THESIS

USE OF IMMUNOTHERAPY TO ENHANCE THE EFFICACY OF CEFTAZIDIME FOR THE TREATMENT OF ACUTE PULMONARY *BURKHOLDERIA PSEUDOMALLEI* INFECTION

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY LISA KELLIHAN ENTITLED "USE OF IMMUNOTHERAPY TO ENHANCE THE EFFICACY OF CEFTAZIDIME FOR THE TREATMENT OF ACUTE PULMONARY *BURKHOLDERIA PSEUDOMALLEI* INFECTION" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

USE OF IMMUNOTHERAPY TO ENHANCE THE EFFICACY OF CEFTAZIDIME FOR THE TREATMENT OF ACUTE PULMONARY *BURKHOLDERIA PSEUDOMALLEI* INFECTION

B. pseudomallei is a soil bacterium endemic to Southeast Asia and northern Australia and is the causative agent of melioidosis. This organism is capable of causing acutely lethal pneumonia in humans when inhaled and is inherently resistant to many commonly used antimicrobials, thus making pulmonary *B. pseudomallei* infection difficult to treat. Effective treatment requires prolonged antibiotic therapy and, even with appropriate treatment, relapse is common. With increasing incidence of this disease in endemic regions and renewed interest in this bacterium as a potential biowarfare agent, more effective treatment approaches are needed. We investigated the ability of immunotherapy to enhance the effectiveness of antimicrobial therapy for the treatment of pulmonary *B. pseudomallei* infection. Immunotherapy with cationic liposome-DNA complexes (CLDC), potent stimulators of innate immunity and pro-inflammatory cytokine production, combined with ceftazidime antimicrobial therapy elicited synergistic inhibition of intracellular *B. pseudomallei* replication in infected alveolar macrophages and protected mice from acutely lethal i.n. challenge with *B. pseudomallei*. Mice treated with CLDC and ceftazidime also had significantly reduced organ bacterial

burdens. We determined *in vitro* that IFN-γ elicited by CLDC administration was the predominant cytokine responsible for the synergistic interaction with ceftazidime. Treatment of alveolar macrophages and mice with recombinant IFN-γ as a substitute for CLDC also significantly increased the efficacy of ceftazidime in the treatment of *B. pseudomallei* infection. As a result, we concluded that immuno-antimicrobial therapy consisting of an IFN-γ-inducing immunotherapeutic and a conventional antimicrobial may improve the treatment of *B. pseudomallei* infection via augmentation of antimicrobial efficacy.

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Chapter 1: Literature Review.

Historical relevance of *Burkholderia pseudomallei*:

Burkholderia pseudomallei, then known as *Bacillus pseudomallei*, was first identified in 1911 by the British pathologist, Captain Alfred Whitmore (1-4). At that time, Captain Whitmore was serving at the Rangoon General Hospital in Burma. He described the disease as a glanders-like illness in the ill and neglected inhabitants of Rangoon, mostly opium addicts. Glanders is an abscess-forming disease of horses, horse-like species, and occasionally humans caused by the bacterium *Burkholderia mallei* (1, 2, 4). In 1913, a disease that was later determined to be caused by *B. pseudomallei* was named melioidosis by Dr. A. Stanton and Dr. W. Fletcher, faculty members and prominent researchers at the Institute for Medical Research in Kuala Lumpur, Malaya. The name melioidosis comes from the Greek meaning "resembling a distemper of asses," referring to this disease's similarity to glanders (4).

Since the time of its identification, many more cases of melioidosis have been reported. Cases are increasingly reported in Southeast Asia, northern Australia, and other tropical areas of the world, where the disease is endemic (1, 3, 5). In addition, several cases were reported during and after World War II, and many more cases were reported in soldiers fighting in Vietnam and the French Indochina War (2, 4). During the Vietnam War, cases reported in apparently healthy America helicopter crew are thought to be a consequence of inhaling soil-derived dusts containing bacteria. Other case reports

include those of a Vietnam veteran and a prisoner in Asia in WWII who developed disease many years after returning to countries where *B. pseudomallei* is not endemic, 26 years later and 62 years later, respectively (5-7). Such cases demonstrate this bacterium's ability to evade the host immune response, persist in the body, and become reactivated later in life (5, 6). In more recent years, melioidosis has become as important a pathogen as *Staphylococcus aureus* or *Escherichia coli* in causing community-acquired septicemias in endemic areas, accounting for 20% of cases in northeastern Thailand, for example. In addition, *B. pseudomallei* accounts for 40% of sepsis-related mortality in the same region. It is speculated that current estimates of the global burden of melioidosis are probably much lower than actual numbers (4, 7).

Multiple characteristics of *B. pseudomallei*, including its presence in soil in endemic areas, high rate of infectivity via aerosols, resistance to many common antibiotics and cellular defenses, and absence of a vaccine make this bacterium an ideal candidate for development into a biological weapon. In fact, several countries have studied *B. pseudomallei* for potential offensive intent (1, 2, 8). It has been suggested that the former Soviet Union military was weaponizing *B. pseudomallei* around the time of conflict in Afghanistan in the early 1980s (1, 9). The United States has also conducted biological weapons research involving *B. pseudomallei* in previous years (1, 8).

In 1972, the "Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction," was introduced, which prohibited any further development of *B. pseudomallei* and other bacteria, viruses, and toxins for offensive intent and ordered their destruction. There have been no reports of the malicious use of *B. pseudomallei* in recent

years (8). However, with the events of September 11, 2001 and following, renewed concern has arisen regarding the potential use of *B. pseudomallei* as an agent of bioterrorism against developed countries. This concern is reflected by the classification of *B. pseudomallei* as a category B agent in the US Centers for Disease Control and Prevention's (CDC) list of potential biological warfare and bioterrorism agents (4, 5, 7, 8). Interest in the pathogenesis of *B. pseudomallei* has increased following this classification (1, 7).

Life history of *B. pseudomallei* and infection:

B. pseudomallei is recognized as an important human and animal pathogen, causing several thousand cases of human melioidosis each year in endemic areas, and is a motile, gram negative, facultative intracellular bacterium (1, 3-5, 10). The bacterium is a soil saprophyte, endemic in Southeast Asia and northern Australia and other tropical areas of the world, as previously mentioned (1, 3, 5, 10). There has recently been an increased incidence of melioidosis in temperate zones, probably due to travelers returning from areas of the world where the disease is endemic (5). *B. pseudomallei* bacilli can be cultured from wet soil and ground water and are often present in rice paddies in Southeast Asia (1, 4, 5).

Humans most likely to contract the disease naturally are those who have prolonged contact with contaminated water and soil, such as farmers and others exposed to the natural environment. A common route of infection is through contamination of cuts and abrasions on the feet, since farming is a common occupation in the region and often the farmers do not wear protective footwear while harvesting rice and other crops.

Aerosols created by heavy rains can also increase the likelihood of inhalation of this pathogen (1, 5, 8). Aspiration of the organism is also possible (7). As a result, melioidosis outbreaks have been linked to typhoon season, tsunamis, and flooding in several endemic areas (1, 3, 6, 7). In addition, infection has been associated with the presence of underlying disease, such as diabetes mellitus, chronic renal disease, liver disease (e.g. cirrhosis), cancer, immunosuppression due to either disease or drug treatment (e.g. steroid therapy), alcoholism, tuberculosis, and thalassaemia (1, 4-6, 8). While *B. pseudomallei* can infect people of all ages, older individuals tend to be at greater risk as a result of the development underlying predisposing illness (4).

B. pseudomallei has been referred to as "the great imitator" of all infectious diseases. As such, melioidosis can present with a broad range of clinical signs and symptoms and has the potential to involve almost any organ of the body (7, 11). The inoculum size, route of infection, virulence of the infecting strain, and immune status of the host most likely determine the type and severity of symptoms that develop (1, 11). Melioidosis may present as either acute or chronic disease. There are no pathognomonic signs of infection and usually there is no evidence of recent trauma or obvious wound infection in affected individuals (1, 4). Symptoms of acute infection include fever, malaise, abscess formation, pneumonia, coughing, and sepsis (1). Acute septicemic melioidosis is the most severe form of disease (11). Even with aggressive antibiotic therapy, septicemia caused by *B. pseudomallei* is rapidly fatal following the onset of symptoms and has a mortality rate of approximately 40% (1, 8). Chronic melioidosis can manifest as a localized infection in almost any organ of the body and typically presents with similar symptoms as the acute disease though they tend to be milder and may last for

months or even years (1, 11). Chronically infected individuals may even be asymptomatic (11). In both forms of the disease, abscess formation in the lungs, liver, spleen, and skeletal muscles are hallmarks of infection (4, 8). Abscess formation in other tissues is being increasingly recognized. In a very small number of cases, melioidosis presents as an uncomplicated localized infection of the skin, subcutaneous tissue, or eye (4). In addition, as previously mentioned, asymptomatic or chronic infections may reactivate and intensify to an acute form of disease after years of dormancy (1, 4-8, 11). Reactivation is often correlated with the onset of other illnesses, such as influenza, diabetes, and cancer (1). Furthermore, recurrence of infection is common despite adequate antimicrobial therapy (7, 11). Despite years of extensive clinical research and improvement of treatment protocols, the overall mortality for *B. pseudomallei* infection in adults is currently about 50% in Thailand and 19% in Australia (4, 6, 7). The mortality rate in Australia has been declining in recent years due to advancements in diagnostic technology, intensive care facilities, and treatment (4).

In mice, disease can also have a broad range of presentations, which are dependent upon the route of infection, dose, and mouse strain. The range of disease in mice can include chronic latent infection, chronic infection with clinical signs, and acute fulminant disease. In small animal models of disease, namely mice and hamsters, *B. pseudomallei* is much more infective by the aerosol or intranasal (i.n.) routes (5). No definitive comparisons of the pathogenesis of disease following aerosol and i.n. delivery of bacteria exist, but preliminary data in BALB/c mice indicate that survival patterns are identical between the two challenge methods, which suggest that i.n. challenge mimics aerosol challenge. I.n. infection of BALB/c mice results in an acute disseminated

infection with features very similar to those of severe human melioidosis including extensive abscess formation in the lungs, development of bacteremia, and dissemination to other organs such as the spleen and liver (5).

Pathogenesis of *B. pseudomallei*:

B. pseudomallei is an efficient pathogen and a difficult organism to kill. It is capable of invading and surviving within several phagocytic and nonphagocytic eukaryotic cell lines and is seen within phagocytic cells in pathological specimens (4). The pathogenesis of *B. pseudomallei* is made possible by its unique intracellular lifestyle: Following internalization, *B. pseudomallei* escapes from membrane-bound phagosomes into the cytoplasm of infected cells. Next, it induces actin polymerization at one pole of the cell to form membrane protrusions. These membrane protrusions are then phagocytosed by nearby cells, thus enabling cell-to-cell spread without exposure to the extracellular environment (4, 7, 12). *B. pseudomallei* can also induce cell fusion, resulting in multinucleated cell formation, which may also contribute to cell-to-cell spread (12). Despite its recognition as a Category B bioterrorism agent by the CDC and knowledge of its intracellular lifestyle, many questions remain regarding the pathogenesis of *B. pseudomallei* and the mechanisms involved (8). However, several factors have been identified that purportedly contribute to the pathogenesis of this organism. These include capsular polysaccharide, lipopolysaccharide, Type III Secretion Systems, quorum sensing, Type IV pili-mediated adherence, flagella, Type VI Secretion Systems, and secreted proteins.

Capsular polysaccharide. *B. pseudomallei* produces an extracellular capsular polysaccharide, the structure of which is -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-. This particular structure of *B. pseudomallei* was initially characterized as a type I O-polysaccharide but, due to its high molecular mass and genetic homology with group 3 capsular polysaccharides of other organisms, has been re-characterized as a capsular polysaccharide (7). *B. pseudomallei*'s capsule is thought to decrease the deposition of complement factor C3b on the bacterial cell surface and act as a barrier, blocking access of complement receptor-1 (CR1) on phagocytic cells to C3b that has been deposited on the surface of the bacterium (7, 13). In addition, this capsule facilitates formation of a biofilm and of microcolonies in which the organism is protected from antibiotic penetration and is rendered less susceptible to antibiotics that do penetrate (4). Experimentally, *B. pseudomallei*'s capsule is required for virulence in animal models of infection. Capsule-deficient *B. pseudomallei* strains display increases in the lethal dose required to cause death in 50% of infected animals (LD_{50}) in the mouse aerosol challenge model, increased phagocytosis, decreased persistence in the blood, and decreased organ bacterial burdens. Addition of purified capsule to capsule-deficient strains increases the LD_{50} in Syrian hamsters and restores the ability of the bacteria to survive in human serum (7, 8). Also, passive immunization of mice with antibodies to *B. pseudomallei* capsule reduces the lethality of infection (4).

Lipopolysaccharide (LPS). Recognition of LPS is necessary for the initiation of a rapid innate immune response to Gram-negative bacteria. This recognition usually occurs through activation of Toll-like receptor (TLR) 4, a type of transmembrane pattern recognition receptor that recognizes conserved sequences typically associated with

pathogens (7). *In vitro* studies in murine macrophages using a LPS mutant strain of *B. pseudomallei* have suggested a possible role for the O-antigenic polysaccharide moiety of LPS in internalization and intracellular survival of the organism (13). The *B. pseudomallei* LPS is highly conserved among its many strains (4, 7). However, there is something different about *B. pseudomallei* LPS compared to that of other Gram-negative bacteria. *B. pseudomallei* LPS exhibits a weaker ability to induce fever in rodents compared with enterobacterial LPS, but a stronger ability to stimulate cell division in murine splenocytes (7). Activation of murine macrophages *in vitro* is slower and much less potent when mediated by *B. pseudomallei* LPS versus *Escherichia coli* LPS, for example (7, 13). *B. pseudomallei*'s LPS may contribute to bacterial pathogenesis by modulating the host response, e.g. becoming less immunogenic, and inhibiting macrophage killing (14).

Type III Secretion Systems (T3SS). T3SS are gene clusters present in Gram-negative bacteria that encode for a secretion apparatus. This secretion apparatus functions like a molecular syringe, which secretes effector proteins into the cytosol of a target-cell in order to subvert that cell's cellular processes (7, 8, 13). These protein secretion systems play an important role in the pathogenesis of other bacterial agents (7, 8. B. pseudomallei encodes three T3SS gene clusters {Larsen, 2009 #25, 13). A gene cluster in *B. pseudomallei*, termed *Burkholderia* secretion apparatus (*bsa*), shares genetic homology with a T3SS found in *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* that is known to be involved in cellular invasion, escape from endocytic vacuoles, intercellular spread, and pathogenesis. Mutation of *bsa* shows that this T3SS is required for intracellular survival and growth and phagosomal escape as mutants have

reduced replication in murine macrophage-like cells, have an inability to escape from endocytic vacuoles, and cannot form membrane protrusions and actin tails (7, 8). Another *B. pseudomallei* T3SS gene product, BopE, shares homology with *S. typhimurium* SopE, a secreted T3SS effector protein involved in the uptake of *S. typhimurium* by nonphagocytic cells. BopE demonstrates the ability to induce cytoskeletal arrangements when expressed in eukaryotic cells and the ability of *bopE* mutants to invade nonphagocytic cells is reduced. Therefore, BopE is likely involved in inducing cytoskeletal arrangements in nonphagocytic cells in order to facilitate cellular invasion by *B. pseudomallei* (7, 8). A third *B. pseudomallei* T3SS gene product, BipD, shares homology with *S. typhimurium* SipD, a T3SS protein that is required for the secretion of effector proteins. *bipD* mutants are significantly attenuated in BALB/c mice after i.n. or intraperitoneal (i.p.) challenge and their replication in the livers and spleens of mice following challenge is markedly reduced (7, 8). Finally, *B. pseudomallei* BipB has been shown to promote the formation of multinucleated giant cells, cell-to-cell spread of bacteria, and apoptosis of infected host cells (7). Taken together, these data indicate that T3SS are major contributors to the pathogenesis of *B. pseudomallei* by facilitating entry into, survival within, and spread between eukaryotic cells (4, 7, 8, 13).

Quorum Sensing. Quorum sensing is a cell-density-dependent communication system present in many Gram-negative bacteria and used to coordinate gene expression. These systems rely on the production of a particular class of *N*-acyl-homoserine lactones, known as autoinducers, to influence the behavior of surrounding bacteria (7, 8, 13). *B. pseudomallei* contains eight genes encoding quorum sensing system homologues: three LuxI gene homologues, whose gene products are responsible for autoinducer

biosynthesis; and five LuxR gene homologues, whose gene products respond to autoinducer concentrations to regulate expression or repression of target genes involved in pathogenesis. The amount of autoinducer present in the environment is dependent on the cell density of the bacteria. Simultaneous disruption of all eight of these quorum-sensing genes in *B. pseudomallei* leads to a significant increase in LD_{50} in Syrian hamsters following i.p. challenge, increased the time to death in aerosol challenged BALB/c mice, and decreased organ colonization in those same mice (7, 8, 15). *B. pseudomallei* PmlI-PmlR, which is a homologue of LuxI-LuxR, directs the synthesis of *N*-decanoyl-homoserine-lactone, and has a role in the regulation of a metalloprotease, is necessary for complete virulence in a mouse model (7, 16). Another homologue found in *B. pseudomallei*, named BpsI-BpsR, is also necessary for maximal virulence and secretion of exoproducts (7, 17). The identity of genes regulated by the *B. pseudomallei* quorum sensing system is as of yet unknown (8).

The extracellular secretion of autoinducers and some quorum sensing-controlled potential virulence factors and processes- such as siderophores (for iron acquisition), phospholipase C, and biofilm formation- are dependent upon the *B. pseudomallei* BpeAB-OprB efflux pump. This is a multidrug efflux pump found in *B. pseudomallei* that is known to be responsible for conferring antimicrobial resistance to aminoglycosides and macrolides (7, 13). This suggests that the *bpe*AB-*opr*B operon might also be regulated by the quorum sensing apparatus. Evidence for this includes the induction of *bpe*AB-*opr*B expression by *N*-decanoyl-homoserine-lactone and *N*-octanoyl-homoserine-lactone and *in vitro* attenuated cell invasion and cytotoxicity in human cell lines by BpeAB mutants (7).

Type IV Pili-mediated Adherence. *B. pseudomallei* contains multiple genes that encode type IV pili. Type IV pili are known to be involved in adherence, which is an important virulence mechanism, in many Gram-negative bacteria. Deletion of *pil*A, a gene that encodes an alleged pilus structural protein called PilA, results in reduced adherence to human epithelial cells and decreased virulence in both the nematode and murine models of *B. pseudomallei* infection. This suggests that type IV pili might also be important in the virulence and pathogenesis of *B. pseudomallei* (7).

Flagella. *B. pseudomallei* is flagellated and, as a result, motile. The role of flagella in the pathogenesis of *B. pseudomallei* is under question, as several studies have shown no difference between wild-type and flagella-defective *B. pseudomallei* strains in their abilities to invade and replicate within human lung cells *in vitro*, and infect diabetic rats and Syrian hamsters *in vivo*; while one study has found flagella-defective mutants to be attenuated in BALB/c mice, relative to wild-type strains, following i.n. and i.p. challenges (3, 7, 13). Other studies have shown *B. pseudomallei* flagella to be important immunostimulatory molecules, causing upregulation of proinflammatory cytokines, and involved in cell invasion of phagocytic and nonphagocytic cells (13).

Secreted Proteins. *B. pseudomallei* produces a number of proteins that are secreted and are thought to be involved in its pathogenesis. Examples of these secreted proteins include proteases, lipase, lecithinase, catalase, peroxidase, superoxide dismutase, and hemolysins (4, 7). In addition, *B. pseudomallei* contains genes for 11 predicted autosecreted proteins, known as autotransporters, which are a class of bacterial proteins that mediate their own secretion and/or membrane localization. In other pathogenic bacterial species, such as *S. flexneri*, *Rickettsia rickettsii*, and *Listeria monocytogenes*,

autotransporters like the ones found in *B. pseudomallei* facilitate the process of actin-based motility, a mechanism that uses components of the eukaryotic host cell's cytoskeleton to propel bacteria through the cell's cytosol, by nucleating and polymerizing actin. *B. pseudomallei* is known to utilize actin-based motility to make possible its intracellular survival and spread. BimA, a predicted *B. pseudomallei* autotransporter, possesses proline-rich regions commonly found in proteins that stimulate actin polymerization. *B. pseudomallei bim*A mutants lack the ability to form membrane protrusions *in vitro*, which implies a role for BimA in actin-based motility (8, 18).

Type VI Secretion Systems (T6SS). T6SS is a recently identified family of protein secretion systems found in Gram-negative bacteria. Thus far, they have been implicated in the pathogenesis of such bacterial species as *Vibrio cholerae* and *Pseudomonas aeruginosa* (8). A series of studies in which hamsters challenged with wild-type *B. mallei* succumbed to infection, while hamster challenged with T6SS mutant strains survived, showed that Hcp1, a protein present in *B. mallei* and secreted in a T6SS-dependent manner, and other T6SS genes are crucial for complete virulence in the hamster model of *B. mallei* infection (8). Given the close relatedness of *B. mallei* and *B. pseudomallei* and that six T6SS have been located in the *B. pseudomallei* genome, at least one of which is thought to be involved in macrophage invasion, T6SS may also be another of the many diverse mechanisms involved in *B. pseudomallei* pathogenesis (8, 13).

Role of innate immunity in the host response to *B. pseudomallei*:

Innate immune mechanisms are vital in determining the outcome of infection with many bacterial pathogens. This is also the case with *B. pseudomallei*. Several innate immune mechanisms are important in host defense against *B. pseudomallei*, including TLRs, pro-inflammatory cytokines, and reactive nitrogen intermediates (RNI).

TLRs are an important first line of defense against many invading pathogens as they detect host invasion by pathogens and initiate cellular innate immune responses (19). *B. pseudomallei* possesses multiple TLR ligands, including peptidoglycan, which binds TLR2; LPS, which usually signals through TLR4; flagellin, which binds TLR5; and CpG DNA, which signals through TLR9 (7). Interestingly, the LPS of *B. pseudomallei* has been found to signal through TLR2 in addition to TLR4 and LPS signaling through TLR2 may actually impair host defense against *B. pseudomallei* infection. This finding was suggested by a strong survival advantage, decreased bacterial loads, reduced lung inflammation, and decreased distant-organ injury in TLR2 KO mice that were inoculated i.n. with *B. pseudomallei* (20). In each case, TLR signal transduction results in translocation of nuclear transcription factor (NF)-κB and induction of a proinflammatory cytokine response from activated tissue-resident macrophages. The proinflammatory cytokines produced, including TNF-α and interleukin (IL)-6, among others, are capable of coordinating local and systemic inflammatory responses (14, 19). In *B. pseudomallei* infection, TLR signaling is essential for early detection of pathogens, but can cause excessive inflammation resulting in septic shock if stimulation is uncontrolled (19).

In regard to pro-inflammatory cytokines, interferon (IFN)-γ is essential for resistance to *B. pseudomallei* infection, while IL-12, IL-18, and tumor necrosis factor

(TNF)-α play additional significant roles (13). Concentrations of each of these cytokines are consistently elevated in the serum of human melioidosis patients (4, 21-23). Experimentally, these cytokines have also been found to be important in animal models of *B. pseudomallei* infection (22-26).

In other intracellular bacterial infections, IFN- γ is known to be an important activator of macrophage microbicidal activity and, thus, is involved in controlling the rate of bacterial growth within the host. Resistance to acute infection with *B. pseudomallei* is dependent upon the production of IFN- γ. Macrophages activated *in vitro* by IFN-γ have been shown to kill *B. pseudomallei*, which can otherwise survive and replicate inside unstimulated cells (27). Neutralization of IFN- γ *in vivo* in Taylor Outbred (TO) mice results in a decrease in the LD50 from > 5 x 10^5 CFU to approximately 2 CFU when challenged i.p., corresponding to a > 100,000-fold increase in susceptibility, and is associated with remarkable increases in the bacterial burdens in the liver and spleen. In addition, the rapid production of IFN- γ within the fist day of infection determines whether the infection proceeds to acutely lethal or chronic disease (22). According to Santanirand et al. (22), IFN-γ is capable of protecting a naïve host from acute sepsis following initial exposure to *B. pseudomallei*. However, much like with *Mycobacterium tuberculosis* and *Toxoplasma gondii*, the host IFN- γ response is unable to eliminate the organism, thereby allowing progression to chronic disease and placing the host at risk of reactivation in the future. Natural killer (NK) cells are the major producers of IFN- γ but neutrophils, macrophages, conventional T cells, and NK T cells also make notable contributions (25, 26, 28). In one study, antibody depletion of NK cells in C57BL/6 mice lead to a 76% reduction in IFN-γ production at 16 h post i.p. *B. pseudomallei* infection,

early mortality, and increased splenic bacterial burdens (26). In another study, antibody depletion of neutrophils resulted in a 91% reduction in pulmonary IFN-γ levels of C57BL/6 mice at 2 d post i.n. challenge and early mortality, and increased pulmonary bacterial burdens (25).

The production of IFN-γ is potently induced and tightly regulated by the endogenous cytokines IL-12 and IL-18, which are derived from monocytes and macrophages. *In vitro*, incubation of whole blood with heat-killed *B. pseudomallei* in the presence of IL-12 or IL-18 neutralizing antibodies leads to significant reductions in IFN-γ production, which was more pronounced in following the neutralization of IL-12. Further reductions in IFN-γ production occur when both IL-12 and IL-18 are neutralized simultaneously, demonstrating the synergistic interaction between IL-12 and IL-18 in the stimulation of IFN-γ synthesis (21). *In vivo* antibody neutralization of IL-12 in TO mice results in increased susceptibility to acute *B. pseudomallei* infection (22). IL-18 knockout (KO) mice display accelerated mortality following i.n. challenge with *B. pseudomallei* and increased organ bacterial burdens in the lungs, livers, spleens, kidneys, and blood (23). IL-12 KO mice and IL-18 receptor (IL-18R)-depleted mice show increased susceptibility to *B. pseudomallei* infection when challenged i.p. as evidenced by rapid mortality compared to wild-type or isotype antibody-treated mice, respectively (26). The increased susceptibility to *B. pseudomallei* infection and rapid mortality are attributed to the reduced production of IFN-γ when IL-12 and/or IL-18 are disrupted.

TNF-α is a proinflammatory cytokine that is produced predominantly by macrophages, neutrophils, and T cells and has a broad range of functions, including upregulation of adhesion molecules and recruitment of macrophages and neutrophils to

the site of inflammation. TNF-α has also been found to work synergistically with IFN-γ to induce macrophage activation (24). The role of TNF-α in the innate immune response to *B. pseudomallei* infection is not fully understood, but several studies have illustrated its importance in host protection. For example, antibody neutralization of TNF-α in TO mice increased susceptibility to infection by the i.p. route (22) (Santanirand et al. 1999). In addition, Barnes et al. (24) discovered that TNF-α and two cell surface receptors that bind and mediate the biological response to TNF-α, TNF receptor (TNFR)-1 and TNFR-2, are all required for optimal control of *B. pseudomallei* infection. To do this, they utilized TNF-α, TNFR-1, and TNFR-2 KO mice and inoculated them i.n. with *B. pseudomallei*. Not only did they observe higher mortality rates compared to wild-type mice in the absence of a TNF-α response, but also increased bacterial loads in the spleens and livers of KO mice and increased inflammatory infiltrates and more extensive necrosis in the organs of KO mice at 2 d PI.

While the inflammatory cytokines IFN-γ, TNF-α, and IL-12 are obviously critical for a protective immune response against *B. pseudomallei* infection, they can also contribute to immunopathology and acute fulminating disease (11, 22). Animal models of septic shock indicate that these cytokines confer resistance when their production is controlled and localized but promote immunopathology when their production is excessive and they are released systemically in response to an overwhelming bacterial load (22). When hyperproduced, TNF-α contributes to immunopathology by initiating a cytokine cascade that leads to increased vascular permeability (24). Hyperproduction of proinflammatory cytokines, especially IFN-γ, in BALB/c mice resulting in septic shock is believed to be responsible for their increased susceptibility to *B. pseudomallei* infection

compared to relatively resistant C57BL/6 mice, which produce only moderate and transient levels of proinflammatory cytokines (1, 11, 28).

Phagocytic cells, namely macrophages, that have been activated by IFN-γ and TNF-α produce reactive nitrogen intermediates (RNI), such as nitric oxide (NO), and reactive oxygen intermediates (ROI), including superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (24, 27, 29). RNI and ROI facilitate killing of intracellular microbes by causing direct damage to the pathogens and by regulating immune responses (29). While both RNI and ROI possess roles in the early innate immune response to *B. pseudomallei* infection, Miyagi et al. (27) concluded that RNI-dependent mechanisms mediate intracellular bacterial killing by macrophages to a greater extent than ROI-dependent mechanisms. The production of NO, a free radical RNI that is toxic to bacteria by causing DNA damage, requires the upregulation of the enzyme inducible nitric oxide synthase (iNOS) by stimulated phagocytes (29, 30). Once iNOS is produced, it reacts with L-arginine to form NO and is also an essential component of the mechanisms that control intracellular persistence and multiplication of *B. pseudomallei* itself (29). However, *B. pseudomallei* has developed a way to interfere with iNOS and NO production as an intracellular survival strategy. Ekchariyawat et al. (31) demonstrated that *B. pseudomallei* is able to activate the expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) in RAW 264.7 cells, a murine macrophage cell line, which correlates directly with a decreased IFN-γ signaling response, reduced iNOS expression, and loss of macrophage bactericidal capacity.

Lack of adequate treatment/prophylaxis for *B. pseudomallei* infection:

Melioidosis is a very difficult disease to treat and, even with the administration of high doses of intravenous (i.v.) antibiotics, response to treatment is often very slow (4). One of the major reasons that melioidosis is so difficult to treat is that *B. pseudomallei* is intrinsically resistant to many commonly used antibiotics (5, 8). The *B. pseudomallei* genome contains multiple drug resistance genes, including drug efflux pumps, β-lactamases, and aminoglycoside acetyltransferases (8, 32). As mentioned previously, several multidrug efflux pumps are implicated in the resistance to aminoglycoside and macrolide antibiotics (7, 8, 13, 32). In addition, *B. pseudomallei* is naturally resistant to penicillins, first- and second-generation cephalosporins, rifamycins, colistin, aztreonam, and fluoroquinolones. Conversely, *B. pseudomallei* is typically susceptible to third-generation cephalosporins (e.g. ceftazidime), carbapenems (e.g. imipenem and meropenem), β-lactam-β-lactamase inhibitor combinations (e.g. amoxicillin-clavulanate, ampicillin-sulbactam, ticarcillin-clavulanate), tetracyclines, ureidopenicillins (e.g. piperacillin), chloramphenicol, and trimethoprim-sulfamethoxazole (TMP-SMZ) (4, 7, 32). It is well-documented that antimicrobial resistance can develop during treatment of melioidosis (32). The current treatment protocol for melioidosis requires a 20-week course of an appropriate combination of antibiotics divided into an i.v. administration phase followed by an oral administration phase. Initial i.v. therapy usually lasts for 10-14 days, or until the patient has improved enough to take oral drugs, and the antibiotics of choice are ceftazidime or a carbapenem (7, 32). Oral therapy consists of a three- or four-drug combination of doxycycline, TMP-SMZ, plus or minus chloramphenicol, and is given until the 20-week course of treatment is completed (4, 32). With the four-drug

combination, chloramphenicol is administered only for the first eight weeks of oral treatment due to its ability to cause severe detrimental side effects in humans (33). The three-drug combination is currently the more common maintenance therapy of choice for melioidosis (32). However, recent *in vitro* data have suggested that doxycycline does not offer any additional benefit to TMP-SMZ in oral maintenance therapy of *B. pseudomallei* infection and may even be antagonistic (34). In addition, amoxicillin-clavulanate can be used as an alternative oral therapeutic in children and pregnant women (4).

Even with early and aggressive treatment, melioidosis still has a relatively high mortality rate for the septicemic form of disease and relapse is common (1, 4, 7, 10). The relapse rate for patients who have completed the 20-week course of antibiotics is approximately 10% and this rate rises to nearly 30% if antibiotic treatment lasts for 8 weeks or less (4, 32). The median time to relapse for melioidosis patients in Thailand is approximately 21 weeks. As a result, apparently recovered melioidosis patients require follow-up with their medical care providers throughout the rest of their lives (4). Interestingly, according to more recent data, up to 25% of melioidosis relapses may actually be a result of infection by multiple strains initially or reinfection with a different *B. pseudomallei* strain rather than a true relapse of the primary infection (1, 35, 36).

Currently, no FDA approved antimicrobials exist for postexposure prophylaxis or treatment of *B. pseudomallei* infection in the event of an intentional release (1, 32). The use of any antibiotics for treatment in this type of situation would be off-label or would require that emergency use authorization status be granted by the FDA (1). In addition, there is presently no vaccine available to protect individuals against infection

with *Burkholderia* organisms, whether naturally or via intentional release of a bioweapon (1, 37).

Review of antimicrobials that have been evaluated for *B. pseudomallei* treatment:

Considering the high intrinsic antibiotic resistance of *B. pseudomallei* and the difficulty of treating melioidosis, a wealth of research has been conducted through the years to characterize drug susceptibilities and discover more effective treatments. Much of this research has been conducted *in vitro*, but there have also been numerous *in vivo* and human patient studies as well. The focus of many *in vitro* experiments has been to determine the minimum inhibitory concentrations (MIC) of various drugs, singly and in combination, against *B. pseudomallei*, while the in vivo experiments have assessed the efficacies of certain drugs and drug combinations in the treatment of *B. pseudomallei* infection in a biological system.

Eickhoff et al. (38) performed a study that evaluated the susceptibilities of 10 strains of *B. pseudomallei* to 20 different chemotherapeutic agents and 12 drug combinations using the 2-fold broth dilution technique. They found that, of the single drug treatments tested, tetracycline was the most active, followed closely by minocycline, another member of the tetracycline class, chloramphenicol, and novobiocin. Both kanamycin and sulfadiazine had slightly higher MICs but were still fairly effective. The penicillins, polymixin B, colistin, and all other drugs assessed were either completely ineffective or only weakly active against *B. pseudomallei*. Of the 12 drug combinations tested, chloramphenicol-kanamycin, tetracycline-kanamycin, and sulfadiazine-chloramphenicol actually exhibited an antagonistic interaction, while sulfadiazine-

kanamycin and ampicillin-dicloxacillin appeared to be synergistic. The authors concluded that tetracycline should be the drug of choice for treating melioidosis based on *in vitro* activity.

Calabi (39) presented the results of a qualitative study that used broth dilution to evaluate the susceptibility of six *B. pseudomallei* strains to four antimicrobial agents, singly and in combination, that were the current recommendation for melioidosis treatment. These included chloramphenicol, kanamycin, novobiocin, and tetracycline. Of the single treatments, only novobiocin appeared to be effective at concentrations below the toxic limit. Of the combinations, novobiocin-tetracycline was considered highly synergistic, achieving bactericidal effect at concentrations far below those that were ineffective when each drug was used alone. Calabi (39) suggested that this synergistic combination may be clinically effective and may provide a means of preventing the emergence of resistant strains.

In 1989, White et al. (33) conducted an open randomized trial in human patients to compare the efficacy of ceftazidime treatment to that of the "conventional therapy," which consisted of chloramphenicol, doxycycline, and TMP-SMZ, for the treatment of severe melioidosis. The rationale for this trial was that the high antibiotic doses of the conventional regimen were associated with potentially serious toxicity and still a high mortality rate in septicemic melioidosis, while ceftazidime was safe, well-tolerated, effective against *Pseudomonas aeruginosa* septicemia, and active against *B. pseudomallei in vitro*. In this trial, ceftazidime treatment was associated with a 50% decrease in overall mortality compared to conventional therapy. From these results, the

authors concluded that ceftazidime should now become the treatment of choice for severe melioidosis.

In another *in vitro* study, Kenny et al. (40) compared the *in vitro* susceptibilities of *B. mallei* to *B. pseudomallei* for 16 antibiotics. They found that *B. pseudomallei* and *B. mallei* have similar antibiotic susceptibility profiles; including ceftazidime, imipenem, doxycycline, ciprofloxacin, and piperacillin but *B. pseudomallei* actually has a broader resistance profile, including gentamicin and azithromycin. The authors recommended that piperacillin may be a suitable candidate to pursue in clinical treatment and also recognized that clinical experience with melioidosis has shown that good *in vitro* activity does not always correspond to good *in vivo* activity.

A 2000 *in vivo* study by Russell et al. (41) compared the efficacies of ciprofloxacin and doxycycline, two antimicrobials which had been reported to be inferior to other antimicrobials in the treatment of human melioidosis and to be associated with high relapse rates, against melioidosis in a mouse model of infection. Both of these antibiotics were previously shown to have *in vitro* activity against *B. pseudomallei* and to penetrate phagocytic cells, where this bacterium resides, but their *in vivo* efficacies for preexposure prophylaxis, immediate therapy post-challenge, and traditional therapy post-challenge had not been investigated. Both antibiotics were effective in preventing symptomatic infection when administered prophylactically or immediately post-challenge, but were ineffective when used therapeutically and resulted in relapses at 5 weeks post-challenge in all treatment groups, which reflected human clinical findings.

In a 2000 *in vitro* study, Vorachit at al. (42) used 4 strains of *B. pseudomallei* to determine the MICs, minimum bactericidal concentrations (MBC), and time-kill curves

with 13 single antimicrobial agents and 33 combinations: 15 expected to be used for acute therapy and 18 for maintenance therapy. Their results provided evidence for the possible clinical efficacy of imipenem, meropenem, and imipenem-azithromycin in the treatment of acute melioidosis and of ciprofloxacin-clarithromycin, ciprofloxacin-azithromycin, and imipenem-azithromycin for the maintenance therapy phase.

A prospective clinical trial in 2001 by Chetchotisakd et al. (43) set out to determine if cefoperazone/sulbactam, a β-lactam/β-lactamase inhibitor combination that has a broader spectrum than ceftazidime, could be used as an alternative to ceftazidime in combination with TMP-SMZ for the treatment of severe melioidosis. They found that mortality rates were not significantly different between treatment groups, both treatments regimens were well-tolerated, and the responses of successfully treated patients were similar. As a result, the authors concluded that cefoperazone/sulbactam plus TMP-SMZ is a possible treatment alternative to ceftazidime plus TMP-SMZ.

Ulett et al. (44) used a mouse infection model to compare different antibiotic regimens in the treatment of acute melioidosis. The purpose of this study was to determine the clinical advantage of adding TMP-SMZ to the standard ceftazidime treatment and to evaluate the activity of newer, fourth-generation cephalosporins, such as cefepime, in the treatment of melioidosis. They found that ceftazidime in combination with TMP-SMZ was more effective than ceftazidime alone as well as cefepime alone or combination with TMP-SMZ.

Thibault et al. (45) determined the MICs of 50 *B. pseudomallei* isolates for 35 antimicrobial agents using the agar dilution technique. Imipenem, ceftazidime, piperacillin, piperacillin/tazobactam, doxycycline, and minocycline yielded the lowest

MICs of the agents evaluated and fluoroquinolones and aminoglycosides had poor activities. They concluded that the recommendations for treatment of *B. pseudomallei* infections at that time were supported by the susceptibility results they obtained.

In 2006, Sivalingam et al. (46) published a letter that discussed the *in vitro* antibiotic susceptibilities of 50 strains of *B. pseudomallei* isolated from clinical cases in Singapore to 4 oral antibiotics commonly used in the treatment of melioidosis. Their study identified some differences in susceptibility patterns of Singapore isolates compared to Thailand isolates, e.g. a lower rate of resistance to TMP-SMZ and a higher susceptibility to ciprofloxacin in Singapore, and determined that doxycycline and amoxicillin-clavulanate were the preferred oral prophylaxes to be considered for local infections.

Also in 2006, Chierakul et al. (47) conducted a prospective pharmacokinetic simulation study, using plasma concentration measurements that they collected and MIC data from 46 clinical isolates and 4 reference strains of *B. pseudomallei*, of oral amoxicillin-clavulanate in human patients with melioidosis to determine the optimal dosage and dosing interval. Their simulation indicated that the dosage and dosing interval of amoxicillin-clavulanate being prescribed at that time, which was 1000 mg/250 mg every 8 h would be expected to maintain plasma concentrations greater than the MIC for long enough periods of time to be effective, thus potentially explaining the relatively high rates of relapse in melioidosis patients treated with amoxicillin-clavulanate. It also determined that the optimal dosage and dosing interval for amoxicillin-clavulanate in patients with melioidosis would be 750 mg/250 mg given every 6 h, but further evaluation would be required to substantiate the clinical efficacy of this regimen.

In a more recent study, Chan et al. (48) explored an interesting hypothesis that phenothiazines may have the ability to augment the antimicrobial activities of substrates of the *B. pseudomallei* BpeAB-OprB and AmrAB-OprB multidrug efflux pumps, such as aminoglycosides and macrolides. Phenothiazines are a class of drugs used as antihistamines and antipsychotics that have also been shown to have modest antimicrobial activities against an array of microorganisms and possibly inhibit drug efflux pumps. They found that, in the presence of phenothiazines, the MICs for numerous macrolides, fluoroquinolones, and amoxicillin-clavulanate were reduced as much as 8,000-fold. In addition, phenothiazines in combination with a subinhibitory concentration of erythromycin protected human lung epithelial cells and macrophages from *B. pseudomallei* infection and cytotoxicity, thus illustrating the role of drug efflux in *B. pseudomallei* antimicrobial resistance and pathogenesis.

Another 2007 study by Karunakaran and Puthucheary (49) investigated the *in vitro* susceptibility of 80 Malaysian *B. pseudomallei* isolates to some antibiotics commonly used to treat melioidosis as well as some new antimicrobials using the E-test method. Their findings supported the *in vitro* efficacies of imipenem, meropenem, and TMP-SMZ against *B. pseudomallei* but also established that moxifloxacin, ertapenem, and azithromycin cannot be recommended for the treatment of melioidosis.

In 2008, Sivalingam et al. (50) utilized an *in vivo* infection system to assess the efficacies of pre- and post-exposure oral antibiotic prophylaxis in the treatment of inhalational *B. pseudomallei* infection. The antibiotics evaluated in this study included amoxicillin-clavulanate, doxycycline, and TMP-SMZ. Their results illustrated the usefulness of doxycycline and TMP-SMZ in preventing acute disease and establishment

of chronic disease, revealed the ineffectiveness of amoxicillin-clavulanate, and suggested that TMP-SMZ may be an effective oral antibiotic as both pre- and post-exposure prophylaxis of *B. pseudomallei* infection.

Finally, Cheng et al. (51) performed a pharmacokinetic simulation study, using time-kill assays that they conducted and other parameters gathered from published literature, to evaluate the adequacies of the dosing regimens of TMP-SMZ for the eradication phase of melioidosis treatment in Thailand and Australia. Their simulation model predicted that the eradication program currently used in Australia would achieve serum concentrations of both drugs high enough to be effective but the regimen formerly used in Thailand would not. However, the new weight-based regimen recently implemented in Thailand would be expected to be effective against most strains but more studies are needed to optimize TMP-SMZ dosing in Thai populations.

Review of immunomodulatory therapies that have been evaluated for *B. pseudomallei* treatment:

The use of immunomodulatory therapies to fight off pathogens by enhancing the body's own natural defense mechanisms is not necessarily a new idea, but the use of these therapies against *B. pseudomallei* is relatively recent. The major reasoning behind this area of research seems to be that since antibiotic therapy can often be ineffective in the treatment of melioidosis and there is no effective vaccine, an alternative approach is needed. The non-specific activation of innate immunity by immunotherapeutic administration represents one such approach.

In 2003, Powell et al. (52) published their research on the use of granulocyte colony stimulating factor (G-CSF) for the treatment of acute disseminated melioidosis in a murine infection model. G-CSF is a growth factor produced by monocytes, macrophages, fibroblasts, stromal cells, and endothelial cells that serve multiple functions, including stimulation of mitogenesis and differentiation of committed stem cells into mature polymorphonuclear leukocytes, including neutrophils; mobilization of mature cells into circulation; enhancement of chemotaxis, phagocytosis, and oxidative activity; and exertion of anti-inflammatory effects on monocytes by reducing the release of pro-inflammatory cytokines while augmenting the release of anti-inflammatory mediators. G-CSF therapy is hypothesized to aid in the treatment of melioidosis because functional neutrophil defects are a risk factor for this infection. This particular study was inspired by a previous study in which the survival of a subgroup of patients in the study who had respiratory failure and septic shock due to *B. pseudomallei* (6 out of 36 patients) was radically enhanced from 5% to 100% by the use of recombinant human G-CSF treatment. While that previous study assessed G-CSF therapy for non-specific community-acquired septic shock, Powell et al. (52) looked at the effectiveness of recombinant murine G-CSF, both as a monotherapy and as an adjunct to antibiotic therapy with ceftazidime, for the treatment of *B. pseudomallei* infection specifically. Unfortunately, their results suggested that immunotherapy using G-CSF would be of limited benefit because it offered no added advantage with regard to survival or splenic bacterial burdens when used as a pre-treatment or when combined with ceftazidime in their murine infection model.

Despite these discouraging results, several more studies have been published since the initial 2003 report further pursuing the use of G-CSF therapy in the treatment of melioidosis. For example, Cheng et al. (53) performed a retrospective study comparing the mortality rates of melioidosis patients during the period of 1989 to 2002. This period encompassed an evaluation of patients from before and after the introduction of G-CSF therapy as an adjunct treatment for melioidosis septic shock at the Royal Darwin Hospital in Australia in December 1998. The purpose of this study was to determine if G-CSF therapy had, in fact, improved or affected mortality in melioidosis patients in any way. They found that the mortality rate had decreased from 95% to 10% since the introduction of G-CSF therapy, while other changes in the management of septic shock were not able to account for the magnitude of benefit observed, leading the authors to conclude that G-CSF may have contributed to the reduction in mortality. Cheng et al. (54) followed up the previous study with one in which they set out to explore the mechanism of action of G-CSF in the treatment of melioidosis. To do this, they employed an *in vitro* whole blood assay where they collected blood from human volunteers who were confirmed to be negative for *B. pseudomallei* antibody via the indirect hemagglutination assay, co-incubated whole blood and sera with recombinant human G-CSF, inoculated G-CSF-treated and untreated whole blood and sera with *B. pseudomallei*, and determined bactericidal activity of the whole blood and sera by calculating and comparing intracellular *B. pseudomallei* burdens at 0 and 60-90 minutes PI. Their results indicated no significant improvements in bactericidal activities of samples following co-incubation with G-CSF, including those from volunteers with chronic renal failure and diabetes mellitus who should theoretically have functional neutrophil defects. Thus, augmentation

of bactericidal activity of blood is likely not the primary mechanism of action through which G-CSF operates in *B. pseudomallei* infection. However, the authors also recognized that the *in vitro* whole blood assay model may not be suitable for investigating *B. pseudomallei* due to several inherent properties of the organism.

Another immunotherapeutic approach to the treatment of *B. pseudomallei* infection involves the administration of immunostimulatory unmethylated CpG oligodeoxynucleotides (ODNs). Unmethylated CpG motifs are a component of bacterial DNA and unmethylated CpG ODNs, synthetic bacterial CpG motifs, have been shown to induce protective immunity in several chronic infectious diseases. They are also known to be potent stimulators of macrophages, dendritic cells, NK cells, and lymphocytes and to preferentially promote the development and differentiation of Type 1 helper T cells (55). In a series of *in vitro* studies in 2002 and 2003, Utaisincharoen et al. (56, 57) demonstrated that CpG ODNs could activate rapid production of iNOS and NO in mouse macrophages as well as amplify phagocytosis of *B. pseudomallei* by mouse macrophages. In a follow-up investigation in 2004, the same group established that CpG ODNs are also capable of conferring protection against *B. pseudomallei* in a mouse model of infection, as evidenced by 90 to 100% survival in treated animals. They achieved this protection by administering CpG ODNs to mice intramuscularly between 2 and 10 d prior to low-dose i.p. *B. pseudomallei* challenge. The bacteremia and cytokine production kinetics observed in this study suggested that protection by CpG ODNs against acute septicemic melioidosis may be associated with a reduction of bacterial loads and interference with the potentially immunopathological effect of excessive proinflammatory cytokine production that often occurs as a result of *B. pseudomallei* replication (55).

In another *in vivo* study, Goodyear et al. (37) successfully protected mice against pneumonic *B. pseudomallei* and *B. mallei* infections by utilizing an inhaled immunotherapeutic. This immunotherapeutic, called cationic liposome-DNA complexes (CLDC), potently and rapidly induces innate immune defenses and in a manner similar to CpG ODNs. When administered mucosally 24 h before or concurrently with bacterial challenge, CLDC were capable of producing complete or nearly complete protection against high-dose inhalational challenge with *B. pseudomallei* and *B. mallei*, as evidenced by increased survival, decreased organ bacterial burdens, and decreased organ pathology in CLDC-treated mice. CLDC-mediated protection was determined to be largely dependent upon IFN-γ production within the lung tissues, partially dependent upon the activation of NK cells, and, surprisingly, independent of iNOS induction. Taken together, these results indicated that mucosal immunotherapy administered at the appropriate time may generate effective non-specific protection against pneumonic *B. pseudomallei* and *B. mallei* infections.

Yet another experimental approach to immunomodulatory therapy in the treatment of *B. pseudomallei* infection involves the administration of exogenous recombinant cytokines. Thus far, this approach has mostly only been investigated *in vitro*. For example, IFN-β is a pro-inflammatory cytokine produced mainly by macrophages infected with or exposed to microbial pathogens and possesses potent antimicrobial functions. This cytokine is thought to enhance iNOS and NO production by mediating the production of interferon regulatory factor 1 (IRF-1), a transcriptional activator that must bind an IRF-1 binding site within the iNOS gene promoter in order to induce iNOS in murine macrophages. However, *B. pseudomallei*-infected macrophages

fail to produce IFN-β at concentrations high enough to stimulate iNOS expression (12). Recall that iNOS induction is necessary for the production of NO, a potent regulator of survival and replication of intracellular bacteria. Utaisincharoen et al. (12) discovered that treatment of mouse macrophages with exogenous IFN-β at the same time as infection with *B. pseudomallei* resulted in enhanced iNOS expression by the infected macrophages as well as reduced intracellular bacterial burdens. Loss of this enhanced antimicrobial activity in the presence of L-NAME, an iNOS inhibitor, indicated that NO was responsible for the intracellular killing of *B. pseudomallei* by macrophages in this study.

In addition, as previously mentioned, exogenous recombinant IFN-γ is another cytokine that has been evaluated for the treatment of melioidosis. Miyagi et al. (27) prestimulated murine macrophages (J774.1) with recombinant IFN-γ for 24 h prior to inoculation with *B. pseudomallei* and found that intracellular bacterial growth was inhibited in a dose-dependent manner. In their assay, IFN-γ-induced microbicidal activity strongly correlated with NO production by the activated macrophages. Similar results were obtained from a different *in vitro* study, in which murine macrophages (RAW 264.7) were pretreated with IFN-γ for 12 h prior to *B. pseudomallei* infection. Once again, IFN-γ activation of macrophages resulted in increased expression of iNOS and, thus, increased NO production, which lead to decreased intracellular survival and growth of the organism (58).

Finally, a more recent approach to immunomodulatory treatment of *B. pseudomallei* infection has involved the pharmacological induction of autophagy in infected cells. Autophagy is an innate immune process by which intracellular bacteria can be targeted for killing. Invading pathogens first become sequestered within

autophagosomes, which then fuse with lysosomes to form autophagolysosomes. Enzymes that were contained within the lysosome become liberated and ultimately cause destruction of the autophagosomal contents. This process has been shown to play an important role in cellular defenses against intracellular pathogens such as *Streptococcus pyogenes* and *Mycobacterium tuberculosis*, but others have developed the ability to actively evade autophagy and survive within host cells. In an *in vitro* study in which autophagy was induced pharmacologically by rapamycin in murine macrophages and mouse embryo fibroblasts prior to infection with *B. pseudomallei*, the bacteria were more frequently found to be sequestered within autophagosomes than in cells in which autophagy had not been induced or that were autophagy-deficient. As a result, intracellular survive of *B. pseudomallei* was reduced in these autophagic cells. The conclusion drawn from these results was that *B. pseudomallei* is one such intracellular pathogen that is capable of evading autophagy but selective induction of autophagy in infected cells or targeting of the proteins that allow *B. pseudomallei* to avoid autophagic killing may represent novel therapeutic strategies against this pathogen (59).

Use of immuno-antimicrobial therapy for fungal and bacterial infections:

With the increasing incidence of life-threatening fungal and bacterial infections, limited ability of antimicrobials to sterilize infected organs of these life-threatening infections, rising emergence of antimicrobial resistance, and growing tendency of antimicrobials to cause side effects due to their properties or the high doses required, immuno-antimicrobial therapy has been a growing area of research in modern times. Immuno-antimicrobial therapy, or the simultaneous administration of an

immunotherapeutic and an antimicrobial, has been evaluated in the treatment of fungal and bacterial infections for years. The idea is that it may become possible to treat challenging infections not successfully treated with antimicrobial agents alone by modulating host immune responses to pathogens with immunotherapeutic and, in turn, increasing the efficacies of antimicrobials and improving clinical outcomes.

Immunotherapy for invasive fungal infections has been an area of significant research and clinical interest since hyperimmune equine immunoglobulin was first used to successfully treat patients with cryptococcal meningitis in the early 1900s. Since then, many effective antifungals have been developed, but complex host disorders producing compromised immune states have also emerged and created a great need for immunotherapy in conjunction with traditional antifungal therapy. Numerous approaches to immunotherapeutic intervention in the treatment of fungal infections have been evaluated, such as recombinant G-CSF-elicited granulocyte transfusions, cytokine growth factors, proinflammatory cytokines including IFN-γ and IL-12, immunoglobulin therapy, and active immunization (60). Extensive *in vitro* and *in vivo* preclinical data have been obtained that support the use of cytokines as an adjunct to antifungal drug therapy against invasive candidiasis infections. In these infections, cytokines are used to modulate anticandidal host defenses; since affected individuals are often immunocompromised or critically ill and functional phagocytes are vital for a positive clinical outcome (61).

The effectiveness of immuno-antimicrobial therapy has also been shown in systemic infection with the fungus, *Cryptococcus neoformans*, which is an important opportunistic pathogen in individuals with HIV/AIDS (62-64). Similar to invasive candidiasis, cell-mediated immunity is critical in the outcome of systemic cryptococcosis.

Clemons et al. (62) investigated the therapeutic potential of IL-12 in the treatment of cryptococcosis because it stimulates IFN-γ production by T cells and NK cells, which then activates macrophages important for host resistance against cryptococcosis. They found that mice infected i.v. with *C. neoformans* and then treated for 10 d with either subcutaneous (s.c.) low- or high-dose IL-12 in combination with fluconazole had markedly reduced levels of brain and liver infection compared to IL-12 or fluconazole alone. Thus, IL-12 enhanced the efficacy of fluconazole for the treatment of cryptococcosis.

Herrmann et al. (63) and Lutz et al. (64) described *in vitro* and *in vivo* systems, respectively, in which the efficacy of rIFN-γ was investigated in combination with the conventional antifungal, amphotericin B. The *in vitro* system utilized murine macrophages to determine the intracellular antifungal activity of this combination of immuno-antimicrobial therapy. They found that macrophages activated with rIFN-γ prior to infection with *C. neoformans* and then treated with amphotericin B had an increased ability to eliminate intracellular organisms. The enhanced activity of amphotericin B was found to be due to the synthesis of RNI by IFN-γ-primed macrophages (63). The *in vivo* system evaluated the effectiveness of rIFN-γ in combination with amphotericin B in a murine model of systemic cryptococcosis. They challenged mice i.v. with *C. neoformans* and treated with rIFN-γ beginning either 7 d prior to infection or after infection, amphotericin B beginning post-challenge, or combinations of treatments. Similar to the above study, they found that rIFN-γ significantly augmented the ability of amphotericin B to reduce *C. neoformans* infection. This was evidence by decreased yeast burdens in several organs, the most important of which was the brain. In addition, this therapeutic

combination was effective following both non-lethal and lethal *C. neoformans* challenges and by both i.v. and s.c. administration of rIFN-γ. However, prophylactic treatment with IFN-γ did not offer any added benefit (64). Both of these studies concluded that IFN-γ may have a role in potentiating the effects of conventional antifungal therapy in the treatment of cryptococcosis.

Much like with fungal infections, research in area of immuno-antimicrobial therapy for the treatment of bacterial infections appears to have started out as a way to improve the clinical outcomes of patients with compromised immune states, in which chemotherapeutic approaches alone were ineffective. As a result, very similar immuno-antimicrobial combinations have been evaluated for the treatment of bacterial infections as for fungal infections. For example, Doherty and Sher (65) investigated the simultaneous administration of antibiotics and recombinant IL-12 for the treatment of opportunistic *Mycobacterium avium* infections in AIDS patients, who tend to be T cell-deficient. They found that *M. avium*-infected SCID mice treated with IL-12 had decreased splenic bacterial loads, but when combined with the antimycobacterial drugs, clarithromycin or rifabutin, lower doses of IL-12 were required to decrease splenic pathogen burdens even more significantly. In fact, the decrease in bacterial numbers observed with the immuno-antimicrobial therapy was significantly greater than those resulting from the administration of either the cytokine or the drug alone. A similar synergistic effect was observed when infected immunocompetent mice were treated with IL-12 and antimycobacterials.

Immuno-antimicrobial therapy has also been assessed against clinical isolates of drug-resistant *Enterococcus faecalis*, an organism that has increasingly caused serious

systemic infections in hosts with weakened immune defenses. Typically this bacterium has been susceptible to the synergistic bactericidal effects of a β-lactam or vancomycin combined with an aminoglycoside, but it has progressively developed resistance to these and other antimicrobial agents. This multidrug-resistant bacterium has presented a major therapeutic challenge, thus an alternative treatment that combined IFN-γ with more traditional therapeutics, vancomycin and gentamicin, has been explored. Onyeji et al. (66, 67) performed as series of *in vitro* and *in vivo* studies evaluating this combination therapy. They found that addition of IFN-γ to vancomycin, gentamicin, or a combination of the two in an *in vitro* system was associated with a significant enhancement of anti-enterococcal activity compared with the effects of any of the agents used alone. The observed anti-enterococcal activity was largely attributable to an activated secretion of microbicidal ROIs by the neutrophils used in the assay. Their *in vivo* system showed very similar results in mice challenged i.p. with gentamicin- and vancomycin-resistant *E. faecalis* and then treated following inoculation with subcutaneous IFN-γ, antibiotic, or a combination of both agents.

Pammit et al. (68) demonstrated the synergistic effect of i.n. treatment with IL-12 and gentamicin in promoting the clearance of pulmonary *Francisella tularensis* subsp. *novicida* infection. *F. tularensis*, like *B. pseudomallei*, is a highly virulent facultative intracellular bacterium that is a potential biological warfare agent and, when inhaled, causes disease that is usually fatal without extensive medical treatment. Their theory was that treatment strategies that directly target the respiratory mucosa may be more effective against pulmonary disease. They challenged mice i.n. with a lethal dose of *F. tularensis* and then treated those mice with i.n. gentamicin, IL-12, or both. They found that IL-12

and gentamicin were each effective alone at promoting bacterial clearance and extending the time to death but did not prevent death, while combination therapy with IL-12 and gentamicin significantly enhanced survival in infected mice. In addition, this synergy was highly dependent upon the production of endogenous IFN-γ and probably also involved the activation of NK cells.

Following a typhoon-related flood in southern Taiwan in 2005, a report exists detailing the successful treatment of a patient with life-threatening melioidosis using recombinant human activated protein C (rhAPC) in combination with meropenem (69). In this case, the patient presented mild symptoms that rapidly progressed to bacteremic pneumonia with acute respiratory distress syndrome, septic shock, and multiple organ dysfunction. He was admitted to the ICU and administered parenteral amoxicillin-clavulanate, a commonly used antimicrobial against standard community-acquired pneumonia in that region of the world. However, his condition continued to deteriorate so his antibiotic was changed to a combination of meropenem and minocycline to achieve better coverage against some less common pathogens that can cause life-threatening community-acquired pneumonia. Due to the persistence of this patient's shock status, rhAPC, which has been reported to reduce the mortality of patients with severe septic shock caused by various other pathogens, was added to the antimicrobial therapeutic regimen. After rhAPC treatment was initiated, the patient's condition began to gradually improve and he was discharged from the hospital after a three week stay. He continued to receive maintenance therapy trough the hospital's outpatient department. This was the first reported case of rhAPC administration in life-threatening melioidosis (69). Keep in mind that this report included the treatment of only a single patient and it was not a

controlled study. Therefore, this patient's survival cannot be definitively attributed to an interaction between the antibiotics and rhAPC administered.

In 2007, Goswami and Jawali (70) had previously discovered that glutathione (GSH), an antioxidant, reduced the antibacterial activity of fluoroquinolone and aminoglycoside antibiotics against *E. coli* so they set out to determine if the activities of other antibiotics against *E. coli* were affected by GSH. They chose to investigate two β-lactam antibiotics, ampicillin and penicillin, and found that the presence of GSH increased the susceptibility of *E. coli* to these antibiotics, as evidenced by significantly decreased MICs. They also found that glutathione in the oxidized state, called GSSG, also augments the activities of ampicillin and penicillin against *E. coli* to the same extent as GSH. The authors concluded that since β-lactams are such valuable antibiotics in the treatment of many infections, further investigations on this topic are warranted to potentially improve treatment regimens.

As previously mentioned, Powell et al. (52) investigated the use of G-CSF as an adjunct to antimicrobial therapy with ceftazidime in the treatment of melioidosis but found that if offered no benefit in their murine infection model. In 2007, Cheng et al. (71) approached this idea again and performed a randomized, placebo-controlled trial that assessed the efficacy of G-CSF administration in ceftazidime-treated patients in Thailand suspected to have severe sepsis caused by melioidosis. Their investigation revealed that, while mortality rates were similar in both the G-CSF- and placebo-treated groups, the duration of survival was significantly longer for patients who received G-CSF than for those who received placebo. As a result, Cheng et al. (71) concluded that G-CSF therapy may "buy time" for severely septic patients but intensive management of sepsis-

associated metabolic abnormalities and organ dysfunction is more likely to improve survival long-term.

Chapter 2: Use of an *in vitro* infection model to evaluate the effectiveness of immuno-antimicrobial therapy in the treatment of *B. pseudomallei* infection.

Introduction:

The difficulties of treating *B. pseudomallei* infections have been well-established. *B. pseudomallei* infection is often fatal without prompt antimicrobial therapy and even with appropriate therapy, many patients are still at risk for relapse of disease or reinfection with *B. pseudomallei*. To even have a chance at clearing the *B. pseudomallei* organism, infected persons are prescribed high doses of potent antimicrobials for prolonged periods of time. The current treatment protocol for melioidosis requires 20 weeks of drug therapy. However, not all antimicrobials can be used in the treatment of *B. pseudomallei* infection as this organism possesses numerous mechanisms by which it is highly resistant to many commonly used antibiotics. In addition, there is currently no effective vaccine available to prevent infection by *B. pseudomallei*. Not only does this organism pose a considerable threat in the areas in which it is endemic, but there is also substantial concern with regard to this organism in the United States, where it is not found naturally, due to its extraordinary potential as a biological weapon.

As a result, newer therapeutic regimens must be developed in order to reduce the mortality rate, duration of treatment, doses of antimicrobials, relapse rate, and emergence of additional antimicrobial resistance. Previously discussed studies have demonstrated the potential of immunotherapeutics that non-specifically activate the innate immune system, such as CpG ODNs and CLDC, in the treatment of *B. pseudomallei* infection

(37, 55). Additional studies that were discussed earlier, while they exhibited variable effectiveness, suggested that there might be potential for the use of immuno-antimicrobial therapies in the treatment of *B. pseudomallei* infection (52, 69, 71). Numerous other studies previously discussed, however, have more definitively shown the effectiveness of immuno-antimicrobial therapy in the clearance of other bacterial pathogens (65-68, 70). Based on these results, we hypothesized that immunotherapy, particularly with an immunotherapeutic capable of stimulating potent IFN-γ release, combined with antimicrobial therapy might be a more effective therapy than either agent alone for the treatment of *B. pseudomallei* infection.

We investigated this hypothesis using an *in vitro* infection model that utilized alveolar macrophages. Alveolar cells were chosen for this infection system to simulate pulmonary *B. pseudomallei* infection, which would be the most likely site of infection if the organism were deliberately released as an aerosol in a biological attack and inhaled (72). Macrophages were selected because they are known to be an important target cell for *B. pseudomallei* infection *in vivo* (27, 58, 73). Ceftazidime was chosen as the antimicrobial for these studies because it is currently a first-line antimicrobial in the treatment of naturally-acquired *B. pseudomallei* infection and it has been evaluated extensively in experimental infection systems. CLDC-elicited cytokines were selected as the immunotherapeutic in these studies, as prior investigations have shown CLDC to be potent inducers of innate immunity and IFN-γ production (74). Alveolar macrophages were infected with *B. pseudomallei*; cultured with ceftazidime, cytokine-rich CLDC supernatants, or both; and intracellular bacterial burdens were compared among the treatment groups. A synergistic increase in the activity of ceftazidime was observed

when combined with CLDC-elicited cytokines *in vitro* and this synergistic increase was determined to be mediated primarily by IFN-γ.

Materials and Methods:

Bacterial strain and culture:

Burkholderia pseudomallei strain 1026b was used in these studies and was kindly provided by Herbert Schweizer, Colorado State University. *B. pseudomallei* 1026b was originally isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue, joint, and spleen involvement and has been studied extensively in the laboratory (20). All procedures involving *B. pseudomallei* 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.

Frozen stocks of *B. pseudomallei* 1026b of known titers were prepared from cultures grown in Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA) by freezing the cultures at -80°C in LB medium containing 15% glycerol. Inocula for *in vitro* infections with *B. pseudomallei* were prepared by thawing a vial of frozen stock and diluting in complete cell culture medium (described below) to the desired concentration.

Cell culture:

The mouse alveolar macrophage cell line, AMJ2 (American Type Tissue Collection, Manassas, VA), was used to investigate the ability of immuno-antimicrobial therapy to inhibit intracellular infection by *B. pseudomallei in vitro*. Harvested cells were cultured at a concentration of 2×10^5 cells per well of 24-well plates or at a concentration of 2×10^6 cells per ml in 15 ml conical centrifuge tubes in complete cell culture medium

without antibiotics until the time of infection. Complete cell culture medium consisted of minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Invitrogen), 1× nonessential amino acids (Invitrogen), and 0.075% sodium bicarbonate (EMD Science, Gibbstown, NJ).

Production of control and Cationic Liposome-DNA Complex (CLDC) spleen supernatants:

Mice were injected intravenously (i.v.) in the lateral tail vein with 200 µl of either phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) or cationic liposome-DNA complexes (CLDC) to produce control or CLDC supernatants, respectively. CLDC were prepared as previously described (74). Briefly, equimolar amounts of DOTIM (octadecanoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride] and cholesterol were combined and extruded through a series of filters, with a final filter diameter of 200 nm, to produce sterile complexes of cationic liposomes. Plasmid DNA was isolated from *E. coli* DH5α using the Qiagen Endo-free Giga kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The plasmid used for the CLDC was a non-coding, ultra-low endotoxin eukaryotic expression plasmid, as previously described (74). CLDC were formed just prior to injection by gently mixing cationic liposomes at 10% v/v with plasmid DNA at 100 µg/ml in Tris-buffered 5% dextrose water (D5W; pH 7.4) to final volume.

Once injected, the mice were sacrificed 3 h later. At that time, their spleens were harvested and the spleen cells were placed to culture in nutrient media overnight. The resulting spleen supernatants were collected and stored by freezing aliquots at -80°C until the time of use. Enzyme-linked immunosorbant assay (ELISA) showed that the control

supernatants contained undetectable concentrations of interferon-γ (IFN-γ), while the CLDC supernatants contained high concentrations of IFN- γ, approximately 3 ng/ml.

Infection of cells:

Adherent AMJ2 cells in triplicate wells of 24-well plates were infected with *B. pseudomallei* at a multiplicity of infection (MOI) of 5 for 1 h in 400 µl complete cell culture medium at 37°C and 5% CO_2. Non-adherent AMJ2 cells were infected in 15 ml conical centrifuge tubes with *B. pseudomallei* in 1 ml complete cell culture medium, with all other conditions the same. A low MOI was chosen to more closely simulate a realistic pulmonary inoculum in the event of a deliberate release of aerosolized organism. Extracellular bacteria were removed by washing the cells three times with PBS, followed by treatment with complete cell culture medium plus 350 µg/ml kanamycin (Sigma-Aldrich) for 1 h. After incubation with kanamycin, the cells were washed three times with PBS and then cultured in triplicate wells of 24-well plates containing complete medium and the appropriate treatment depending on the experiment, as described below, for an additional 24 h.

Preparation of therapeutics and treatment of infected cells:

Ceftazidime. Ceftazidime (Sigma-Aldrich) stocks were prepared by reconstituting the powder at 10 mg/ml in PBS with 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Aliquots were frozen at -20°C until the time of use. Working concentrations of the drug were prepared fresh for each infection by thawing stock ceftazidime and diluting it to the desired concentration(s) in complete cell culture medium.

CLDC and control supernatants. Working concentrations of CLDC and control supernatants were prepared fresh by thawing stock supernatants of each and diluting them to the desired concentration(s) in complete cell culture medium.

Neutralizing antibodies. Neutralizing antibodies were used to determine the role of IFN-γ in generating the observed antibacterial activity of supernatants from CLDC-stimulated spleen cells. To do this, spleen supernatants were treated with 10 μg/ml anti-IFN-γ antibodies (clone R4.6A2) (eBioscience, San Diego, CA) or 10 μg/ml of irrelevant isotype-matched control antibodies (clone eBRG1) (eBioscience) at 4°C for 30 minutes prior to the addition of the supernatants to the cells.

Recombinant IFN-γ. Murine recombinant IFN-γ (rIFN-γ; Peprotech, Rocky Hill, NJ) was reconstituted to a concentration of 1 mg/ml in 10 mM sodium phosphate (Fisher Scientific, Fairlawn, NJ) in PBS and then diluted to 100 μg/ml in RPMI with 10% FBS, all according to the manufacturer's instructions. The reconstituted cytokine was then aliquoted and frozen at -20°C to be used as stock aliquots. Working concentrations of rIFN-γ were prepared fresh by thawing a vial of stock cytokine and reconstituting the contents to 1 μg/ml in PBS with 0.1% BSA. Further dilutions were performed in complete cell culture medium to achieve desired concentrations.

Cell treatment. For ceftazidime dose titration studies, ceftazidime was added to the infected macrophages at the indicated concentrations after bacterial infection and elimination of extracellular bacteria with kanamycin, approximately 1 h post-infection (PI). For spleen supernatant dose titration studies, supernatants from control or CLDC-stimulated spleen cells were added to infected cell cultures at the indicated dilutions at 1 h PI. For rIFN-γ dose titration studies, rIFN-γ was added to the macrophages at the

indicated concentrations at 1 h PI. For combination studies, to investigate the ability of CLDC-elicited cytokines to enhance the activity of antimicrobial drugs, ceftazidime, CLDC supernatants or rIFN-γ were added to the infected macrophages alone or in combination at 1 h PI. For neutralizing antibody studies, to assess the ability of anti-IFN-γ antibodies to influence the interaction between ceftazidime and CLDC supernatants, ceftazidime, CLDC supernatants or neutralized supernatants were added to the cell cultures alone or in combination at 1 h PI. All treatment groups denoted as "untreated" received complete cell culture medium at 1 h PI. In all studies, the macrophages were cultured for an additional 24 h after the addition of the therapeutics, at which time intracellular bacterial burdens were determined as described below.

Determination of intracellular B. pseudomallei burdens:

Following the 24-hour incubation with appropriate therapeutics, the treatment medium was removed from the cells and the cells were washed three times with 2 ml sterile PBS. The cells were then lysed with 1 ml sterile ddH_2O with 0.01% Triton X-100 (Sigma-Aldrich) in order to release the intracellular bacteria for quantitation. Serial dilutions of the lysates were performed in PBS and plated on LB agar (Becton, Dickinson, and Company, Sparks, MD) quad plates. The plates were incubated at 37°C for 48 hours and then colony growth was counted. The number of colonies counted was multiplied by the dilution factor for that particular quadrant to determine the total number of intracellular *B. pseudomallei* colony forming units (CFU), since the volume of the lysates was 1 ml. In several early experiments, bacteria present in the final PBS wash were quantitated in the same manner and subtracted from the cell lysate counts to control for any extracellular bacteria not removed by thorough washing. In all treatment groups,

the quantity of bacteria present in the last PBS wash was negligible compared to the quantity present in the cell lysates. Therefore, lysate counts accurately reflect intracellular bacterial burdens.

Statistical analyses:

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Intracellular *B. pseudomallei* (in Log_{10} CFU/well) were evaluated for statistical differences between groups. For experiments involving three or more treatment groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple means comparison test to compare significance among groups. For experiments involving combined therapies, a two-way ANOVA was performed, followed by Bonferroni post-tests, to determine if a synergistic interaction existed between the monotherapies when combined (75). Data were considered statistically significant for $P < 0.05$.

Results:

In order to determine if immunotherapy is capable of enhancing the effectiveness of ceftazidime therapy at inhibiting intracellular replication of *B. pseudomallei*, an *in vitro* alveolar macrophage (AMJ2) infection assay was utilized. Cytokine-rich supernatants generated from spleens of mice treated with CLDC *in vivo* were used as the immunotherapeutic in these studies, as spleen cells are a major source of cytokine production in CLDC-treated mice (74). Before the combination therapy could be evaluated, the individual effects of ceftazidime and CLDC supernatants on intracellular

B. pseudomallei replication and appropriate subtherapeutic concentrations of each had to be elucidated.

The effect of ceftazidime dose on inhibition of intracellular *B. pseudomallei* replication was determined by performing dose titration studies. AMJ2 cells were infected with *B. pseudomallei* for 1 h, treated with ceftazidime for 24 h, and then the numbers of intracellular bacteria were determined as described in Materials and Methods. The results of the dose titration studies demonstrated the dose-dependent nature of the inhibitory effects of decreasing concentrations of ceftazidime on intracellular *B. pseudomallei* replication (Figure 2.1). Concentrations of ceftazidime 25 μg/ml or greater very effectively inhibited *B. pseudomallei* replication in infected macrophages, while concentrations of 10 μg/ml or less failed to exhibit significant inhibition. The 10 μg/ml ceftazidime concentration was chosen to be the subtherapeutic antimicrobial dose used in future combination experiments because, as already mentioned, it did not significantly inhibit intracellular *B. pseudomallei* replication but it is well within the range of clinically achievable concentrations of ceftazidime *in vivo* (76).

Similar dose titrations were performed to determine the inhibitory effects of various dilutions of CLDC supernatant on intracellular replication of *B. pseudomallei*. Alveolar macrophages were treated with CLDC supernatants or supernatants from untreated control mice following infection with *B. pseudomallei* and intracellular bacteria were quantitated 24 h later. As Figure 2.2 illustrates, dilutions of CLDC supernatant at 1:5 to 1:100 and control supernatant at 1:5 significantly decreased intracellular *B. pseudomallei*. This effect diminished with greater dilutions of the CLDC supernatant. The 1:10 dilution of CLDC supernatant was chosen for use in future combination

experiments as the subtherapeutic dose of immunotherapeutic because that dilution most consistently produced partial reduction in intracellular *B. pseudomallei* numbers.

Once the subtherapeutic doses of ceftazidime and CLDC supernatant were selected, they were then combined to treat macrophages for 24 h following their infection with *B. pseudomallei*. When compared to each therapeutic agent alone, the two agents in combination produced synergistic inhibition of intracellular replication in *B. pseudomallei*-infected cells (Figure 2.3). To illustrate this finding, immuno-antimicrobial therapy consisting of CLDC supernatant and ceftazidime reduced intracellular *B. pseudomallei* numbers by greater than 4 Log_{10} compared to untreated cells, from 5.2×10^5 CFU to approximately 27 CFU, while the CLDC supernatant and ceftazidime monotherapies each only reduced intracellular *B. pseudomallei* burdens by about 1 Log_{10} compared to untreated cells, to 6.0×10^4 CFU and 7.9×10^4 CFU, respectively. Therefore, cytokines elicited by CLDC immunotherapy synergistically inhibited intracellular replication of *B. pseudomallei* when combined with subtherapeutic ceftazidime antimicrobial therapy.

We next wished to determine which cytokine present in the CLDC supernatant was primarily responsible for the observed synergistic inhibition of intracellular *B. pseudomallei* elicited by immuno-antimicrobial therapy *in vitro*. To do this, we pretreated CLDC supernatants with neutralizing antibodies and then repeated the *in vitro* combination therapy infection assay using these pretreated supernatants. Prior research had identified IFN-γ as a very important component of the CLDC-stimulated immune response, so we chose to focus the current study on neutralization of this cytokine (37, 74, 77). The neutralization of IFN-γ resulted in the elimination of antibacterial synergy

observed with immuno-antimicrobial therapy for *B. pseudomallei* infection of alveolar macrophages, therefore confirming IFN-γ to be the predominant cytokine responsible for the synergistic interaction with ceftazidime (Figure 2.4). Neutralization of TNF-α in CLDC supernatants, another cytokine released in response to CLDC administration, was also evaluated in this system but was found to have a negligible effect on the observed synergy between ceftazidime and CLDC supernatants (data not shown).

Having determined CLDC-elicited IFN-γ to be the primary mediator of the synergistic activity produced by our immuno-antimicrobial therapy, we then investigated the ability of recombinant murine IFN-γ to reproduce the effect of CLDC supernatant in the *in vitro* infection assay. But first we needed to determine the effect of rIFN-γ dose on inhibition of intracellular *B. pseudomallei* replication. Dose titration studies similar to those conducted for ceftazidime and CLDC supernatants were performed. Briefly, infected AMJ2 cells were treated with varying doses of rIFN-γ for 24 h following *B. pseudomallei* infection and then intracellular bacteria were quantitated. The results of the dose titration studies established that, much like with ceftazidime and CLDC supernatants, the inhibitory effects of decreasing concentrations of rIFN-γ on intracellular *B. pseudomallei* replication were dose-dependent (Figure 2.5). Concentrations of rIFN-γ 100 ng/ml or greater markedly inhibited *B. pseudomallei* replication in infected macrophages, while concentrations of 50 ng/ml or less failed to display significant inhibition. The 10 ng/ml rIFN-γ concentration was selected to be the subtherapeutic dose of immunotherapeutic used in this series of combination therapy experiments because it is within the range of clinically achievable and safe serum concentrations of IFN-γ *in vivo* (78).

Finally, the selected subtherapeutic dose of rIFN-γ was combined with 10 μg/ml ceftazidime to treat infected alveolar macrophages and the effect on intracellular *B. pseudomallei* replication was evaluated. Immuno-antimicrobial therapy consisting of rIFN-γ and ceftazidime evoked potent synergistic inhibition of *B. pseudomallei* replication *in vitro* (Figure 2.6). Intracellular bacterial counts were reduced by about 4 Log_{10}, from 1.98×10^6 CFU in untreated cells to 177 CFU in cells treated with both rIFN-γ and ceftazidime. Cells treated with the rIFN-γ and ceftazidime monotherapies only decreased intracellular *B. pseudomallei* loads by less than 1 Log_{10} compared to untreated cells, to 8.8×10^5 CFU in IFN-γ-treated cells and to 3.7×10^5 CFU in ceftazidime-treated cells. The synergistic inhibition of intracellular *B. pseudomallei* replication obtained with immuno-antimicrobial therapy consisting of rIFN-γ and ceftazidime was very similar to that observed with immuno-antimicrobial therapy consisting of CLDC supernatant and ceftazidime, thus, implying that rIFN-γ is capable of reproducing the effect of CLDC supernatant in immuno-antimicrobial therapy.

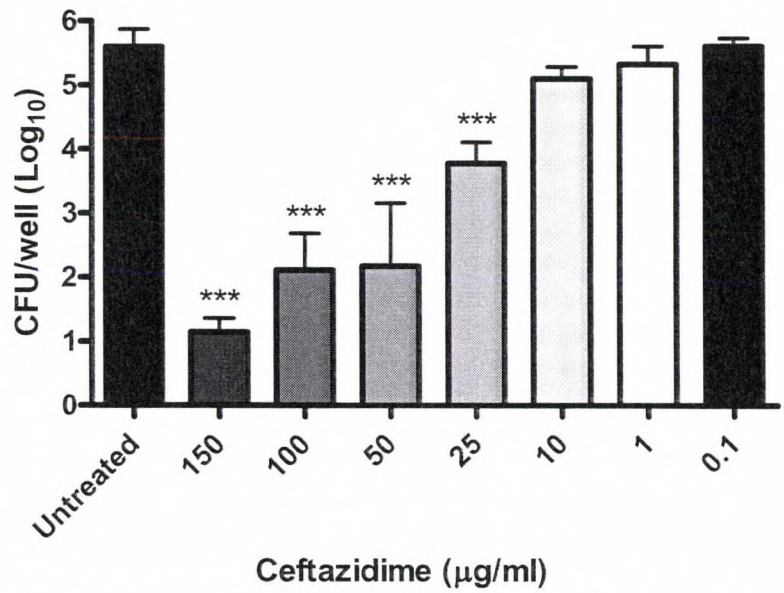

Figure 2.1: Ceftazidime elicits dose-dependent inhibition of intracellular *B. pseudomallei* replication *in vitro*. Alveolar macrophages (AMJ2) were infected with a low MOI of *B. pseudomallei* and then treated 1 h later with varying doses of ceftazidime. Intracellular *B. pseudomallei* were quantitated 24 h later. Ceftazidime treatment significantly reduced (***, $P < 0.001$) intracellular *B. pseudomallei* burdens in cultured alveolar macrophages, and this effect was dose-dependent.

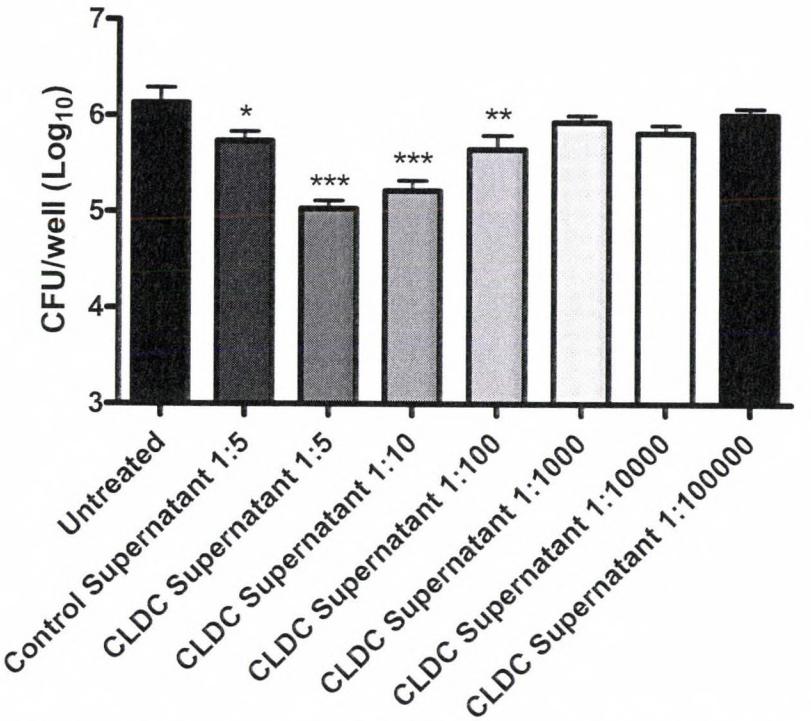

Figure 2.2: Spleen supernatant elicited by i.v. CLDC administration inhibits intracellular replication of *B. pseudomallei in vitro*. AMJ2 cells were infected with a low MOI of *B. pseudomallei* and then treated with dilutions of supernatant generated from CLDC-treated mouse spleen cells at 1 h PI. At 24 h after infection, cells were lysed and the numbers of intracellular *B. pseudomallei* organisms were determined. The treatment of AMJ2 cells with CLDC supernatant resulted in significant reductions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) in the numbers of intracellular *B. pseudomallei* organisms versus untreated cells. This effect diminished at higher dilutions of the supernatant.

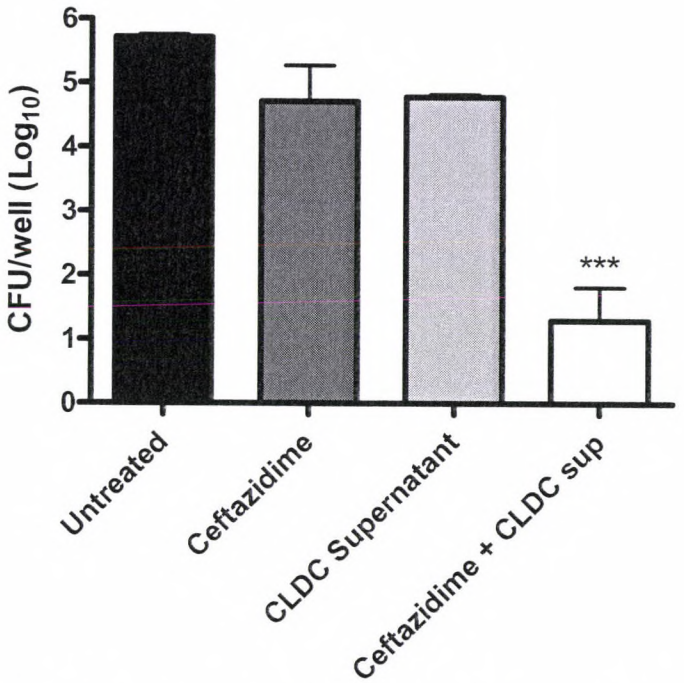

Figure 2.3: Immuno-antimicrobial therapy with CLDC supernatant and ceftazidime elicits synergistic inhibition of *B. pseudomallei* replication in alveolar macrophages *in vitro*. At 1 h after infection with a low MOI of *B. pseudomallei*, AMJ2 cells were left untreated or treated with ceftazidime at 10 ug/ml, a 1:10 dilution of CLDC supernatant generated by CLDC-treated mouse spleen cells, or both and cultured for 24 h, at which time cells were lysed and intracellular *B. pseudomallei* were quantitated. Synergistic reduction (***, $P = 0.0005$) in intracellular *B. pseudomallei* replication at 24 h PI was observed in cells treated simultaneously with both CLDC supernatant and ceftazidime.

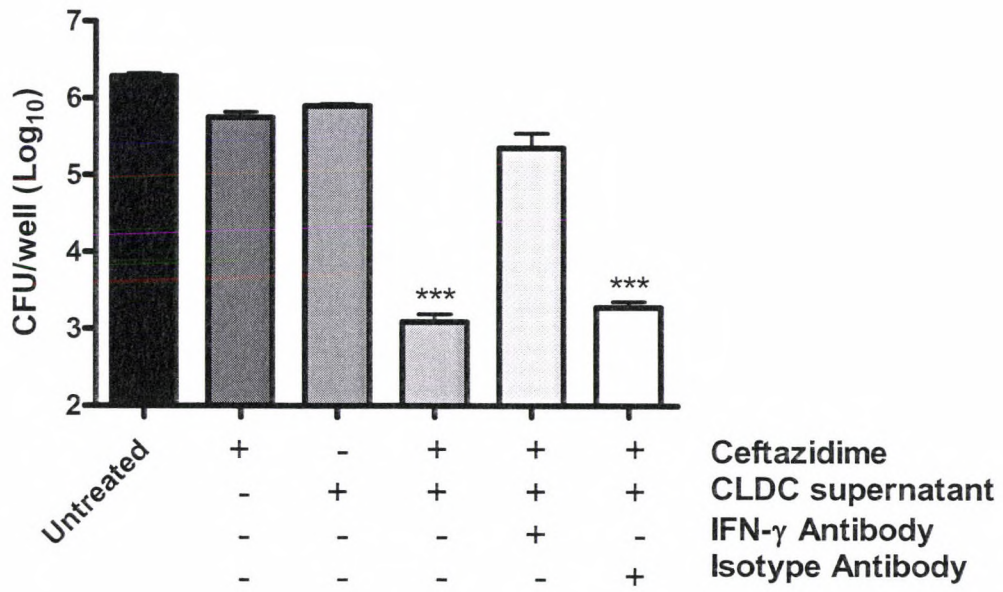

Figure 2.4: IFN-γ elicited by i.v. CLDC treatment is responsible for the synergistically inhibitory activity of immuno-antimicrobial therapy *in vitro*. AMJ2 cells infected with a low MOI of *B. pseudomallei* were left untreated or treated with ceftazidime, CLDC supernatant prepared as described previously, or both at 1 h post-infection. In addition, neutralizing antibodies were utilized to determine the role of IFN-γ in the inhibitory effect of CLDC supernatant in immuno-antimicrobial therapy. The neutralization of IFN-γ resulted in the elimination of antibacterial synergy (***, $P < 0.001$) observed with immuno-antimicrobial therapy for *B. pseudomallei* infection of alveolar macrophages.

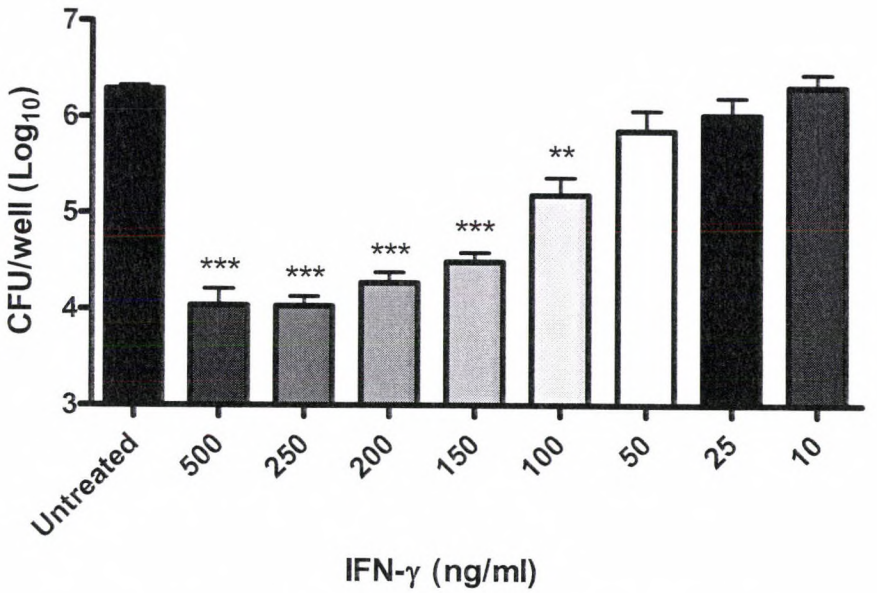

Figure 2.5: Recombinant IFN-γ elicits dose-dependent inhibition of intracellular *B. pseudomallei* replication in alveolar macrophages *in vitro*. AMJ2 cells were stimulated with varying concentrations of rIFN-γ at 1 h after infection with a low MOI of *B. pseudomallei*. Intracellular bacteria were quantitated at 24 h post-infection. The treatment of AMJ2 cells with 100ng/ml rIFN-γ or greater resulted in significant (**, $P < 0.01$; ***, $P < 0.001$) reduction in numbers of intracellular *B. pseudomallei* organisms versus untreated cells.

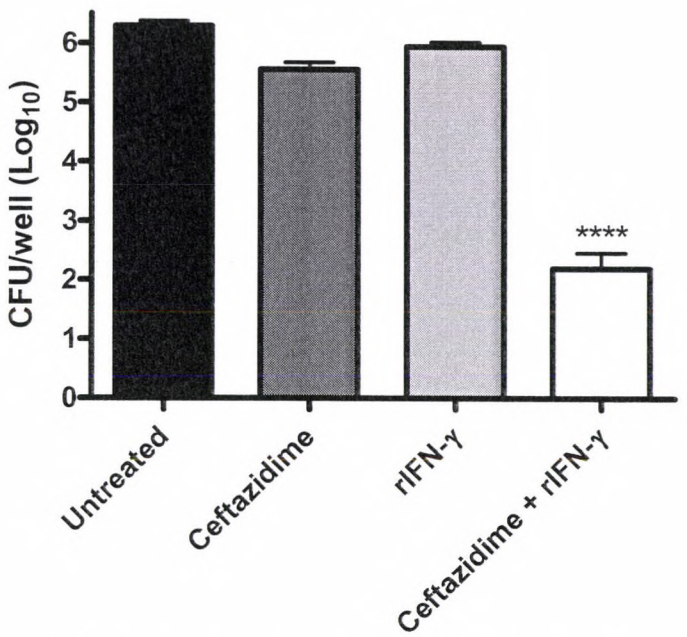

Figure 2.6: Combination therapy with ceftazidime and rIFN-γ synergistically inhibits intracellular *B. pseudomallei* replication in alveolar macrophages *in vitro*. AMJ2 cells were infected with *B. pseudomallei* at a low MOI and then left untreated or treated with ceftazidime at 10 μg/ml, rIFN-γ at 10 ng/ml, or both at 1 h PI. At 24 h PI, numbers of intracellular *B. pseudomallei* organisms were quantitated. Ceftazidime and rIFN-γ interacted synergistically (****, $P < 0.0001$) to reduce intracellular *B. pseudomallei* infection.

Discussion:

Due in large part to *B. pseudomallei*'s intrinsic resistance to many antimicrobials, treatment of naturally-occurring melioidosis is undeniably challenging. Consequently, it is reasonable to speculate that infections resulting from an intentional release of *B. pseudomallei*, should it ever occur, would also be difficult to treat. For the benefit of those who suffer from melioidosis in endemic areas each year and for the safety of those who might be at risk of exposure should an intentional release ever be attempted, more effective treatment options for *B. pseudomallei* infection are needed. In this chapter, we presented a new approach to the treatment of such infections, i.e. CLDC immunotherapy combined with ceftazidime antimicrobial therapy, and demonstrated its remarkable potential in an *in vitro* macrophage infection system. We found that IFN-γ elicited by CLDC administration was capable of interacting with ceftazidime, an antibiotic used routinely in the treatment of melioidosis, to produce a synergistic inhibition of *B. pseudomallei* infection of macrophages.

As seen in Figure 2.2, a 1:5 dilution of control supernatant, which came from spleen cells harvested from non-CLDC-treated mice, yielded a significant inhibition of intracellular *B. pseudomallei* replication. While this result was unexpected, it was not unreasonable. The spleens used to generate the control supernatants were collected from intact biological systems (mice) and, therefore, may have had residual cytokines in the tissues that had been previously produced in response to minor immunological insults. These low levels of cytokine may also have been produced and/or released due to cellular injury during tissue processing to harvest individual cells for culture. Dilutions of control supernatant greater than 1:5 failed to significantly reduce intracellular *B. pseudomallei*

burdens and none of the control supernatant dilutions generated a synergistic interaction with ceftazidime against *B. pseudomallei* (data now shown), indicating that the quantities of any residual cytokines that may have been present in the control supernatants were insufficient to interact with ceftazidime and, as a result, were negligible in our *in vitro* combination therapy trials.

As previously described, Figure 2.6 illustrates the ability of rIFN-γ to reproduce the effect of CLDC supernatant in immuno-antimicrobial therapy for *in vitro B. pseudomallei* infection. However, the synergy observed with rIFN-γ and ceftazidime is slightly less dramatic than that observed with CLDC supernatant and ceftazidime. This difference could be attributable to the effects of other cytokines in addition to IFN-γ likely present in the CLDC supernatant, since CLDC has been shown to stimulate the production of multiple cytokines (74).

A similar series of experiments to the ones described in this chapter were performed with *B. mallei* as well. The same *in vitro* infection model utilizing AMJ2 alveolar macrophages was implemented. Not surprisingly, due to the close relatedness of the two pathogens, very similar results were obtained. Dose-dependent effects of ceftazidime, CLDC supernatants, and rIFN-γ on *in vitro* inhibition of intracellular bacterial burdens comparable to those observed in *B. pseudomallei* were observed in *B. mallei*. However, *B. mallei* was found to be more sensitive to the antibacterial effects of ceftazidime (data not shown). This is not surprising, as the MIC_{90} of ceftazidime for *B. mallei* is 8 μg/ml, while that of *B. pseudomallei* is > 64 μg/ml (40). In addition, *B. mallei* displayed mildly increased susceptibilities to in inhibitory effects of spleen CLDC supernatant and rIFN-γ compared to *B. pseudomallei* in our *in vitro* infection system

(data not shown). Perhaps these two susceptibilities are related, as IFN-γ is a major cytokine produced in response to CLDC stimulation (74). We also observed the inhibition of *B. mallei* infection in macrophage cultures with supernatants from the lungs of CLDC-stimulated mice but the effect was much less potent than that with spleen supernatants so we did not pursue this any further (data not shown). Finally, IFN-γ was determined to be the CLDC-elicited cytokine primarily responsible for the inhibition of intracellular *B. mallei* replication *in vitro* as well (data not shown).

Given the profound improvement in intracellular *B. pseudomallei* inhibition observed when CLDC supernatant and IFN-γ were combined with low-dose ceftazidime in our alveolar macrophage infection system; this combination of immuno-antimicrobial therapy warranted further investigation. The appropriate next step was to implement a murine model of pulmonary *B. pseudomallei* infection in which to confirm an *in vivo* role for this therapeutic regimen in the treatment of melioidosis. Such investigations are presented in the following chapter.

Chapter 3: *In vivo* assessment of the effectiveness of immuno-antimicrobial therapy for treatment of pulmonary *B. pseudomallei* infection.

Introduction:

Once we discovered that CLDC-elicited cytokines, particularly IFN-γ, were capable of augmenting the anti-*B. pseudomallei* activity of ceftazidime in an *in vitro* infection model, we wished to determine if immuno-antimicrobial therapy would also be effective *in vivo*. To do this, we utilized a murine model of acute pulmonary *B. pseudomallei* infection. The efficacy of CLDC immunotherapy against multiple pulmonary pathogens, including *F. tularensis* and *B. pseudomallei*, has been previously established (37, 77). However, both of these studies used mucosally-administered CLDC to induce protection against pulmonary infection by these pathogens in mice. In the present study, we chose to administer all treatments to mice i.p. even though our goal was to protect from pulmonary *B. pseudomallei* challenge. The reason i.p. administration was preferred was to enable us to administer larger volumes of each therapeutic and to administer both therapeutics in the same biological space where they would potentially have a greater chance of interacting and stimulating a powerful systemic response.

To assess the efficacy of immuno-antimicrobial therapy in a murine model, mice were infected with *B. pseudomallei* and then treated with ceftazidime, CLDC, or both beginning at 6 h after challenge. The mice were evaluated for survival of acute pulmonary disease and organ bacterial burdens were also assessed. Our investigations

revealed that *in vivo* treatment with ceftazidime and a potent IFN-γ-inducing immunotherapeutic, such as CLDC, generates significant protection against lethal pulmonary *B. pseudomallei* infection.

Materials and Methods:

Mice:

Female BALC/c mice were used in this study (Jackson Laboratories, Bar Harbor, ME). All mice were 8 to 12 weeks of age at the time of infection and were housed under specific pathogen-free conditions. Protocols for these experiments were approved by the Animal Care and Use Committee (ACUC) at Colorado State University.

Preparation of B. pseudomallei stocks and animal infections:

B. pseudomallei 1026b was used in these studies and was kindly provided by Herbert Schweizer, Colorado State University. All procedures involving *B. pseudomallei* were performed in a BSL-3 facility, in accordance with select agent regulations and with Institutional Biosafety Committee oversight at Colorado State University.

Frozen stocks of *B. pseudomallei* 1026b were cultured and prepared as described in the Materials and Methods section for Chapter 2. Inocula for *in vivo* infections with *B. pseudomallei* were prepared by thawing a vial of frozen stock and diluting in sterile PBS to the desired challenge dose. The LD_{50} dose of *B. pseudomallei* in BALB/c mice by the intranasal (i.n.) route was previously determined by the Reed-Muench method to be 900 CFU (37). The targeted challenge dose for these *in vivo* studies was $8 \times LD_{50}$ or approximately 7500 CFU. The actual challenge dose was confirmed by retrospective plating on LB agar.

Prior to infection, mice were anesthetized by intraperitoneal (i.p.) injection with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) and 10 mg/kg xylazine (Ben Venue Laboratories, Bedford, OH) solution prepared in sterile water. Mice were infected with *B. pseudomallei* i.n. using a total volume of 20 µl of inoculum delivered in alternating nostrils. Thus, each nostril received approximately 10 µl of the inoculum.

Preparation and administration of antibiotics:

Ceftazidime treatments were prepared by reconstituting the drug at the desired concentration(s) in PBS with 0.1% BSA. Aliquots were drawn up into 1 ml tuberculin syringes (Becton, Dickinson, and Company, Franklin Lakes, NJ), capped, and frozen at -80°C until the time of use. For survival studies, ceftazidime treatments were administered to mice i.p. beginning at 6 h post-challenge and continuing every 12 hours for three days (a total of 7 treatments administered at 6, 18, 30, 42, 54, 66, and 78 h after infection). For organ bacterial burden studies, ceftazidime treatments were administered to mice i.p. beginning at 6 h post-challenge and continuing every 12 hours until the mice were sacrificed at 48 h post-challenge (a total of 4 treatments administered at 6, 18, 30, and 42 h after infection).

Preparation and administration of CLDC:

CLDC were prepared as previously described in Chapter 2 Materials and Methods. Preformed CLDC were diluted in Tris-buffered 5% D5W to the indicated concentrations. A one-time treatment was administered to mice i.p. in a total volume of 200 µl at 6 h post-challenge.

Preparation and administration of rIFN-γ:

Murine rIFN-γ was reconstituted, aliquoted, and stored as previously described (Chapter 2 Materials and Methods). For *in vivo* treatments, rIFN-γ stocks were diluted in PBS with 0.1% BSA to the indicated concentrations. Treatments were administered to mice i.p. in a total volume of 200 µl at 6 h and 18 h post-challenge. All *in vivo* studies involving rIFN-γ were performed by Katie Propst and Ryan Troyer.

Combination therapy treatment groups:

For immuno-antimicrobial studies, combined therapy (i.e. ceftazidime with CLDC or rIFN-γ) was compared to each of its monotherapy components by looking at survival and organ bacterial burdens. Untreated mice received no treatment after challenge with *B. pseudomallei*. Ceftazidime-treated mice received 25 mg/kg ceftazidime i.p. at each treatment interval, as described above. CLDC-treated mice received a one-time administration of 20 µl CLDC i.p., as described above. Recombinant IFN-γ-treated mice received two i.p. injections of 3×10^3 IU rIFN-γ, as described above. Combined therapy-treated mice received 25 mg/kg ceftazidime i.p. at the dose intervals described above in addition to either one i.p. injection of 20 µl CLDC or two i.p. injections of 3×10^3 IU rIFN-γ at the treatment times described above.

Determination of survival:

For all survival studies, infected mice were observed twice daily for disease symptoms and humanely euthanized when they reached pre-determined endpoint criteria. Endpoint criteria included anorexia, loss of $\geq$ 15% of their pre-challenge body weight, severe dehydration, CNS signs (e.g. head tilt, circling, paralysis), nonresponsiveness,

respiratory distress, and splenic lesions. Mice that had not been euthanized by day 7 post-challenge were considered to have survived acute *B. pseudomallei* infection.

Determination of bacterial burdens in tissues:

Viable *B. pseudomallei* were quantitated in lungs, livers, and spleens of infected mice at 48 h post-challenge to determine tissue bacterial burdens. To do this, lungs, livers, and spleens were aseptically removed and homogenized in 5 ml sterile PBS using a Stomacher 80 Biomaster (Seward, Bohemia, NY). Serial 10-fold dilutions of the organ homogenates were performed in sterile PBS and plated on LB agar quad plates. The plates were incubated at 37°C for 48 h, at which time the number of colonies were enumerated. The number of colonies counted was multiplied by the dilution factor for that particular quadrant and then multiplied by 5 to determine the total *B. pseudomallei* burden for that organ, since the volume of the homogenates was 5 ml. The individual tissue bacterial burdens were expressed as CFU/organ. The limit of detection for determination of bacterial burdens in organ homogenates was 50 CFU/organ.

Statistical analyses:

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows. Survival and tissue bacterial burdens were evaluated for statistical differences between groups. For experiments involving three or more treatment groups, a one-way ANOVA was performed, followed by Tukey's multiple means comparison test to compare significance among groups. Data were considered statistically significant for $P < 0.05$, except in survival studies where Bonferroni's correction factor was taken into account. To determine Bonferroni's correction factor, the significance cutoff value of P

< 0.05 was divided by the total number of relevant comparisons made within the survival study. The resulting value then became the new significance cutoff value (P).

Results:

The purpose of these studies was to determine if immuno-antimicrobial therapy consisting of CLDC and ceftazidime is effective against pulmonary *B. pseudomallei* infection *in vivo*. Much like for the *in vitro* system used to assess immuno-antimicrobial therapy against *B. pseudomallei*, each of the components had to be evaluated individually before they could be combined. Assessing the monotherapeutics individually allowed us to measure the effectiveness of each when used alone against *B. pseudomallei* infection in an *in vivo* system as well as to determine the appropriate subtherapeutic doses of each to be administered in combination therapy studies. Efficacy of a therapeutic was established based on percent survival of the animals receiving that treatment through acute pulmonary *B. pseudomallei* challenge.

The effect of ceftazidime dose on survival of mice acutely infected with *B. pseudomallei* was determined by performing antibiotic dose trials. BALB/c mice were infected i.n. with approximately $8 \times LD_{50}$ (approximately 7500 CFU) of *B. pseudomallei* and treated i.p. with 0, 50, 100, or 200 mg/kg ceftazidime beginning at 6 h PI. Ceftazidime administration was repeated every 12 h for a total of 7 treatments. The mice were observed for survival throughout the acute infection period. All of the ceftazidime doses assessed in these trials generated significant protection of mice against pulmonary *B. pseudomallei* infection compared to untreated mice, with 200 mg/kg, 100 mg/kg, and 50 mg/kg resulting in 100%, 67%, and 25% survival, respectively (Figure 3.1). Our goal

in choosing a subtherapeutic ceftazidime dose was to select a dose that would consistently protect ≤ 20% of acutely infected mice. Since the 50 mg/kg ceftazidime dose evaluated in these trials was still able to protect 25% of acutely infected mice, we chose 25 mg/kg as our subtherapeutic ceftazidime dose for use in future *in vivo* immuno-antimicrobial studies.

Dose titration trials were conducted in a similar manner in order to determine the effect of CLDC dose on mouse survival. For these trials, BALB/c mice were infected i.n. with approximately 8 × LD_{50} *B. pseudomallei* and administered a single i.p. injection of 10, 50, or 100 µl CLDC at 6 h PI. We believed that a single dose of CLDC would be sufficient to stimulate an adequate immune response, as previous research has shown that CLDC-mediated immune stimulation persists for a relatively long period of time (37). Figure 3.2 shows that both the 100 µl and 50 µl i.p. CLDC doses protected 25% of mice from acute death compared to untreated mice, while the 10 µl dose failed to offer any protection. As a result, we chose 20 µl administered i.p. at 6 h PI as our subtherapeutic CLDC dose to be used in upcoming immuno-antimicrobial studies, with the goal of protecting ≤ 20% acutely infected mice.

Now that we had selected our subtherapeutic doses of ceftazidime and CLDC, we combined them to determine if combination therapy was capable of yielding enhanced protection in mice against lethal pulmonary *B. pseudomallei* infection compared to each therapeutic alone. Combination therapy trials were conducted by challenging BALB/c mice i.n. with approximately 8 × LD_{50} *B. pseudomallei* and then treating 6 h later i.p. with low-dose ceftazidime, CLDC, or both. As in the monotherapy trials, ceftazidime treatment was repeated every 12 h from the initial injection for a total of 7 treatments and

CLDC was administered as a one-time treatment. Immuno-antimicrobial therapy consisting of CLDC and low-dose ceftazidime completely protected mice from acute pulmonary infection with *B. pseudomallei*, while survival rates for ceftazidime and CLDC monotherapies were only 20% and 0%, respectively (Figure 3.3). While CLDC monotherapy failed to protect any mice from death, it did prolong their survival time from 2.5 d to 4 d. These results lead us to conclude that CLDC immunotherapy significantly enhanced the efficacy of low-dose ceftazidime for the treatment of lethal pulmonary *B. pseudomallei* infection.

Having observed the profound effect of combined CLDC and ceftazidime therapy on survival of *B. pseudomallei*-infected mice we next wanted to determine if this immuno-antimicrobial therapy also had an effect on bacterial burdens in the organs of infected mice. To do this, mice were inoculated i.n. with *B. pseudomallei* and treated as described for the combination therapy survival studies. Mice were then euthanized 48 h PI, at which time their lungs, livers, and spleens were harvested and bacterial burdens in these organs were quantitated. Combined CLDC and ceftazidime therapy elicited significant reductions in bacterial burdens in all three organs analyzed compared to untreated mice and the CLDC and ceftazidime monotherapies (Figure 3.4). Thus, immuno-antimicrobial therapy inhibited intracellular *B. pseudomallei* replication in these organs much like what was observed in the *in vitro* studies described earlier in this report. It is also possible that spleen and liver bacterial numbers were decreased because the combined therapy inhibited dissemination of *B. pseudomallei* to these organs (79).

Since CLDC and ceftazidime were found capable of interacting in the *in vivo* infection system to increase survival and decrease organ bacterial burdens of mice

challenged with lethal *B. pseudomallei*, we next were interested in determining if, like in the *in vitro* infection system, rIFN-γ was capable of producing the same effects *in vivo* when combined with ceftazidime. Once again, we first had to perform rIFN-γ dose trials in mice to determine the most appropriate dose and dosing interval to use in our combination studies. Mice were challenged i.n. with a lethal dose of *B. pseudomallei* and then treated i.p. with 10^3, 3×10^3, 5×10^3, or 10^4 IU rIFN-γ at 6 h PI and again 12 h later. We chose to administer two injections of rIFN-γ, instead of one injection as with CLDC, because the immune stimulatory effects of rIFN-γ are not as prolonged as with CLDC. Each of the rIFN-γ doses used in this study significantly increased survival time of *B. pseudomallei*-infected mice versus untreated mice, but the high dose of 10^4 IU rIFN-γ administered at 6 h and 18 h was the only treatment able to protect any of the mice from death (Figure 3.5, Data courtesy of Katie Propst and Ryan Troyer). Since 80% of the mice receiving the 10^4 U dose survived, we decided that a lower dose that would protect fewer mice would be more appropriate for the combined therapy studies. As a result, we chose 3×10^3 IU administered at 6 h and 18 h PI as our subtherapeutic rIFN-g dose to use in future immuno-antimicrobial therapy studies.

Our final investigation involved combining our subtherapeutic dose of rIFN-γ with the low-dose ceftazidime used in earlier *in vivo* combined therapy studies. As mentioned previously, the purpose of this investigation was to determine if rIFN-γ, like CLDC, is capable of increasing the effectiveness of ceftazidime in an *in vivo* infection system and protecting mice against lethal pulmonary *B. pseudomallei* infection. Just as with previous *in vivo* combined therapy studies, mice were infected i.n. with approximately $8 \times LD_{50}$ *B. pseudomallei* and then treated i.p. with low-dose ceftazidime,

rIFN-γ, or both beginning at 6 h PI. Ceftazidime treatment was repeated every 12 h for a total of 7 treatments and a second dose of rIFN-γ was administered at 18 h PI. Compared to untreated mice, 80% of mice receiving immuno-antimicrobial therapy consisting of rIFN-γ and low-dose ceftazidime survived acute pulmonary *B. pseudomallei* infection, while only 20% of mice receiving rIFN-γ or ceftazidime monotherapies survived (Figure 3.6, Data courtesy of Katie Propst and Ryan Troyer). The protection observed with this immuno-antimicrobial therapy was significantly enhanced when compared to untreated mice and was nearly significantly enhanced when compared to mice receiving rIFN-g or ceftazidime monotherapies when the Bonferroni correction factor is taken into account. Nevertheless, the trend illustrates that immuno-antimicrobial therapy with rIFN-g and low-dose ceftazidime increases survival in mice following acute inhalational challenge with *B. pseudomallei*. Thus, suggesting that IFN-γ is the CLDC-elicited cytokine largely responsible for the antibacterial activity of immuno-antimicrobial therapy *in vivo* as well. While immuno-antimicrobial therapy with rIFN-γ and ceftazidime was not as effective as immuno-antimicrobial therapy with CLDC and ceftazidime, rIFN-γ could also be used to effectively augment the antibacterial activity of ceftazidime and provide protection against *B. pseudomallei* infection.

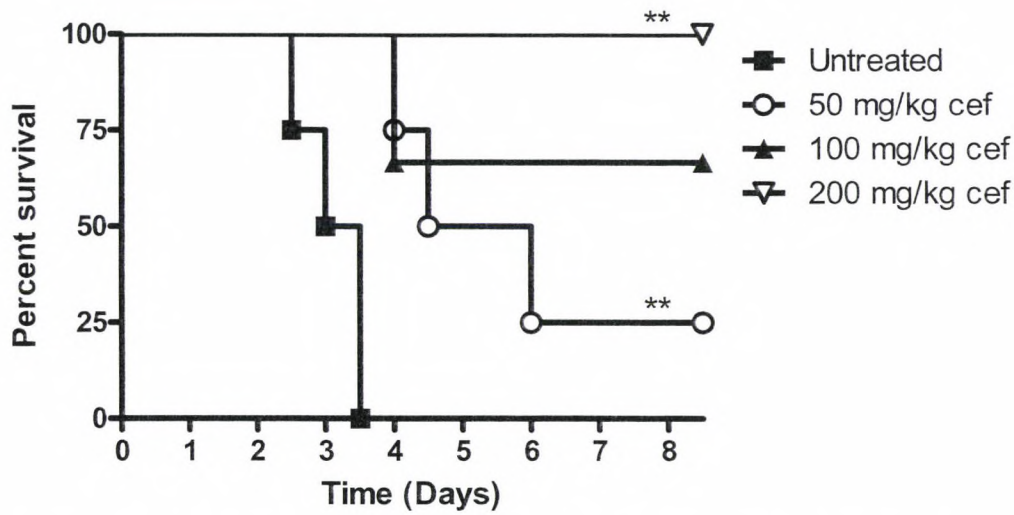

Figure 3.1: *In vivo* ceftazidime (cef) dose titration. BALB/c mice (n = 4 per group) were infected i.n. with 4000 CFU *B. pseudomallei* and then left untreated or treated i.p. with various doses of ceftazidime beginning at 6 h post-challenge and continuing every 12 h for 3 days (a total of 7 treatments). The mice were observed twice daily and humanely euthanized when they reached endpoint criteria. Since the 50 mg/kg ceftazidime dose assessed in this trial was able to significantly increase (**, P = 0.0091) survival of *B. pseudomallei*-infected mice, we chose 25 mg/kg as our subtherapeutic ceftazidime dose to use in future immuno-antimicrobial therapy studies.

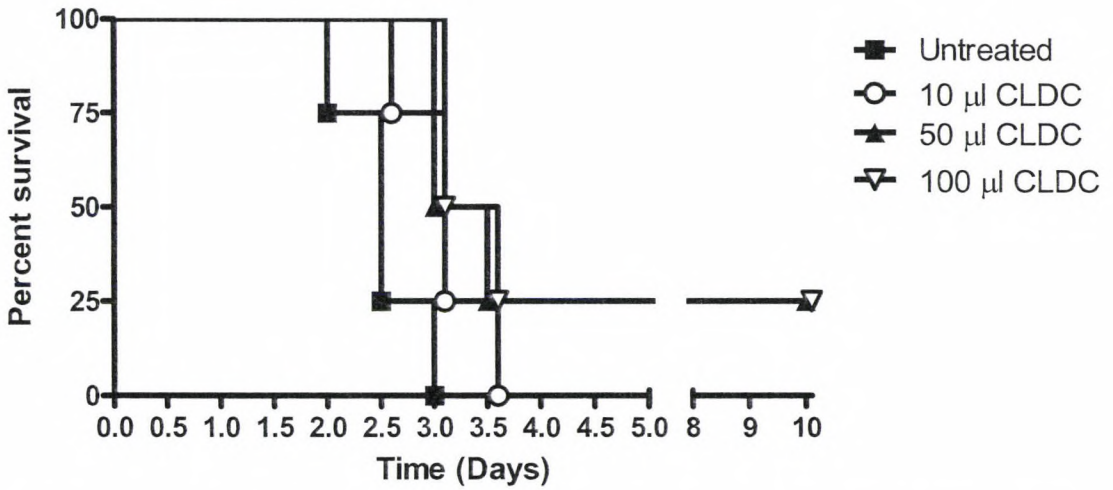

Figure 3.2: *In vivo* CLDC dose titration. BALB/c mice (n = 4 per group) were infected i.n. with 7500 CFU *B. pseudomallei* and then left untreated or administered a one-time i.p. treatment of various doses of CLDC at 6 h post-challenge. CLDC were formed just prior to injection and containted 10% v/v cationic liposomes and 100 μg/ml plasmid DNA. The mice were observed twice daily and humanely euthanized when they reached endpoint criteria. Each of the CLDC doses used in this study failed to significantly increase survival of *B. pseudomallei*-infected mice once the Bonferroni correction was taken into account (cutoff, P = 0.01). However, both the 50 μl and 100 μl treatments protected one mouse each from acute death (ns, P = 0.0311), while the 10 μl treatment failed to protect any mice. As a result, we chose 20 μl as our subtherapeutic CLDC dose to use in future immuno-antimicrobial therapy studies.

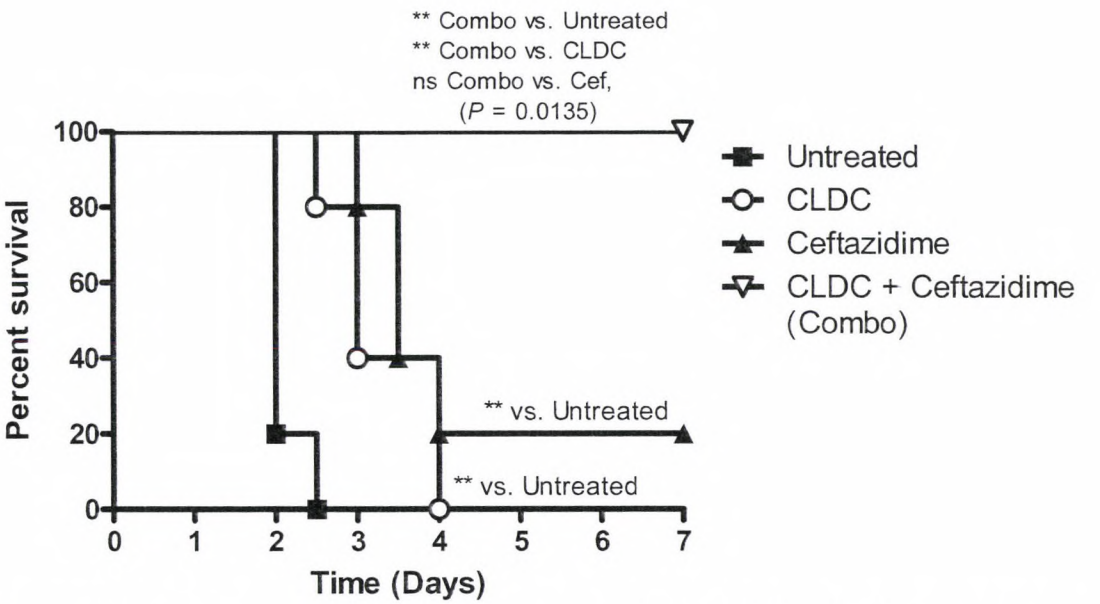

Figure 3.3: Immuno-antimicrobial therapy with CLDC and ceftazidime protects mice from acute death due to inhalational *B. pseudomallei* infection. BALB/c mice (n = 5 per group) were infected i.n. with 7500 CFU *B. pseudomallei* and then left untreated or treated i.p. with 20 μl CLDC, 25 mg/kg ceftazidime, or both at 6 h post-challenge. Ceftazidime treatments continued every 12 h after the initial injection for 3 days (a total of 7 treatments). The mice were observed twice daily and humanely euthanized when they reached endpoint criteria. Immuno-antimicrobial therapy offers enhanced survival over no treatment and CLDC monotherapy (**, P = 0.0016 and 0.0026, respectively). The increase in survival observed with immuno-antimicrobial therapy is nearly significant versus ceftazidime monotherapy (ns, P = 0.0135) when the Bonferroni correction is taken into account (cutoff, P = 0.01).

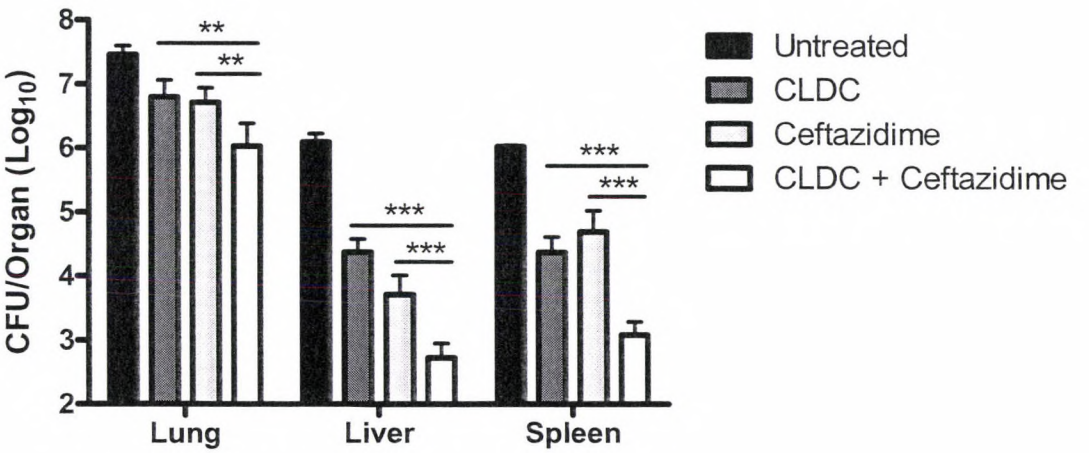

Figure 3.4: Immuno-antimicrobial therapy with CLDC and ceftazidime significantly reduces bacterial burdens in lungs, livers, and spleens of mice following inhalational challenge with *B. pseudomallei*. BALB/c mice (n = 5 per group) were challenged intranasally with 8000 CFU *B. pseudomallei* and then left untreated or treated with CLDC, ceftazidime, or both beginning at 6 h post-challenge. Ceftazidime treatments continued every 12 h after the initial injection for 2 days (a total of 5 treatments). At 48 h post-*B. pseudomallei* challenge, the mice were humanely euthanized and lungs, livers, and spleens were harvested for tissue bacterial burden determinations. Combined CLDC and ceftazidime therapy elicited significant reductions (**, $P < 0.01$; ***, $P < 0.001$) in bacterial burdens versus the component monotherapies in all three organs analyzed.

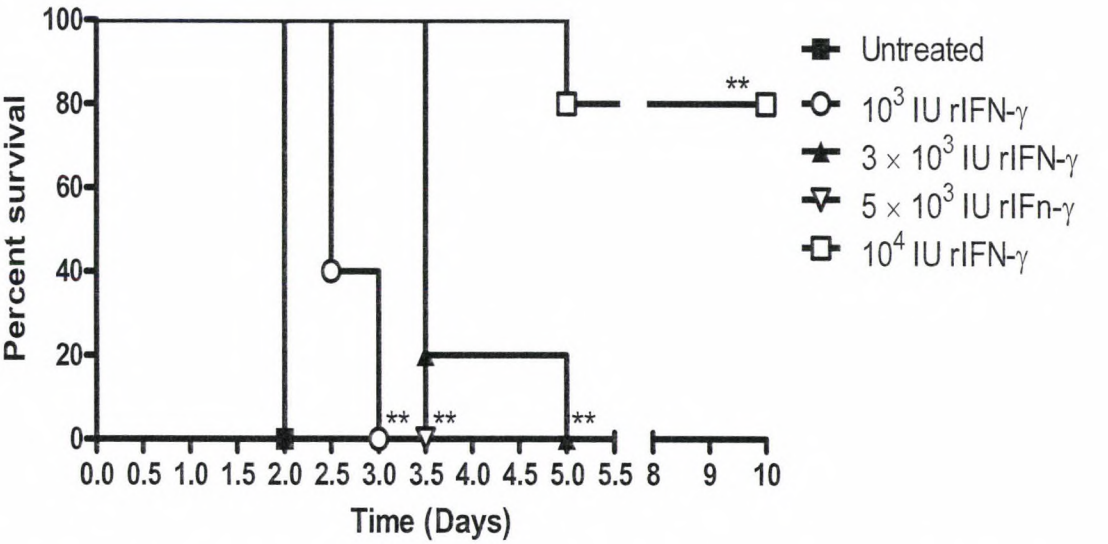

Figure 3.5: *In vivo* recombinant IFN-γ dose titration. BALB/c mice were infected i.n. with 8700 CFU *B. pseudomallei* and then left untreated or treated i.p. with different doses of rIFN-γ at 6 h post-challenge and again 12 h later. The mice were observed twice daily and humanely euthanized when they reached endpoint criteria. Each of the rIFN-γ doses used in this study significantly increased (**, $P = 0.0027$) survival time of *B. pseudomallei*-infected mice versus untreated mice, though the 10^4 IU rIFN-γ dose was the only treatment to protect any of the mice from death. As a result, we chose to use two injections of 3×10^3 IU as our subtherapeutic rIFN-γ dose to use in future immuno-antimicrobial therapy studies. Data courtesy of Katie Propst and Ryan Troyer.

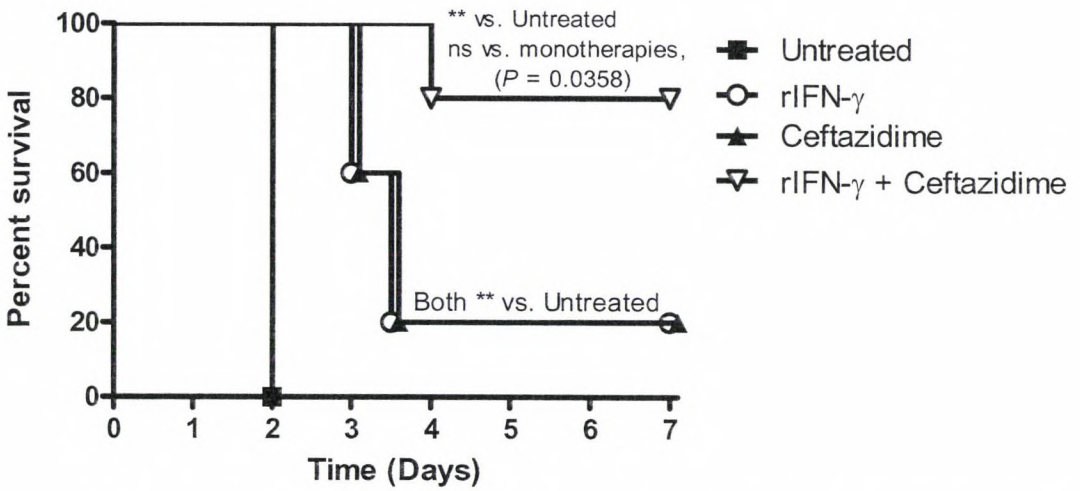

Figure 3.6: Recombinant IFN-γ therapy is capable of partially supplementing the activity of CLDC in immuno-antimicrobial therapy *in vivo*. BALB/c mice (n = 5 per group) were infected i.n. with 7800 CFU *B. pseudomallei* and then sham treated i.p. with PBS or treated i.p. with 3×10^3 IU rIFN-γ, 25 mg/kg ceftazidime, or both at 6 h post-challenge. The rIFN-γ treatment was repeated at 18 h post-challenge and the ceftazidime treatments continued every 12 h after the initial injection for 3 days (a total of 7 treatments). The mice were observed twice daily and humanely euthanized when they reached endpoint criteria. Immuno-antimicrobial therapy offers enhanced survival over no treatment (**, P = 0.0027). The increase in survival observed with immuno-antimicrobial therapy is nearly significant versus rIFN-γ and ceftazidime monotherapies (ns, P = 0.0358) when the Bonferroni correction factor is taken into account (cutoff, P = 0.01). Immuno-antimicrobial therapy with rIFN-γ and ceftazidime increases survival in mice following acute inhalational challenge with *B. pseudomallei*, thus suggesting that IFN-γ is the CLDC-elicited cytokine responsible for the antibacterial activity of immuno-antimicrobial therapy *in vivo*. Data courtesy of Katie Propst and Ryan Troyer.

Discussion:

Having first shown the efficacy of immuno-antimicrobial therapy with CLDC and ceftazidime *in vitro*, we have now confirmed its relevance *in vivo*. This is not the first report in which the effectiveness of antimicrobial therapy has been augmented by the adjunct administration of immunotherapy. As described previously, both IL-12 and IFN-γ have been used as adjunct therapy to the conventional antifungals, amphotericin B and fluconazole, and have been shown to increase their efficacies in the treatment of the fungal infection, cryptococcosis (62-64). Immuno-antimicrobial therapy with various immunotherapeutics and antibiotics has also previously been shown to be effective in the treatment of bacterial infections with *M. avium* (65), *E. coli* (70), *E. faecalis* (66, 67), and *F. tularensis* (68). A series of earlier studies in *B. pseudomallei* that looked at using rG-CSF as an adjunctive therapy to antibiotics showed inconsistent results. A retrospective study in human melioidosis patients suggested that G-CSF therapy in addition to standard antimicrobial therapy dramatically decreased mortality (53). However, an *in vitro* study showed that G-CSF did not increase bactericidal activity of human blood against *B. pseudomallei*, even in specimens from volunteers with neutrophil deficiencies (54). Additionally, an *in vivo* study failed to show a benefit to survival or bacterial inhibition in *B. pseudomallei*-infected mice from G-CSF therapy, alone or in combination with ceftazidime (52). Finally, a human study found that while G-CSF therapy adjunctive to ceftazidime treatment offered longer duration of survival in melioidosis patients, mortality rates were not improved in the end (71). Owing to these inconsistent and generally discouraging results, G-CSF therapy has been deemed ineffective in the treatment of melioidosis. Therefore, this is the first report that we are aware of in which

immuno-antimicrobial therapy has been successfully applied in the treatment of acute *B. pseudomallei* infection both *in vitro* and *in vivo*. In our studies, IFN-γ produced in response to CLDC stimulation clearly enhanced the effectiveness of ceftazidime against *B. pseudomallei* infection and improved overall treatment success.

The mechanism by which CLDC and ceftazidime interact to produce synergistic inhibition of *B. pseudomallei* replication is currently unknown but is under investigation. Previous studies have shown the importance of ROI and RNI, especially NO, in host defense against *B. pseudomallei* infection (27, 29, 30). In addition, numerous studies have demonstrated iNOS and NO upregulation in response to immunotherapy with CpG ODN (55-57), IFN-β (12), and IFN-γ (27, 58), which lead to greater control of *B. pseudomallei* infection. Even though a recent study in our lab showed that, while IFN-γ is critical for the protection elicited by mucosal CLDC against pneumonic *Burkholderia* infection, iNOS induction is not involved (37), we thought that this mechanism might be valid in the protection observed with combination therapy involving CLDC. Our hypothesis was that the RNI and/or ROS induced by IFN-γ increase the susceptibility of *B. pseudomallei* to the bactericidal activity of ceftazidime. However, preliminary *in vitro* experiments involving inhibition of iNOS, and subsequent NO production, with aminoguanidine and inhibition of ROS with several commonly used ROS inhibitors suggest that RNI and ROS are not involved, as synergy was not abolished (R. Troyer, unpublished data). These findings are supported by a series of *in vitro* and *in vivo* studies by Breitbach et al. (80), in which *iNOS*$^{-/-}$ and *gp91phox*$^{-/-}$, a gene necessary for a functional NADPH oxidase, mice and bone marrow macrophages were used to show that

iNOS- and ROS-independent killing mechanisms are responsible for combating infection with *B. pseudomallei*, even in the presence of IFN-γ stimulation.

The synergistic effects of cytokine and antimicrobial combinations have been previously attributed to the enhanced uptake of antimicrobial (53, 68, 71), therefore, another potential mechanism involves increased intracellular concentrations of ceftazidime as a result of increased permeability to the drug in IFN-γ-treated macrophages. Preliminary data evaluating intracellular ceftazidime concentrations following treatment with CLDC or rIFN-γ via high performance liquid chromatography imply that this also is not the mechanism, as intracellular ceftazidime concentrations were not increased in IFN-γ-treated cells (R. Troyer, unpublished data).

Building upon known actions of IFN-γ, we also explored the possible role of GSH in our observed synergy, as IFN-γ is an inducer of GSH (81). In preliminary *in vitro* experiments, GSH exhibited bactericidal activity against *B. pseudomallei* in an MIC-type setup. However, the GSH concentrations necessary to inhibit *B. pseudomallei* replication were greater than what is physiologically achievable. The addition of GSH to ceftazidime in an alveolar macrophage infection system was unable to reduce the concentration of GSH needed to produce inhibition of intracellular *B. pseudomallei* replication to a physiologically achievable level and a synergistic interaction between GSH and ceftazidime was not observed (R. Troyer, unpublished data). Additional preliminary *in vitro* and *in vivo* experiments involving inhibition of GSH with diethylmaleate (DEM) and L-buthionine-sulfoxamine (BSO) gave conflicting results. For example, addition of DEM to *in vitro* cultures resulted in complete inhibition of the synergistic interaction between IFN-γ and ceftazidime. Moreover, *in vivo* treatment of

mice with DEM also abolished immuno-antimicrobial synergy (R. Troyer, unpublished data). In contrast, addition of BSO *in vitro* or *in vivo* had no effect on immuno-antimicrobial synergy. The reasons for these conflicting results are currently unknown but are being investigated.

Another function of IFN-γ includes induction of autophagy. As mentioned previously, autophagy is an innate immune process by which intracellular bacteria can be targeted for killing (59). Much like with GSH, using rapamycin, a stimulator of autophagy, as a substitute for rIFN-γ in combination with ceftazidime, failed to elicit synergy against intracellular *B. pseudomallei* replication *in vitro* (data not shown). In addition, the use of wortmannin, an inhibitor of autophagy, in addition to immuno-antimicrobial therapy with ceftazidime and rIFN-γ exhibited no suppression of the synergistic interaction (R. Troyer, unpublished data). These results suggest that autophagy is also not the mechanism by which ceftazidime and IFN-γ interact to produce synergistic inhibition of *B. pseudomallei* infection.

The final potential mechanism that has been explored thus far is drug efflux. As described earlier, *B. pseudomallei* possesses several multidrug efflux pumps that enable the bacterium to actively remove drugs, such as antibiotics, from the cell's interior. For preliminary investigations on this concept, a mutant strain of *B. thailandensis*, an avirulent relative of *B. pseudomallei* that is very similar both phenotypically and serologically (10), was used. The mutant strain, called Bt38, is deficient in two drug efflux pumps. *In vitro* infections using this bacterium were performed just as they were with *B. pseudomallei*, except that they did not require a BSL-3 facility. Thus, the use of *B. thailandensis* provided us with a more convenient and rapid screening tool. Infection

of alveolar macrophages with Bt38 and then overnight treatment with rIFN-γ and ceftazidime resulted in less profound synergy than is seen with WT *B. thailandensis* E264. We are currently conducting additional experiments to investigate the role of efflux pump inhibition in immuno-antimicrobial synergy.

Despite having not yet identified the mechanism(s) by which IFN-γ, stimulated by CLDC, and ceftazidime interact to synergistically inhibit intracellular *B. pseudomallei* replication, the potential implications of this discovery are multiple. For one, it offers a strategy for improving the efficacy of antimicrobial therapy for the treatment of *B. pseudomallei* infection. This could translate to lower doses and a shorter duration of antimicrobials needed to treat infection, which could result in reduced side effects and toxicities, better patient compliance, fewer cases of relapse, and possibly decreased mortality. Additionally, being that CLDC is a very potent but non-specific stimulator of innate immunity, immuno-antimicrobial therapy involving CLDC may have applicability beyond the improvement of melioidosis therapy. That is to say, this therapeutic regimen may be effective in the treatment of other bacterial infections. For example, our lab is currently working on a similar model of immuno-antimicrobial therapy for the treatment of *Staphylococcus aureus* infection (S. Dow, personal communication).

Chapter 4: Conclusions and Future Directions.

Conclusions- Chapter 2:

Our *in vitro* data clearly illustrated the potential for immuno-antimicrobial therapy as a new approach to the treatment of *B. pseudomallei* infection. Cytokines elicited by CLDC immunotherapy were capable of increasing the efficacy of low-dose ceftazidime, resulting in synergistic inhibition of intracellular *B. pseudomallei* replication. IFN-γ was identified as the CLDC-elicited cytokine predominantly responsible for the interaction with cytokine and resulting synergy against *B. pseudomallei in vitro*. These *in vitro* findings deserve further investigation in an animal model of infection to determine if there is *in vivo* applicability for immuno-antimicrobial therapy with CLDC/IFN-γ and ceftazidime in the treatment of *B. pseudomallei* infection.

Conclusions- Chapter 3:

Our *in vivo* data correlated well with our *in vitro* data. Mice treated with low-dose CLDC immunotherapy in combination with low-dose ceftazidime therapy were protected from acute pulmonary *B. pseudomallei* infection. Therefore, these results indicated that CLDC immunotherapy enhanced the effectiveness of ceftazidime antimicrobial therapy *in vivo*, possibly by suppressing bacterial replication and dissemination. Immunotherapy with IFN-γ was also capable of interacting with ceftazidime *in vivo* to increase survival of mice acutely infected with *B. pseudomallei*,

but the survival advantage was less profound. Thus, rIFN-γ could be substituted for CLDC in immuno-antimicrobial therapy to augment the efficacy of low-dose ceftazidime treatment. However, the extended production of IFN-γ elicited by CLDC immunotherapy, or the induction of other cytokines in addition to IFN-γ, may have provided greater protection against *B. pseudomallei* infection.

Conclusions- Summary:

The work presented in this thesis describes a novel therapeutic regimen that may improve the treatment of *B. pseudomallei* infection. We demonstrated a powerful synergistic interaction between IFN-γ, stimulated by CLDC immunotherapy, and low-dose ceftazidime that restricted *B. pseudomallei* infection in an *in vitro* alveolar macrophage infection model as well as in an *in vivo* murine lethal bacterial challenge model. To our knowledge, this is the first time that immuno-antimicrobial therapy has successfully improved the treatment of acute *B. pseudomallei* infection. The enhanced antimicrobial efficacy seen with immuno-antimicrobial therapy may reduce the dose and duration of antimicrobials necessary to clear infection, thereby reducing the risk for potential side effects and toxicities, increasing the likelihood of patient compliance, decreasing the risk for relapse after antimicrobial therapy has been completed, and, ultimately, reducing mortality. In addition, the potent innate immune activation generated by CLDC immunotherapy represents a non-specific approach for improving the efficacy of antimicrobial therapy for melioidosis and may also be relevant in the treatment of infections in which the pathogen is unknown or in which the pathogen has been identified but is difficult to treat and/or infection is potentially life-threatening. The

mechanism by which CLDC-elicited IFN-γ and ceftazidime interact synergistically to suppress *B. pseudomallei* infection is as of yet unidentified but is still under investigation.

Future Directions:

Future directions for this project will undoubtedly involve further exploration of the mechanism of synergy between IFN-γ and ceftazidime. According to Onyeji et al. (67), exposure of bacteria to a subinhibitory concentration of antibiotic may cause alterations in the organisms leading to increased susceptibility to the microbicidal activity of IFN-γ or IFN-γ-stimulated cells, resulting in a greater than additive killing of the bacteria. Therefore, this hypothesis should be explored in the context of ceftazidime and rIFN-γ combination therapy. Through the initial use of a cell-free *in vitro* system followed by an *in vitro* infection system with alveolar macrophages, ceftazidime-induced enhancement of susceptibility to IFN-γ can be evaluated. Treatment of *B. pseudomallei* organisms in nutrient medium with a subinhibitory dose of ceftazidime prior to treatment with decreasing doses of rIFN-γ will suggest if the amount of IFN-γ necessary to kill the organisms in the presence of subinhibitory ceftazidime is less than that required when organisms are treated with rIFN-γ alone. Likewise, infection of alveolar macrophages with *B. pseudomallei* followed by treatment with a subinhibitory dose of ceftazidime prior to treatment with decreasing concentrations of rIFN-γ may suggest if the organisms are more susceptible to IFN-γ-potentiated microbicidal activity of the cells as a result of ceftazidime-induced increased susceptibility. These results could offer interesting insight into the mechanism by which IFN-γ and ceftazidime interact to synergistically suppress *B. pseudomallei* replication.

Mucosal CLDC immunotherapy has been shown in our lab to provide protection against acute lethal inhalational infection with both *B. pseudomallei* and *B. mallei* (37). Immuno-antimicrobial therapy with CLDC and ceftazidime should be evaluated in a murine model of acute pulmonary *B. mallei* infection to confirm that this therapeutic regimen is also applicable in the treatment of both of these organisms. *B. pseudomallei* and *B. mallei* are closely-related pathogenic organisms that cause diseases which are difficult to distinguish quickly based on clinical signs and that need to be treated promptly in order to give the infected individual the best chance for survival. These findings would be especially important in the event of an intentional release where immediate action in the face of a potentially unknown infecting organism would be required.

Adjunctive G-CSF therapy has potentially influenced the treatment of septic shock due to melioidosis, as mortality rates have declined significantly since this therapeutic was adopted for use in patients with septic shock in Australia in 1989, but experimental data have failed to confirm this finding (52-54, 71). Considering that neutrophils are known to play a critical role in resistance to *B. pseudomallei* infection partly by producing IFN-γ (25), G-CSF administration increases neutrophil production and enhances neutrophil function (52, 53), CLDC administration stimulates production of IFN-γ (37, 74), and IFN-γ is capable of potentiating the microbicidal activity of neutrophils (25, 67), immuno-antimicrobial therapy for *B. pseudomallei* infection involving G-CSF, CLDC, and ceftazidime may be more effective than just CLDC and ceftazidime. Pre-treatment of *B. pseudomallei*-infected mice with G-CSF prior to administration of CLDC and ceftazidime could lead to a greater influx of neutrophils, a

resultant increase in the response to CLDC stimulation, and enhanced synergy between CLDC-elicited IFN-γ and ceftazidime. Several questions arise concerning this therapeutic regimen, mostly regarding timing of treatments. For example, should ceftazidime treatment be started at the same time as G-CSF pre-treatment? Will IFN-γ stimulated by CLDC administration still synergize with ceftazidime if CLDC immunotherapy is initiated after ceftazidime therapy? Would waiting to administer CLDC and ceftazidime until after the G-CSF pre-treatment period allow *B. pseudomallei* too much time to establish infection and be too late to get ahead of the infection or would the G-CSF treatment provide enough benefit to keep infection from getting out of control before the CLDC therapy is started? Would the increased IFN-γ production from neutrophils result in immunopathology? Would this therapeutic regimen have any relevance as a post-exposure therapy in the case of natural exposure or intentional release? Would this therapy be cost-prohibitive in the regions of the world where melioidosis is endemic? Many of these questions are important but may be unanswerable without applying this therapeutic regimen in the context of an experimental *in vivo* infection system. The benefit of this therapeutic regimen to the treatment of *B. pseudomallei* infection could be tremendous if G-CSF administration were capable of further enhancing the suppression observed with CLDC-ceftazidime immuno-antimicrobial therapy.

Finally, chronic infection with *B. pseudomallei* or any pathogenic organism presents additional challenges concerning treatment due to altered metabolic status of the bacteria, immune evasion within macrophages, and protection deep within tissues. Preliminary data generated from a model of chronic *B. mallei* infection in C57BL/6 mice

showed encouraging results for the use of repeated CLDC immunotherapy in the treatment of chronic *B. mallei* infection. In these studies, C56BL/6 mice, which are know to be more resistant to infection by *Burkholderia* species (11, 28, 82), were infected i.n. with a low-dose inoculum of *B. mallei* and then left untreated for 7 d to allow for any acute disease to subside and for chronic disease to become established. At 7 d PI, mice were left untreated or treated i.p. with CLDC. CLDC treatment was repeated every 7 d thereafter until 60 d PI, at which time any surviving mice were humanely euthanized and organs were harvested to compare organ bacterial burdens between chronically infected mice that received no treatment and those that received repeated CLDC immunotherapy. In addition, the mice were tail bled at approximately 40 d PI to compare bacteremia between the treatment groups. The mice that received repeated CLDC immunotherapy had a reduced frequency of observable splenic lesions, decreased organ bacterial burdens in their lungs and livers, and reduced bacteremia (data not shown). Based on these preliminary results with chronic *B. mallei* infection, CLDC immunotherapy shows potential to improve treatment of chronic infection. Combining ceftazidime treatment with repeated CLDC administration may exhibit synergistic suppression and clearance of chronic *B. pseudomallei* infection similar to that seen in our model of acute infection. Thus, immuno-antimicrobial therapy with CLDC and ceftazidime should be assessed in a murine model of chronic *B. pseudomallei* infection to determine if it is possible to achieve complete clearance of organisms that are latent within macrophages and deep within tissues.

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