale bar represents 25 microns in all images.