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Effects of Diabetic State and Gender on Pro-Inflammatory Cytokine Secretion by
Human Macrophages Infected with *Burkholderia pseudomallei*

Annette J. Blam

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Effects of Diabetic State and Gender on Pro-Inflammatory Cytokine Secretion by Human Macrophages Infected with *Burkholderia pseudomallei*

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Burkholderia pseudomallei is a gram-negative opportunistic soil pathogen that causes the life-threatening disease melioidosis. It is endemic in Northern Australia and Southeast Asia but can be found throughout many other regions in the world. Diabetes mellitus is a predisposing risk factor for infection with this organism and it has been demonstrated that diabetic males are particularly susceptible to severe infection. Previous research suggested that monocytes isolated from the whole blood of diabetic males demonstrated a decreased ability to produce the proinflammatory cytokines IL-1 β and IL-8. We hypothesized that monocyte-derived macrophages from diabetic males would also secrete lower levels of pro-inflammatory cytokines and that this difference between gender and diabetic state would be more pronounced compared to those seen previously with monocytes. Twenty volunteer with type I diabetes mellitus (ten males and ten females), along with twenty healthy age- and gender-matched controls donated blood for this study. Monocytes were collected from whole blood and allowed to differentiate into macrophages with the use of human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Macrophages were then divided into groups and infected with *B. pseudomallei*, *B. thailandensis* (a closely related by non-pathogenic bacterium that inhabits similar niches), and *E. coli*. An uninfected control was used as well. At six hours post-infection, mRNA was collected from all cells and qPCR was performed to determine cytokine expression levels. All mRNA values collected from cells which had been infected with bacterial agents were normalized against the corresponding concentrations of mRNA from mock-infected cells. Mean log fold increases in both IL-1 β and IL-8 were computed and compared. Preliminary testing showed decreased levels of both IL-1 β and IL-8 from *B. pseudomallei*-infected macrophages isolated from a diabetic male compared to the healthy, age-matched male control. Surprisingly, results from all forty donors demonstrated that gender and diabetic state were not significant factors in the proinflammatory responses of macrophages infected with *B. pseudomallei*, although further testing is needed to determine if these results were influenced by experimental parameters.

Keywords: *Burkholderia pseudomallei*, melioidosis, diabetes mellitus, proinflammatory cytokine

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Introduction

Burkholderia pseudomallei is a gram-negative environmental saprophyte. It is non-spore-forming, aerobic, and motile by way of a polar tuft of two to four flagella (26). *B. pseudomallei* has a large genome divided between two chromosomes that are 4.07 Mb and 3.17 Mb in size (a total of 7.24 Mb) (101). The larger of the two chromosomes contains the house-keeping genes, such as those needed for cellular growth and metabolism including gene clusters encoding the lipopolysaccharide and capsular polysaccharide, while the smaller of the two chromosomes contains accessory genes; those needed for adaptations and survival under harsh conditions, including those encoding the putative type III secretion system (77). It is believed that the large size of *B. pseudomallei*'s genome accounts for the organism's adaptability and versatility (31). The genome has a G+C content of 68%, which is relatively high compared to many other bacteria (101). *B. pseudomallei* produces many cell-associated and secreted substances associated with virulence, including hemolysin, phospholipase C, proteases, type III secretion systems, a siderophore, and other factors (53). It is resistant to damage from complement, lysosomal defensins, and cationic peptides (101). *B. pseudomallei* grows aerobically on most agar media and visible colonies are produced in about 24 hours at 37 °C, but colony morphology can be highly variable: with morphologies ranging from smooth to the more common rough type, and sometimes mucoid (84). A Gram stain of this organism shows bipolar staining. It is oxidase positive and can reliably be identified by its biochemical profile with kit-based systems (101). *B. pseudomallei* is currently listed by the Center for Disease Control and Prevention (CDC) as a category B select agent because of its high potential for bioweaponry and its extensive antibiotic resistance. The United States Department of Agriculture (USDA) also has this organism listed as a select agent, due to its significant animal pathogenicity (108).

Epidemiology

B. pseudomallei is readily cultured from the soil, water, and importantly, rice paddies in endemic areas including South East Asia, northeast Thailand, and northern Australia. It can also be found throughout the tropics and subtropics, specifically countries that are located within 20° north and 20° south of the equator (74). Sporadic cases have been described in Mexico, Papua New Guinea, the Philippines, South America and Africa (44). It is rare in North America. Most of the few cases that have been reported are due to either a laboratory-acquired infection or involve an individual who has traveled to an endemic area. In Taiwan, it was found that *B. pseudomallei* could be cultured from depths of 300-600 mm under crop-covered fields (17). There has been little evidence found to support the idea of natural vector-borne transmission of *B. pseudomallei* (94). While *B. pseudomallei* is associated with many plant species, a specific, natural niche for this organism remains to be identified (24).

Melioidosis

Melioidosis is the infectious disease caused by *B. pseudomallei*. It was first described in 1911 by pathologist Alfred Whitmore and his assistant C. S. Krishnaswami in Rangoon, Burma (19). Septicemic melioidosis accounts for approximately one-fifth of all community-acquired septicemias in northeast Thailand. In rice-farming communities, 80% of children have acquired anti-*B. pseudomallei* antibodies by the time they reach five years of age, and while most of the population in endemic areas have antibodies against *B. pseudomallei*, these antibodies have not been shown to be protective (42, 101). Based on indirect hemagglutination assays, 80% of the population in endemic areas have been exposed to this organism without manifesting clinical symptoms (89). Also, in northeast Thailand, the vast majority of melioidosis cases occur in rice

farmers and their families (64). In Singapore, melioidosis accounts for 7% of community-acquired pneumonias (101). In Northern Territory, Australia, there was a calculated mean annual incidence of 19.6 per 100,000 people in 2004 (25). Not only are humans susceptible to infection with *B. pseudomallei*, but many animals are as well. The list of organisms susceptible to infection includes nematodes, amoeba, rodents, cats, dogs, swine, camels, horses, sheep, goats, kangaroos, koalas, deer, alpacas, sea mammals (particularly dolphins), and large primates—most notably highland gorillas (19, 21, 33, 46, 49, 88). Host body temperature does not appear to be a factor in infection as this organism has also been reported to infect fish, snakes, and crocodiles (90).

Humans are most often infected by cutaneous inoculation or inhalation, and less often through ingestion, although transmission via sexual contact and birth have been reported (101). Inoculation of environmental organisms into minor cuts or abrasions is also thought to be an important route of infection. Melioidosis primarily affects those who come in contact with contaminated soil and water and is a disease that is associated with rainy seasons. Heavy rain fall may bring the bacterium to the surface and then aerosolize it from the soil and stagnant water. Near drowning victims in endemic areas are highly susceptible to a pneumonic form of melioidosis, presumably due to inhalation of a large inoculum from contaminated water. In Thailand, exposure frequently occurs during rice farming since rice farmers often sustain many minor cuts and abrasions while working in the rice paddies.

There are several predisposing conditions to infection by *B. pseudomallei*: diabetes mellitus, renal disease, thalassaemia, alcoholism, cirrhosis, and immunosuppression are a few. Patients with diabetes mellitus are particularly susceptible to melioidosis. About 60% of

melioidosis cases in Thailand have pre-existing or newly diagnosed diabetes, usually with indication of poor blood glucose control before infection occurred (16).

Pulmonary melioidosis was more common in U.S. soldiers working as helicopter crewman in Vietnam and it was thought that this was because the rotor blades of the helicopter dispersed *B. pseudomallei* into the air from the soil and the bacteria were then inhaled by the soldiers. These observations are supported by the low rate of melioidosis seen in Japanese troops that returned from South East Asia after World War II, who did not use helicopters (2). *B. pseudomallei* is known to have a long latency period and a high rate of reactivity, with a 6-13% chance of relapse among patients (1, 55). A report of reactivated melioidosis in a World War II veteran 62 years after exposure has been documented (107). Reactivation is usually concurrent with an immunocompromised state, often triggered by an infection or injury (44). Because of the organism's ability to sequester itself in the host and then reactivate much later in the host's life, the disease it causes has earned the name "Vietnam Time Bomb" (101). This organism has been shown to be able to survive in triple distilled water for more than three years (104).

Clinical Manifestations

Clinical manifestations of melioidosis are highly variable and none are disease specific, leading to frequent misdiagnosis. Symptoms can range from a chronic debilitating localized infection to an acute fulminant septicemia. Other presentations can include soft tissue or skin abscesses, peritonitis, genitourinary infections or neurological presentations (73, 101). Acute infections can be divided into the pulmonary and septicemic forms, and either of these left untreated can be fatal in a few days. The overall mortality for adult patients in Thailand is 50%, while in Australia it is 20% (19, 101, 102). Most of the patients seeking therapy are septicemic, and show no obvious infected wounds. The lung is most commonly infected, but abscess

formation in the spleen, liver, skeletal muscle, and prostate are also frequently seen (101). Localized abscess formation, soft tissue infection, and asymptomatic disease with seroconversion are common (43). In Thailand, children with melioidosis often present with acute suppurative parotitis (fever with pain, and swelling over the parotid gland). This is more common in Thailand (15%) than in northern Australia (4%), while presentation as brainstem encephalitis or flaccid paraparesis is more common in Australia (4%) than in southeast Asia (0.2%) (101). The most common misdiagnosis for pulmonary melioidosis is tuberculosis, as the manifestations are quite similar (97). The incidence of melioidosis may be higher than current data implies because melioidosis is difficult to diagnose. While melioidosis is more commonly seen in males in endemic areas, studies have shown that there is higher seropositivity among females (50).

Diagnosis can be ascertained in many ways. *B. pseudomallei* is readily cultured from infected sites and blood. Splenic abscesses are less commonly seen with other bacterial infections, and in endemic areas, are more likely to occur than liver abscesses; especially since in northeast Thailand, 95% of splenic abscesses are caused by *B. pseudomallei* (101). Culturing from clinical samples is currently considered the “gold standard” in diagnosis (60). Serology is not used for diagnosis due to the delayed seroconversion and the high seropositivity in endemic regions (60).

Treatment of *B. pseudomallei* infections can prove difficult due to many factors: the bacterium is intrinsically resistant to many antibiotics, there is a long latency period, and predisposing conditions often contribute to infection. Because clinical manifestations are highly variable, correct diagnoses may not always be timely, and this can also impede treatment. *B. pseudomallei* is susceptible to chloramphenicol, the tetracyclines, trimethoprim-sulfamethoxazole, amoxicillin-clavulanate, third generation cephalosporins, and carbapenems.

The current, most effective treatment consists of either a combination of four antibiotics (chloramphenicol, doxycycline, and trimethoprim-sulfamethoxazole), or of ceftazidime alone. This treatment is carried out for 20 weeks, with a relapse rate of 10% (101). Resistance to penicillins, macrolides, aminoglycosides, rifamycins, first and second generation cephalosporins, and colistin is an intrinsic property of the bacterium (101).

Host Immune Response

The underlying immunological mechanisms responsible for the variable clinical manifestations in melioidosis are still unclear. The ability of *B. pseudomallei* to infect, survive in, and replicate inside both phagocytic and non-phagocytic cells may play a role in the different disease symptoms. Development of adaptive cell-mediated immune responses have been shown both clinically and experimentally to be essential for host survival following infection with *B. pseudomallei*. Individuals that are exposed to this organism but do not become ill, may seroconvert and develop a strong cell-mediated immune response that may protect them from a progressive infection (50).

In both mice and humans, IFN- γ is essential for early control of *B. pseudomallei* infection (12). CD4⁺ T cells specific for *B. pseudomallei* secrete INF- γ following exposure to the organism (89). For many intracellular pathogens, caspase-1 is crucial for host resistance. Caspase-1 is a member of the inflammatory caspases and is involved in the maturation of IL-1 β and IL-18, which are both pro-inflammatory cytokines (12). In macrophages, caspase-1-dependent mechanisms are factors in the control of intracellular replication of *B. pseudomallei* (12). An important part of the host defense against infection with this organism is caspase-1-dependent IL-18-mediated IFN- γ production (12). Human natural killer (NK) cells are also

important producers of IFN- γ . In a study by Tippayawat, et al, it was shown that NK cells provided 80% of the IFN- γ in the first few hours of infection (89).

Polymorphonuclear leukocytes (PMNL) are important in containing the initial infection of *B. pseudomallei* as supported by the use of granulocyte-colony stimulating factor (G-CSF) in patients with melioidosis septicemia; an increase in survival was shown with those receiving G-CSF (25). Some studies, such as one by Egan, et al, have shown that *B. pseudomallei* is resistant to destruction by PMNLs (30).

Killing by macrophages is an important component of the innate immune system. *B. pseudomallei* stimulates macrophages weakly; this enables the organism to evade killing by these phagocytes (67). The importance of macrophages in combating an infection with *B. pseudomallei* has been demonstrated by Wikraiphat, et al, who, using dichloromethylene biphosphonate-containing liposomes, depleted mice of their macrophages. When these mice were infected with *B. pseudomallei*, they were more highly susceptible to active infection (103). Macrophages use reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) to destroy intracellular bacteria. *In vitro*, it has been shown that *B. pseudomallei* is susceptible to both ROI and RNI (16). Miyagi, et al, found that IFN- γ -activated macrophages use ROI- and RNI-dependent killing mechanisms to prevent the growth of this organism intracellularly (61). As it was also found that *B. pseudomallei* was able to multiply intracellularly within unstimulated macrophages, stimulation to activate ROI and RNI may be important in the clearing of this organism from within these phagocytes (61).

Classical activation of macrophages, such as activation by microbial products, leads to type-1 macrophages (M-1), which produce proinflammatory cytokines. The production of these cytokines can then, in turn, lead to an active immune response against the invading

microorganism and promote cell-mediated immunity. Alternative activation of macrophages can lead to the production of M-2 macrophages. These cells produce anti-inflammatory cytokines, such as IL-10, and are poor antigen presenting cells in comparison with M-1's. This leads to a poor cell-mediated immune response. Verreck, et al, has demonstrated that the priming of human blood monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) generates pro-inflammatory type-one macrophages (M-1) (98). GM-CSF is known to act on monocytes, enhancing not only phagocytosis, but intracellular killing mechanisms as well (82).

Cytokines

A few key cytokines are specifically involved in the host response to an infection with *B. pseudomallei*: IFN- γ , TNF- α , and IL-1 β (70). Cytokines found to be increased in the serum of patients suffering from melioidosis include IFN- γ , TNF- α , IL-6, IL-8, IL-18, IL-12 p40, IL-15, IFN- γ -inducible protein 10, and monocyte IFN- γ -inducible protein (70). IFN- γ produced by natural killer (NK) cells and CD8⁺ T cells is critical in host defense. Cells of the monocyte lineage produce IL-1 β , a strong pro-inflammatory cytokine, in response to many different microbial products, including LPS. The increased levels of IL-1 β seen in melioidosis patients may be due to the proliferation of monocytes, as it has been reported that the LPS of *B. pseudomallei* has strong mitogenic activity (70). Studies on macrophage cell lines demonstrated a decrease in the production of TNF- α when these cells were stimulated with *B. pseudomallei* LPS, and no inducible nitric oxide synthase (iNOS) was produced. Opposite effects were seen when macrophages were stimulated with *E. coli* LPS, indicating that the LPS of *B. pseudomallei* may be interrupting critical macrophage functions (70).

IL-8 is an important cytokine in host defenses against bacterial pathogens. It is a powerful neutrophil chemoattractant and is involved in the trafficking of these immune cells

across the endothelium. This cytokine is secreted by activated macrophages, phagocytosing neutrophils, endothelial cells, and fixed tissue cells. In a study done by Friedland, et al, it was demonstrated that plasma concentrations of both IL-6 and IL-8 were a better predictor of patient mortality than plasma concentrations of TNF- α in *B. pseudomallei* infections (34).

Putative Virulence Factors

Epithelial Attachment

It has been shown by Kanaphun and others that there is a relationship between pharyngeal colonization by *B. pseudomallei* and pulmonary infection (40). This organism expresses functional adhesins. Adhesins are surface molecules that mediate the attachment to specific receptors present on the surface of a host cell. It has been shown by Gori, et al, using thin layer chromatography, that *B. pseudomallei* binds to the asialoganglioside aGM1-aGM2 (40). The surface molecule on *B. pseudomallei* that mediates this attachment is still unknown, however *Pseudomonas aeruginosa*, a closely related organism, mediates this same attachment via type IV pili (40). A study done with a *B. pseudomallei* K96243 type IVA mutant strain showed reduced adhesion to epithelial cell lines, providing evidence that perhaps *B. pseudomallei* uses type IV pili to attach to host cells in a manner similar to that used by *P. aeruginosa* (1). It has been shown that the level of attachment of *B. pseudomallei* is quite low and that this may account for the need for greater bacterial loads in order to cause infection, and why this organism is often an opportunistic pathogen (2).

Intracellular Survival and Spread

Intracellular pathogens have adopted many strategies to evade host defenses. This includes invading non-professional phagocytes, escaping into the cytoplasm of host cells,

interfering with reactive oxygen intermediates, inhibition of fusion of the phagosome with the lysosome, and resistance to cationic peptides and lysosomal contents (44). *B. pseudomallei* is able to actively invade and multiply in both phagocytic and non-phagocytic cells. Initially after cellular uptake, the bacteria can be seen in vacuoles and later in the cytoplasm, where they replicate (1). Within *B. pseudomallei*-infected human macrophages, lysosome fusion is detected, but to a small degree, and eventually the proliferation of bacteria that survived the fusion overwhelms the macrophage. Once inside the cytoplasm, the organism can induce actin rearrangement. This polymerization of host cell actin is initiated at one pole of the bacterium leading to actin tail formation and actin-associated peripheral membrane protrusions (69). This host cell actin utilization allows the bacterium to spread from cell to cell. The Arp2/3 complex is involved in *B. pseudomallei*-induced actin polymerization much like the actin-based motility seen in *Listeria*, *Shigella*, and the vaccinia virus (11). However, unlike either *Shigella* or Vaccinia, activation of the Arp2/3 complex is N-WASP independent. *Listeria monocytogenes* uses a surface protein, ActA, to activate Arp2/3. The ActA-like protein of *B. pseudomallei* mimics WASP/Scar family proteins in that it activates and recruits Arp2/3 (11). Stevens, et al, found that induction of the rearrangement of host actin was caused by an effector of the *Burkholderia* secretion apparatus (Bsa) type III secretion system, termed BopE (85).

Capsular Polysaccharides

Capsule production has been shown to be an important virulence factor for many bacteria that are pathogenic in humans. In *B. pseudomallei*, the capsular polysaccharide is encoded by the *wcb* operon (100). Through electron microscopy, it has been established that there are three distinct morphological states of *B. pseudomallei*: those possessing a macrocapsule (0.1-0.25 μ m thick), those possessing a microcapsule (0.086 μ m thick) and those lacking a capsule (1). The

capsular polysaccharide, designated as type I O-PS, is a polymer of -3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1- (74). It protects the bacterium from serum killing by reducing levels of complement C3 deposition. Although C3 deposition is not completely eliminated, as has been shown by the use of a mouse monoclonal antibody against human C3, the decrease in adherence is enough to limit the effectiveness of opsonization and phagocytosis (74). It was also demonstrated, by Reckseidler-Zenteno, et al, that in the presence of human serum, a *B. pseudomallei* mutant strain lacking a capsule was phagocytosed more readily than the wild type (74). The capsule has been shown to be important for the pathogenesis of *B. pseudomallei* in that mutants unable to produce functional capsule polysaccharides are attenuated significantly (100). Capsules also function as a form of protection against antibiotics. *B. pseudomallei* is able to form microcolonies: this not only aids in protection against antibiotics, but also phenotypically alters the bacteria themselves, making them less susceptible to some antibiotics. It has also been shown that the capsule may play a role in intracellular survival of the bacterium (103).

Biofilm Formation

A biofilm is an aggregate of bacteria where cells have either adhered to a surface or simply to each other by using an extracellular polymeric substance that they are able to produce themselves. Many bacteria are able to form biofilms, and it is thought that the formation of a biofilm is an important factor in many chronic infections. Cells within a biofilm are physiologically and phenotypically distinct from planktonic cells. One characteristic of bacteria in a biofilm is an increased resistance to antibiotics as compared to the planktonic bacteria; this resistance can be increased by as much as 1,000 times (87). *B. pseudomallei*'s highly hydrated glycocalyx capsule facilitates the formation of biofilms. In electron micrographs of *B. pseudomallei*-infected lung tissue from guinea pigs and humans, biofilms have been

demonstrated (1). Although biofilm formation appears not to be essential for virulence of *B. pseudomallei*, it almost certainly plays a role in survival in harsh environments (1).

Lipopolysaccharide

The lipopolysaccharide (LPS) of *B. pseudomallei* is also known as type II O-PS and has a structure of β -D-glucopyranose-(1-3)-6-deoxy- α -L-talopyranose-(1- (74). There are three distinct antigenic types of LPS described for *B. pseudomallei*: two smooth, serotypes A and B, and a rare rough serotype. There is no cross reactivity seen between the three types, but they all possess similar endotoxic levels and similar macrophage activation levels (1). Much like the capsular polysaccharide, the LPS is important for virulence of *B. pseudomallei*. In mice and diabetic rats, there is a reduction in lethality of infection when the animals are treated with antibodies against the LPS (77). The LPS of *B. pseudomallei* has different characteristics than those from other gram negative bacteria: *B. pseudomallei*'s LPS possesses weaker pyrogenic activity in mice than enterobacterial LPS but has stronger mitogenic activity in the splenocytes of mice (4). Arjcharoen, et al, also showed that the LPS of *B. pseudomallei* exhibits weaker and slower activation kinetics for mouse macrophage cell lines; this could be due in part to the unique structure of the LPS, as the chemical composition of the acid-stable inner core, which is attached to a lipid A moiety, is atypical (4).

O-Antigen

The O-polysaccharide moiety of *B. pseudomallei*'s LPS has been shown to play an important role in evasion of the innate immune system. It is involved in the invasion and survival in macrophages and is able to modulate macrophage activation (4). Since activation of iNOS may be an important early step in the destruction of this bacterium, the ability to inhibit

macrophage activation may be important in its survival. Studies have shown that mutant strains with alterations in the O-antigen structure are attenuated in animal models of melioidosis (77).

Flagella

Motility is an important virulence factor in many bacterial species as it aids their ability to invade and replicate within both phagocytic and non-phagocytic cells. Flagella are also important in the dissemination of bacteria from localized sites of infection. *B. pseudomallei* is motile by means of flagella and thus contains the *fliC* gene, which encodes flagellin, the protein from which flagella are constructed. Flagella may not be critical for virulence as no attenuation was seen in experiments using diabetic rats and Syrian hamsters infected with a mutant strain of *B. pseudomallei* with an altered *fliC* gene (22). Bacterial flagellin is recognized as a strong stimulator of the immune system and it has been shown that anti-flagellin antibodies, used to passively immunize diabetic rats, can reduce the median lethal dose of *B. pseudomallei* (18). While flagella may not play an essential role in the virulence of *B. pseudomallei* in animal models, it has been shown to be important for adherence to initiate bacterial entry into amoeba (1, 23).

Pili

Both adherence to, and colonization of host cell surfaces are important first steps in the establishment of an infection. Pili often mediate non-intimate adherence to host cells. Boddey, et al, established that *B. pseudomallei* uses a type IV A pilin, encoded by the *pilA* gene, to adhere to eukaryotic cells *in vitro* (specifically, human cervical carcinoma cell line ME180) (8). Type IV pili typically aid bacteria in forming biofilms and microcolonies, which are both important for virulence as discussed previously, however, it has been demonstrated that *pilA* is not required for biofilm formation (8). In the *B. pseudomallei* genome, eight loci have been identified that

encode seven putative pilin subunits, which may explain this organism's ability to infect such a broad range of hosts (32).

Quorum-Sensing

Quorum sensing is a form of cell-to-cell communication that is density dependent and modulates many diverse biological functions of gram-negative bacteria, such as exopolysaccharide and extracellular enzyme production, biofilm formation, and expression of extracellular virulence factors. At high densities, cells produce and respond to *N*-acylhomoserine lactone (AHL) autoinducers. This process relies on two proteins: an AHL synthase, of the LuxI family, which directs synthesis of AHL; and, belonging to the LuxR family, a transcriptional regulator, which binds AHL and activates or represses targeted genes (96). Two quorum-sensing systems have been characterized for *B. pseudomallei*: the PmlI-PmlR system and the BpsI-BpsR system, both of which are required for full virulence of this organism. The PmlIR system modulates the production of a protease, MprA, and at high autoinducer concentrations, PmlR appears to repress expression of MprA (96). The BpsIR quorum-sensing system controls the secretion of exoproducts in *B. pseudomallei* (83). This pathogen has at least two efflux pumps, BpeAB-OprB and AmrAB-OprA. BpeAB-OprB is a multidrug efflux pump that is responsible for conferring resistance to macrolides and aminoglycosides (15). It is thought that it is quorum-regulated and in addition to antibiotic resistance, it also is necessary for production of quorum-sensing-controlled virulence factors, such as a siderophore and biofilm components (15). Because of the importance of BpeAB-OprB in the production of certain virulence factors, it is recognized as an important virulence determinant.

Type III Secretion System

Type III secretion systems (T3SS) are large assemblies of proteins spanning the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space and penetrating the host cell membrane. These systems are used to transport effector proteins into host cells under specific conditions in an ATP-dependent manner (31). The genome of *B. pseudomallei* contains three putative T3SS with the locus designated Bsa for *Burkholderia* secretion apparatus (1). The T3SS involves an injectosome, a hollow tube which acts as a needle, through which the effectors travel. This subunit is made up of repeating units of BsaL (108). The translocon is the ring-like assembly that spans the host cell's membrane, through which translocator proteins travel into the host cell, causing inhibition of certain functions which aid the bacterium in its survival (31). The Bsa effector proteins are termed Bop (*Burkholderia* outer protein) and the translocators are termed Bip (*Burkholderia* invasion protein) (99). The T3SS has been shown to be important in virulence of *B. pseudomallei* in many of the animal models used. It has been shown that mutations in either *bopA* and *bopE* cause attenuation of virulence in animals, while disruption of the *bipD* gene reduces the organism's ability to invade eukaryotic cells and reduces its virulence in mice (31). BipD is believed to act as an extracellular chaperone which aids the translocator proteins in entering the target cell, where they then form a pore (47). *B. pseudomallei* is unique among facultative intracellular bacterial pathogens in that it is able to induce host cell fusion, which leads to multinucleated giant cell (MNGC) formation in tissue culture models. By inactivating the *bipB* gene, MNGC formation was reduced as was intracellular spread; this mutant was also significantly diminished in its ability to cause infection in mice (86). Bsa T3SS mutants have been shown to be attenuated as well; both in the mouse and Syrian hamster infection models.

These mutants have a reduced ability to escape from endocytic vesicles and decreased intracellular replication, and are not able to form actin tails or MNGC; all of these factors are associated with *B. pseudomallei* virulence (41). Serum from melioidosis patients has been shown to react with purified translocation proteins (BipB, BipC, and BipD), indicating that the T3SS is functional *in vivo* (1).

Type VI Secretion System

Type VI secretion systems (T6SS) have been recently characterized as protein transport mechanisms used by many gram-negative bacteria, and Burtnick, et al, has demonstrated that they are key virulence determinants (14). Although not much is yet known about the structure and function of these secretion systems, evidence suggests that they have features similar to bacteriophage tail complexes (14). The genome of *B. pseudomallei* contains six putative T6SS coding regions, which is more than any other bacterium known. It is likely that these T6SSs play a role in virulence and it is likely that having such a large number of them (approximately 2.3% of the genome) allows the organism to occupy a large range of niches (80). It has also been demonstrated by Shalom, et al, that one of the T6SS is important for the uptake by, and invasion of macrophages (79).

Siderophore

Iron is essential for bacterial growth. In order to establish and persist in a host, bacteria must be able to acquire iron. In mammalian hosts, most of the iron is bound to proteins, such as lactoferrin (in mucosal secretions) and transferrin (in plasma) or is located intracellularly and is not accessible to the bacterium. To counter this, many bacteria secrete low-molecular-weight iron chelators, called siderophores, which compete with host proteins for iron. Siderophores have been implicated in the virulence of many different pathogenic bacteria. *B. pseudomallei* is

no exception and needs iron to survive within a host, and thus possesses a siderophore which is able to remove iron from both lactoferrin and transferrin (105, 106). This siderophore was termed malleobactin by Yang, et al (105). Since *B. pseudomallei* infections are initiated at the mucosal surface, its ability to obtain iron from lactoferrin may be important in initiating an infection (106).

Protease

A serine metalloprotease, MprA, has been described for *B. pseudomallei*, but it is not required for infection in mice (1, 51). A 52 kDa calcium-dependent serine protease has also been observed in this organism, and it has been shown to cause tissue necrosis (1). A 42 kDa and a 65 kDa protease have also been discovered, but their roles in pathogenesis are currently unknown (1, 36).

Homology to species in the *Burkholderia* genus

It has been suggested that *B. pseudomallei*, *B. mallei*, and *B. thailandensis* represent three states of ecological niche adaptation: opportunistic pathogen, obligate pathogen, and saprophyte, respectively (56). It has also been proposed that Australian members of this species may be ancestral to those from Southeast Asia: this is known as the Gondwana hypothesis (24).

Taxonomy

The *Burkholderia* genus comprises more than 30 species. These species are able to inhabit many different and diverse ecological niches and have been isolated from soil, water, plants, infected humans and animals, hospital and industrial settings, and insects. This genus was defined to accommodate most of the former rRNA group II pseudomonads (29).

Burkholderia thailandensis

B. thailandensis is a gram-negative saprophyte that is readily found in the environment in Southeast Asia. It is closely related to both *B. pseudomallei* and *B. mallei* but shows reduced virulence in comparison, with a much larger infectious dose of *B. thailandensis* needed to cause disease in animal models (72). The 16S rRNA sequences of *B. pseudomallei* and *B. thailandensis* differ by 15 nucleotides which are lacking in *B. thailandensis* (70, 75). The LPS of *B. thailandensis* shares structural similarities to the LPS of *B. mallei* and *B. pseudomallei*, and it has been demonstrated that antibodies against the LPS of *B. pseudomallei* can be detected using the LPS of *B. thailandensis* in indirect immunofluorescent assays (72). Both *B. pseudomallei* and *B. thailandensis* are motile by means of flagella and the *fliC* gene shows 99% amino acid homology (23). Also highly conserved between the two species is the Bsa T3SS-encoding region, although in arabinose-containing media, the T3SS of *B. thailandensis* is negatively regulated (41). *B. thailandensis* is also able to induce cell fusion and the formation of MNGCs after ingestion by phagocytes (63).

Key differences between *B. pseudomallei* and *B. thailandensis*

The gene cluster that encodes the capsular polysaccharide in *B. pseudomallei* is absent in *B. thailandensis*, even though much of the *wcb* capsule operon genes are present in its genome; thus it does not express a capsule (77). Even with the lack of a capsule, it is shown to be resistant to serum killing, yet is unable to cause bacteremia in a Syrian hamster model (74). The lack of a capsular polysaccharide contributes to its lower virulence. The *B. thailandensis* genome contains an arabinose-assimilation operon that is lacking in *B. pseudomallei* (37). While the genome of *B. thailandensis* encodes the *fliC* gene, it also possesses another flagellin gene,

flagellin D, which is absent in *B. pseudomallei* (23). *B. pseudomallei* produces significantly higher amounts of biofilm compared with *B. thailandensis*.

Differences between *B. pseudomallei* and *B. mallei*

B. mallei is an obligate mammalian pathogen and is the causative agent of glanders, a disease affecting primarily equines but humans and other animals as well. Human cases are rare and are usually laboratory acquired or associated with the handling of infected animals. It is believed to be endemic in Southeast Asia, some parts of Africa, Central and South America, and the Middle East (3). Using multi-locus sequence typing, it has been demonstrated that *B. mallei* evolved from *B. pseudomallei* (76). The quorum-sensing systems of *B. mallei* are very similar to those of *B. pseudomallei* with the exception of one *luxIR* pair (29). Both *B. pseudomallei* and *B. mallei* are intrinsically resistant to many antimicrobials. However, while the former is resistant to gentamicin, *B. mallei* is susceptible due to the deletion of the AmrAB-OprA efflux pump-encoding genes (78). Although there are some differences in antimicrobial susceptibilities, treatment for both melioidosis and glanders is the same. *B. mallei* expresses a heterogeneous mixture of acylated lipid A species, instead of the lipid A expressed by *B. pseudomallei* (13, 72). *B. mallei* contains the gene cluster which encodes the capsular polysaccharide, but does not contain the regions coding for the type III or type IV secretion systems. Because these two secretion systems are conserved in *B. thailandensis*, it is thought that perhaps they play a role in environmental survival (77).

Homology to other species

The exopolysaccharides of gram negative bacteria are divided into two groups, I and II, on the basis of modes of expression and structural features. The exopolysaccharide of *B. pseudomallei* is placed in group I, based on knowledge about its high molecular mass (>150

kDa) and the temperature at which it is able to be expressed (below 20°C) (84). As has been seen with *Salmonella*, *Shigella*, and *Listeria*, *B. pseudomallei* is able to induce caspase-1-dependent cell death in monocytes and macrophages (12). Two of the putative T3SS of *B. pseudomallei* show similarities to those of the plant-like pathogen *Ralstonia solanacearum* (41). It is thought that these secretion systems allow the organism to interact with the plants in rice paddies, where *B. pseudomallei* is typically found. The other T3SS (Bsa) shares significant homology to the T3SS found on the *Shigella flexneri* virulence plasmid and *Salmonella enteric* serovar Typhimurium pathogenicity island 1 (41, 108). Also, several *Salmonella* T3SS-delivered effector homologues have been found in the T3SS gene cluster of *B. pseudomallei*; this includes homologues to SopA and SopE, SipB and SipC, and IacP and IagB (99). One gene identified in *B. pseudomallei* showed a weak sequence homology to a *P. aeruginosa* glycosyltransferase that is important in capsule synthesis, while other genes involved in capsule synthesis show a strong homology to those of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Escherichia coli* (75).

Animal Models

Although melioidosis manifests itself in many different forms, animal models currently focus on pathogenesis of acute disease. Syrian hamsters and diabetic rats are incredibly susceptible to infection, and are used as models for acute disease (107). BALB/c mice are highly susceptible to infection with *B. pseudomallei*, while C57BL/6 mice are 10⁴ times more resistant to disease (90, 93). Assays for determining virulence levels of different strains of this organism are performed using BALB/c mice. It has also been shown that levels of mRNA for certain cytokines, such as IFN- γ , are higher in BALB/c mice than in C57BL/6 (90, 95). While mice are often used as models of infection, few studies have been attempted to study long-term latency.

While a study in 1982 described experimental *B. pseudomallei* infection in goats, there are not many reports of large animal models of infection with this organism (90).

Vaccination

Both *B. pseudomallei* and *B. mallei* are listed by the CDC as category B select agents because of their high potential for weaponization and their high antibiotic resistance (39). Despite this, there is currently no licensed vaccine available for either organism. Many successful human vaccines are based on the use of capsular polysaccharide and capsular polysaccharide conjugates. This has led to the belief that the capsular polysaccharide of *B. pseudomallei* could be used to form part of a useful human vaccine (100). In addition, the capsule is the dominant structure recognized by the immune system of patients that show seropositivity (57, 100). Immunization with purified solutions of this polysaccharide were shown to provide protection against intraperitoneal inoculation with *B. pseudomallei* in mouse models, however, no protection was seen against airborne challenge (10, 100). The high genetic similarity between *B. pseudomallei* and *B. mallei* strengthens the belief that a single vaccine may be able to prevent or treat both melioidosis and glanders. Previous studies have shown that subunit vaccines based on certain common polysaccharides were protective against both organisms (76). However, due to the high degree of antigenic variation between strains of *B. pseudomallei* alone, a vaccine that is based on a single antigenic determinant may not be protective against all strains (5). It has also been shown that vaccines using inactivated bacteria failed to induce the same level of immunity seen when live bacteria were used; thus the presence of living bacteria may be important to generate sufficient protection (10). *B. thailandensis* has been investigated as a potential vaccine candidate. Guinea pigs vaccinated with this organism were shown to be protected against challenge with a lethal dose of *B. pseudomallei* (41).

However, since *B. thailandensis* has been shown to be able to cause disease in humans, using this organism as a live vaccine may not be feasible.

Diabetes

Type-1 diabetes mellitus is an autoimmune disease caused by the destruction of the insulin-producing beta cells in the pancreas by autoreactive T cells. It manifests often during childhood or early adulthood when at least 70% of the beta cell mass has been destroyed and there is no longer sufficient insulin being produced (65). This disease is characterized by blood glucose dysregulation, insufficient insulin production, hyperglycemia, and other long-term complications (52). Type 1 diabetes mellitus has been studied extensively, yet the complete immunological mechanisms responsible for the destruction of the pancreatic beta cells is not completely clear; genetic predisposing factors and possible environmental triggers are likely involved (38).

Many types of infections are seen more frequently in diabetic patients than in those without diabetes (45). The innate immunity of patients with diabetes mellitus has been shown to be altered, especially in terms of PMNL function, when compared to the innate immunity of non-diabetic patients. Diabetic PMNLs show reduced migration in response to IL-8 compared to those from healthy subjects and have been shown to have reduced phagocytic capacity (16). Macrophages have been shown to be impaired as well, possibly causing the increased susceptibility to many gram-negative infections seen in those with diabetes (71). Poor glycemic control may have an effect on cell function, as immune function has been seen to improve as glycemic control improves (45, 59). Phagocytosis is greatly impaired in cases of poor glycemic control, as well as the ability of granulocytes to kill engulfed bacteria (6, 66). HbA_{1c} levels are used to determine the average plasma glucose concentration. While the American Diabetes

Association recommends having an HbA_{1c} level below 7.0, those with diabetes often have levels above this.

The Current Study

Previous research by Laura Dickey (27) showed that cytokine profiles of monocytes infected with *B. pseudomallei* are highly dependent on both gender and diabetic state. Monocytes from diabetic males were shown to produce significantly less proinflammatory cytokines, especially IL-1 β and IL-8, than those from healthy, age-matched controls, when cells were infected with not only *B. pseudomallei*, but *B. thailandensis* and *E. coli* as well. Monocytes from diabetic females were shown to actually produce significantly more IL-1 β when infected with *B. pseudomallei* than those from healthy, age-matched controls. The study by Dickey involved a small sample population and the cells used were monocytes, precursors to macrophages. It was hypothesized that if macrophages were used for infections instead of monocytes, the differences that were shown to be dependent on diabetic state and gender would be more robust. It was also decided that doubling the sample population would give more conclusive results. The purpose of this study was to determine the effect of gender and diabetic state on levels of proinflammatory cytokines produced by M1 macrophages infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli*, compared with uninfected controls. Preliminary studies were performed on macrophages from a diabetic male to prove feasibility. Results from these studies were similar to those reported by Dickey. Therefore, the full study was subsequently undertaken.

Materials and Methods

Blood Donors

Persons with type I diabetes mellitus and healthy controls, matched for age and gender, were recruited using fliers and personal communication. Protocols involving human blood donors followed federal regulations and were approved by the Brigham Young University Institutional Review Board (IRB).

Bacteria

Bacteria strains used were *Escherichia coli* ATCC 11229, *Burkholderia thailandensis* ATCC 700388, and *Burkholderia pseudomallei* NCTC 13178. *B. pseudomallei* 13178 was originally isolated from a patient admitted to the Townsville General Hospital (Townsville, Queensland, Australia) and its virulence has been previously documented (7).

Relationship of Turbidity to Cell Concentration

Bacteria were grown overnight in 5 ml of Brain Heart Infusion (BHI) broth in 15-ml conical tubes at 37 °C in a shaking incubator at 250 rpm. Cultures were centrifuged at 3,838 xg, washed, and resuspended in 5 ml of supplemented RPMI media. Serial dilutions were made for each species (1:2, 1:3, 1:4, 1:6, 1:8, 1:10, 1:16, and 1:64). Spectrophotometer readings were recorded for each dilution and 1-ml samples of dilutions 1×10^{-5} , 1×10^{-6} , and 1×10^{-7} (for *E. coli*), 1×10^{-6} , 1×10^{-7} , 1×10^{-8} (for *B. thailandensis*), and 1×10^{-7} , 1×10^{-8} , and 1×10^{-9} (for *B. pseudomallei*) were plated in triplicate on Columbia agar plates using membrane filtration. Plates were incubated at 37 °C. Colony forming units (CFU) were counted 24-48 hours later. Regression lines of percent

transmittance versus log cell concentration were created using MiniTab 16 Statistical Software (MiniTab Inc.) and used to determine bacterial concentrations for all subsequent infections.

Monocyte Separation from Whole Blood

Fifty ml whole blood was collected from donors in K2 EDTA (K2E) vacutainers (Becton Dickinson). Twenty-five ml of whole blood was diluted in 10 ml of Hanks Balanced Salt Solution (HBSS; HyClone). Approximately 35 ml of blood-HBSS was carefully layered over 15 ml of Lymphocyte Separation Medium (LSM; Cellgro) and centrifuged at 600 xg for 20 minutes at 20 °C. Approximately 15 ml of peripheral blood mononuclear cells (PMBCs) were removed and mixed with 4 ml of OptiPrep reagent (Sigma Aldrich). This was overlaid with 7.5 ml and 20 ml of Optiprep solutions with densities of 1.084 g/ml and 1.068 g/ml, respectively. Cells were centrifuged at 800 xg for 25 minutes at 20 °C. The top 20 ml were then removed, centrifuged and washed twice. Cells were counted and resuspended in the appropriate amount of RPMI medium (HyClone) supplemented with 20% heat-inactivated FBS (Atlanta Biologicals), 1% sodium pyruvate (HyClone), 0.2% HEPES (OmniPur), 0.02% gentamicin (Atlanta Biologicals), and 10% human serum (pooled from four to five healthy donors) to give 1×10^6 cells per well of a Costar 12-well cell culture plate (Corning).

GM-CSF Preparation

Granulocyte-macrophage-colony stimulating factor (GM-CSF; Millipore) was dissolved in sterile water. A stock solution at a concentration of 0.1-1.0 mg/ml was made and aliquots were stored at -20 °C until use.

Macrophage Culturing

GM-CSF, at a concentration of 0.5 ng/ml, was added to each well of separated monocytes. The cell culture dishes were incubated at 37 °C with 5% CO₂ for 24 hours.

Fluorescence Microscopy

Once monocytes were separated from human whole blood and allowed to differentiate into macrophages for 24 hours, fluorescence microscopy was performed to assure that adherent cells were indeed macrophages. Cells were washed twice with 1 ml of Dulbecco's Phosphate Buffered Saline (DPBS; Atlanta Biologicals). Macrophages were stained with anti-CD14 antibody by flooding each well with 500µl of DPBS to which 10µl of anti-CD14 antibody conjugated with R-Phycoerythrin (PE; Invitrogen) was added. Cells were incubated at room temperature for 15 minutes and then washed twice with 500µl of DPBS. A final volume of 500µl of DPBS was added and cells were visualized under fluorescence microscopy.

Bacterial Cultures

Overnight cultures of each of the three bacteria were grown in 5 ml of BHI broth at 37 °C in a shaking incubator at 250 rpm. Suspensions of approximately 10⁷ cfu/ml were made in supplemented RPMI media (10% FBS, 1% HEPES, and 1% sodium pyruvate) using the turbidity regression lines.

Infection

Infections were performed using the three aforementioned bacterial strains and a mock infection (in which no bacteria were added) was included as a control. Cells were washed three times with

DPBS prior to addition of 1 ml of bacterial suspension (at 10^7 cfu/ml). Infections were performed at a multiplicity of infection (MOI) of 10:1 (bacteria to macrophages) in RPMI media. After 90 minutes, 200 μ g/ml of kanamycin was added to each well. Total RNA was extracted from each well at six hours post-infection.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the macrophage cultures using SurePrep TrueTotal RNA Purification Kit (Fisher Scientific) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop (ND-1000, V3.3.0) and cDNAs were synthesized using a QuantiTect Reverse Transcriptase (RT) Mix (Quiagen). The cDNA was used as the template in qPCR reactions.

Real-Time PCR and Determination of Relative Expression Levels

All PCR reactions were performed using an ABI Prism 7900HT (Applied Biosystems). Reagents used in reactions were from the QuantiTect Probe RT-PCR kit and included RNase-Free water and RT-PCR Master Mix. Expression of both IL-1 β and IL-8 mRNA was determined using oligonucleotide primers from Integrated DNA Technologies (TABLE 1). The TATA binding protein (TBP) mRNA was used as an internal control (TABLE 1). The results were presented as Ct values. Ct is defined as the cycle at which the amplified product crosses the threshold. Δ Ct was the difference in the Ct values of the gene of interest (IL-1 β or IL-8) and the TBP. The Δ Ct for each experimental sample (infected samples) was subtracted from the Δ Ct of the control sample (the uninfected, or mock, samples). This difference is designated $\Delta\Delta$ Ct. The

relative level of the difference of the gene of interest mRNA expression from that of a mock infection was reported as $\log(2^{-\Delta\Delta C_t})$, or the log of the fold expression (54).

ELISA Assays

Cells were infected with bacterial cultures, as described above, and at 6 hours post infection, supernatants were collected and filtered using Acrodisc Syringe Filters (Pall Corporation) to ensure removal of cells. Supernatants were stored at -80 °C until use. Supernatants from select samples were measured for IL-1 β and IL-8 using a QuantiGlo Chemiluminescent Immunoassay (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

The IL-1 β and IL-8 PCR data were transformed to the logarithmic (base 10) scale before analysis. Data were analyzed using mixed linear models with species of bacteria, sex of subject, diabetic status of subject, and age of subject, as well as interactions of these factors as predictors. Subjects were used as a random effect in the models. Residual plots were used to assess goodness of fit of the models. Models with interactions were compared to main effect models using likelihood ratio tests. Means for significant factors were compared using pairwise t-tests.

Results

Monocytes differentiate to macrophages are produced after a 24 hour incubation with GM-CSF

Many protocols and culture conditions were tested to determine those best suited for producing type-1 macrophages from monocytes separated from human whole blood. Monocytes from healthy donors (both male and female) were separated from whole blood using density gradients. These cells were incubated with GM-CSF for 24 hours. Examination of the cultures using phase microscopy revealed adherent cells with morphologies characteristic of macrophages. *In situ* staining with PE-labeled anti-CD14 antibody confirmed the cells to be macrophages (Figure 2 A and B). These cells were infected for a period of six hours using one of four infection schemes. The six-hour time-point was chosen based on similar macrophage infection studies and preliminary testing (70).

Macrophages produce IL-1 β and IL-8 when infected with B. pseudomallei, B. thailandensis, and E. coli

A preliminary infection (Figure 3) was performed to insure that macrophages isolated from a healthy subject and infected with bacteria were able to produce both IL-1 β and IL-8 at levels higher than those produced by uninfected cells. Monocytes were isolated from human whole blood, differentiated to macrophages for 24 hours in the presence of GM-CSF, and then infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli* in triplicate. After six hours, mRNA was collected and qPCR was performed. Infected macrophages produced greater than a 2.5 log fold increase of IL-1 β and almost a two log fold increase of IL-8, with *B. pseudomallei*-infected cells producing slightly less proinflammatory cytokines than macrophages infected with either *B. thailandensis* or *E. coli*.

Use of kanamycin does not affect cytokine production

Kanamycin was added 90 minutes after infection with each organism to inhibit extracellular growth of the bacteria. To be certain that the use of kanamycin was not affecting the production of mRNA for either cytokine, a six hour infection with *E. coli* was performed with and without kanamycin. As seen in Figure 4, the use of kanamycin did not appear to significantly alter the production of either IL-1 β or IL-8.

Preliminary testing with a single diabetic male and an age-matched, healthy control showed lower proinflammatory cytokine production by the diabetic

As seen in Figure 5, a preliminary infection using macrophages isolated from both a diabetic male and an age- and gender-matched control showed a significant decrease in the production of mRNA for both IL-1 β and IL-8. Macrophages from the diabetic patient produced significantly lower proinflammatory cytokines than those from the healthy control in response to all three infectious agents.

mRNA transcript levels of IL-1 β and IL-8 correlate generally with protein production

The first full set of infections (one male diabetic, one female diabetic, and their age- and gender-matched controls, Figure 6 A and B) produced results that differed from those of the preliminary infection reported above. Infected macrophages from the diabetic male showed little difference in comparison to those from the healthy control subject, with regards to cytokine production. However, cells from the diabetic female produced lower levels of IL-1 β and IL-8 compared to those from the healthy control. To confirm that this was true of both the mRNA and the protein itself, an ELISA for IL-1 β was performed on culture supernatants. As seen in Figure 6 C, the production of both IL-1 β transcript and protein correlated significantly with each other ($p < 0.012$). All samples of both mRNA and protein collected from cells which had been

infected with bacterial agents were normalized against the corresponding concentrations of mRNA and protein in mock infected cells.

Diabetes and gender showed no statistically significant difference with respect to proinflammatory cytokine profiles of macrophages infected with B. pseudomallei

A total of 40 volunteers were included in this complete study: ten diabetic males and ten healthy male age-matched controls, and ten diabetic females and ten healthy female age-matched controls. When the means of the complete data were analyzed, diabetes and gender were not significant factors in the production of IL-1 β and IL-8 in response to infection with *B.*

pseudomallei, *B. thailandensis*, or *E. coli* (Figure 7).

Age of subject may be a significant factor in the mean fold increase in IL-8 production in response to infection

Subject ages ranged from 18 to 57 (Table 2). Age was a significant factor in the production of IL-8 in response to infection, with the mean production of transcript increasing by a factor of 1.066 per year of age ($p < 0.01$). However, this affect was based upon the participation of only one pair of older females, both 57 years of age. Data from a larger number of older subjects are needed to confirm this trend.

Cytokine production differed depending on infectious organism

As expected, cytokine responses varied depending on the organism used to infect monocyte-derived macrophages. In the case of IL-1 β transcript production, a significantly lower response was seen when macrophages were infected with *B. thailandensis* as compared to either *B. pseudomallei* or *E. coli* (Figure 8 A). Production of IL-8 mRNA was upregulated most when the infectious agent was *E. coli*, while little differences were seen between *B. pseudomallei* and *B. thailandensis* (Figure 8 B).

HbA_{1c} levels of volunteers used in this study were all similar

As seen in Table 3, the HbA_{1c} levels of all donors who participated in the study were similar, with a mean HbA_{1c} of 7.5, and a range from 6.0-13.0. Three participants did not know their HbA_{1c} levels.

Discussion

Previous research (Laura Dickey) from our lab showed that there were significant differences in the production of both IL-1 β and IL-8 by monocytes infected with *B. pseudomallei* based upon gender and diabetic state (27). It was demonstrated that diabetic males produced significantly less IL-1 β and IL-8, as well as less TNF- α and IL-6, when compared to healthy, age-matched males, while monocytes derived from diabetic females showed an increase in the production of IL-1 β when compared to those from healthy, age-matched females. Since blood monocytes may not be the first cell most commonly encountered by *B. pseudomallei* early in the infectious process, we wanted to determine whether macrophages would respond similarly.

In this study, similar work to that of Laura Dickey was performed except macrophages and a larger sample size (twice the number used before) were used, and levels of IL-1 β and IL-8 were followed using qPCR. Macrophages are known to have greater immune response capabilities when challenged with microbial agents. Using these parameters, we hypothesized that significant effects due to gender and diabetic state would be amplified, possibly clarifying the dramatic susceptibilities of male diabetic patients to disease caused by *B. pseudomallei*.

Type I diabetes mellitus is a disease that affects multiple body systems. It is believed to be an autoimmune disease that is caused by the destruction of the insulin-producing beta cells in the pancreas. This leads to insufficient insulin production and blood glucose dysregulation, which affects immune function (52). Patients with diabetes mellitus appear to be more susceptible to certain bacterial infections than those who are healthy (45). The innate immunity of patients with diabetes mellitus has been shown to be altered, especially key neutrophil functions. For example, neutrophils from diabetics show not only reduced migration, but

reduced phagocytic capacity as well, compared to those from healthy subjects (16). Critical functions of macrophages from diabetics have been shown to be negatively altered as well (71). Though past research demonstrated an impairment of immune function in monocytes from diabetic males, the results of the current study show that macrophages differentiated in the presence of GM-CSF and normal human serum for 24 hours do not demonstrate these same impairments. These results suggest that macrophages that have differentiated outside of the diabetic environment seemed to have regained functions lost by diabetic monocytes, leading to monocyte-derived macrophages that now behave similar to those that came from healthy donors.

It has been shown that glycemic dysregulation can influence immune cell function, making it probable that once removed from the diabetic environment, macrophages are able to regain normal immune functions. Macrophages and neutrophils from patients with poorly managed diabetes mellitus have impaired immune functions (71). This was demonstrated by both Bagdade, et al, and Nolan, et al, who showed increases in both phagocytic and killing abilities in cells from diabetics, once the patient's serum glucose levels were brought to normal (6, 66). Nolan, et al, also showed that the intracellular killing of *Staphylococcus aureus* by granulocytes from diabetics was markedly decreased in an inverse proportion to serum glucose levels: the higher the glucose levels of the diabetic patient, and thus the less controlled the diabetic state, the less able the cells were to eliminate the bacteria (66). In addition, a study by McMahon, et al, showed that leukocytes from diabetic patients exhibiting poor glycemic control had impairments not only in phagocytosis and intracellular killing, but chemotaxis and adherence as well (59). However, once serum glucose levels were reduced through treatment, phagocytic cells recovered their normal abilities to phagocytose and eliminate bacteria (6, 45, 59, 66).

In the current study, the average HbA_{1c} level of all 20 diabetic donors was 7.5 (Table 3). While this is slightly higher than the level recommended by the American Diabetes Association, who recommends an HbA_{1c} level below 7.0, all donors closely monitored their serum glucose levels and took insulin daily.

Diabetics living within many of the regions in which *B. pseudomallei* is endemic most likely have glucose levels less well managed, as many do not have access to adequate health care. Without proper treatment and education, glycemic control would be less well managed and elevated serum glucose levels would likely reduce the ability of those infected with *B. pseudomallei* to fight infection. This may at least partly explain why diabetes mellitus is a risk factor for melioidosis. Diabetes is also well documented as a risk factor for many other gram negative infections (9, 45, 59, 71).

In Australia, indigenous status is a risk factor for melioidosis. In the northern part of the country, aboriginal patients account for approximately half of all melioidosis cases (20). This risk factor may be due to the high incidence of diabetes in the Aboriginal population. In a study done by McDermott, et al, it was discovered that the incidence of diabetes in two groups of indigenous people in Australia was almost four times higher than that reported in the general population (58). Depending on location, proper diabetes care may not always be available, which implies that within the Aboriginal population, diabetes may run unchecked, leading to poor blood glucose control (81). The aboriginals also tend to live traditionally which puts them in close contact with the environment and the affects of the changing season, particularly heavy rain (20).

Taking all of this into account, the normal responses of the macrophages used in this study may be due to not only the donor's excellent glycemic control, but by the fact that the cells

were cultured for 24 to 30 hours outside of the diabetic environment. These factors all could have influenced the perceived ability of macrophages from diabetic donors to regain more normal immune response capabilities.

Hormones and certain types of medications, such as steroids, have been shown to alter the immune response. Production of cytokines, such as IL-1, IL-6, TNF- α , and TGF- β , by monocytes and macrophages, can be affected negatively by estrogen (48, 62). Glucocorticoids (GCs), which are commonly used to treat many illnesses such as asthma and allergies, as well as other common medications, can also interfere with immune responses. GCs repress pro-inflammatory mediators and induce the synthesis of anti-inflammatory molecules (91). GCs are known to suppress cytokine and chemokine production and cell adhesion molecules, and as Tuckermann, et al, demonstrated, GCs can suppress macrophage functions (91, 92). GCs interfere with macrophage function at many levels: they can hinder differentiation, proliferation, and production of certain cytokines, including IL-1 and IL-8 (91). Although a short survey was given to each of the diabetic donors to better understand their diabetic condition, no questions regarding hormones or current medications were asked of either diabetic or non-diabetic donors. Because estrogen is a common hormone used in birth control, and GCs, as well as other medications, are prescribed to treat common maladies, any of these unknown factors could have affected the results of this study.

It is known that age is also a predisposing risk factor for melioidosis, with individuals over the age of 45 being more susceptible to infection with *B. pseudomallei* (19, 25, 73). It is also well known that age is associated with a decline in immune function (28, 35, 68). In a study done by Chen, et al, it was demonstrated that adults over the age of 60 are especially at risk for infection with *B. pseudomallei* (17). In the previous research done by Laura Dickey, the sample

population examined consisted of individuals that were over the age of 40. This study involved donors that were between the ages of 19 to 27 years old, with only one pair of females over the age of 40 (Table 2). The lower average age for those in this study may have also factored into the results seen here.

The current study did show significant differences in cytokine production depending on the infectious organism (Figure 8). Production of IL-1 β mRNA was significantly lower ($p < 0.001$) in macrophages infected with *B. thailandensis*, while production of IL-8 mRNA was significantly higher ($p < 0.0001$) in those infected with *E. coli*. Infection with *B. thailandensis* produced the lowest increase of both cytokines. *B. thailandensis* is rarely pathogenic to humans and a significantly higher dose is needed for infection in animal models (41). It creates a strong innate immune response and because of this response, *B. thailandensis* is able to be quickly cleared from the host.

Results from this study were consistent with those from studies with *E. coli*, in that this organism is a strong stimulator of the innate response due to its Lipid A component. Lipid A plays an important role in stimulating the immune system, and *E. coli* has one of the most biologically active forms of this molecule (67). The LPS of *B. pseudomallei* stimulates both human and murine macrophages to produce less IL-6, IL-10, and TNF- α than does the LPS of either *B. thailandensis* or *E. coli*, as demonstrated by Novem, et al (67). It was shown that the LPS of *B. pseudomallei* causes a response from the host immune system that is different from those generated by other gram negative bacteria: the LPS of *B. pseudomallei* has weaker pyrogenic activity in mice (4). Arjcharoen, et al, also showed that the LPS of *B. pseudomallei* exhibits weaker and slower activation kinetics for mouse macrophage cell lines (4). While this study showed that IL-8 mRNA production was decreased in both *Burkholderia* species as

compared to *E. coli*, the stimulation of production of IL-1 β by *B. pseudomallei* was actually higher than that of *B. thailandensis* (Figure 8). The macrophage culturing conditions used in this study may have altered the cells responses to infection and may explain why the results from this part of the study differ from what has been described previously.

Nearly all people in endemic regions are seropositive for *B. pseudomallei*, yet not all have active disease. This reinforces the belief that host susceptibility may have a role in melioidosis. While diabetes is a risk factor for melioidosis, male diabetics are known to be particularly susceptible to infection with *B. pseudomallei* (25). Previous research has demonstrated that monocytes from male diabetics produce a blunted proinflammatory cytokine response when infected by *B. pseudomallei* (27). In the current study, monocytes were isolated from diabetic and age-matched healthy controls using density gradients and cultured in media containing normal human sera and GM-CSF. The addition of GM-CSF to the media has been shown to produce M1 macrophages, which are known to produce proinflammatory cytokines (98). After 24 hours, *in situ* staining of adherent cells with PE-labeled antibody against human CD14 demonstrated the presence of macrophages. After a 6 hour infection with either *B. pseudomallei*, *B. thailandensis*, or *E. coli*, total RNA was extracted from cells, quantified, converted into cDNA, and qPCR was performed for the presence of IL-1 β and IL-8 transcript. All levels of mRNA were standardized to the levels of mock infected (uninfected) cells that were run as a control for each experiment. When the means of the major factors were analyzed, it was determined that under the conditions and parameters established for this study, diabetic state and gender did not have an effect on the IL-1 β and IL-8 responses of infected monocyte-derived macrophages.

The results of this study in combination with the research performed by Laura Dickey indicate the importance of maintaining the diabetic condition throughout the infection process. Once removed from the diabetic environment, macrophages seem to regain immune functions similar to the functions of cells isolated from healthy donors. Thus, it is imperative that the conditions under which differentiation and infection take place are as close as possible to the original diabetic environment. This outcome will be valuable to future research investigating effects of the diabetic state on immune cell functions and may have important implications towards the treatment of diabetes.

Further testing will need to be performed to determine the effects of the diabetic environment on the ability of macrophages to respond to infection. Studies using older diabetic donors that have poor glycemic control and healthy age- and gender-matched controls will need to be performed. By using serum from these donors in the differentiating environments in a reciprocal crossover study, in which each type of macrophage will be incubated in each type of serum, perhaps the true effects of the diabetic state can be determined. These studies are currently underway.

Table 1. Sequences of primers and probes used for q-PCR detection of log fold increases in proinflammatory cytokines.

GENE	FORWARD PRIMER	REVERSE PRIMER	PROBE
IL-1 β	5'-TGGAGCAACAAGTGGTGT-3'	5'-TTGGGATCTACTCTCCAGC-3'	5'-CCTTGAGGCCCAAGGCCACA-3'--FAM
IL-8	5'-GCCAACACAGAAATTATTGTAAGCTT-3'	5'-AATTCTCAGCCCTTCAAAAATT-3'	5'-AGAGCTCTGTCTGGACCCAAGGAAAAC-5'--TET
TBP	5'-CACGGCACTGATTTTCAGTTCT-3'	5'-TTCTTGCTGCCAGTCTGGACT-3'	5'-TGTGCACAGGAGCCAAGAGTGAAGA-3'--HEX

Table 2. Ages of blood donors used in this study.

Male		Female	
Diabetic	Non-Diabetic	Diabetic	Non-Diabetic
24	24	23	24
24	24	19	20
20	22	19	21
20	18	21	21
21	22	27	26
24	24	24	24
21	23	22	20
21	22	57	57
29	27	22	20
19	18	25	27

Table 3. HbA_{1c} levels of diabetic donors.

Subject	A1c level
M prelim	7.8
F1	6
M1	7.1
F2	8.6-9
M2	7.2
F3	unknown
M3	unknown
F4	6.9
M4	7.0-8.0
F5	7.6
M5	6.1
F6	6.0-8.0
M6	13
F7	8.4
M7	unknown
F8	7.9
M8	6.2
F9	7.4
M9	6.7
F10	7.2
M10	7.5

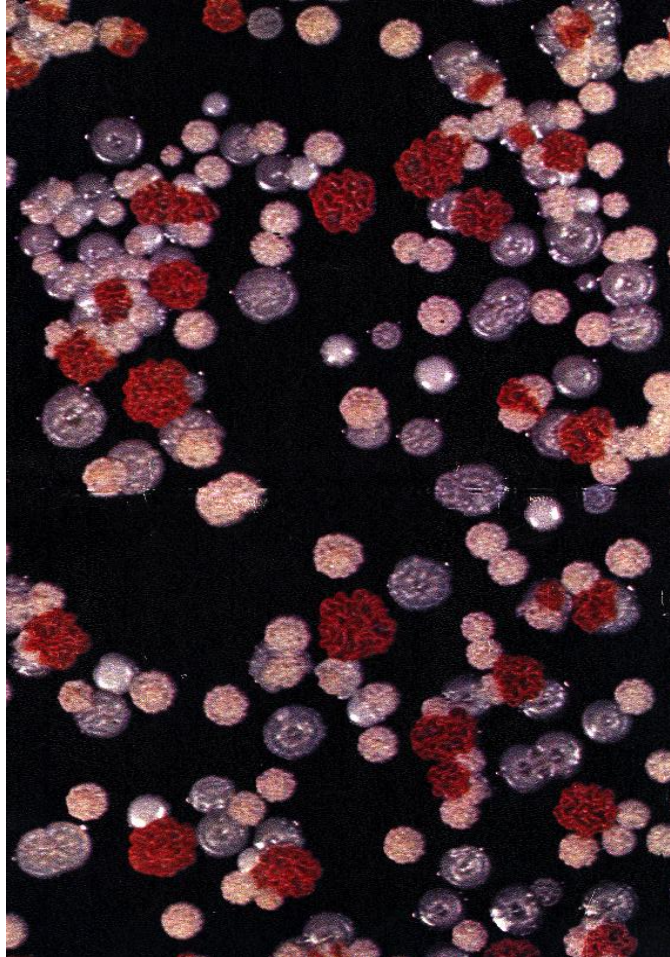


Figure 1. The variable colony morphology of a pure culture of *Burkholderia pseudomallei*.

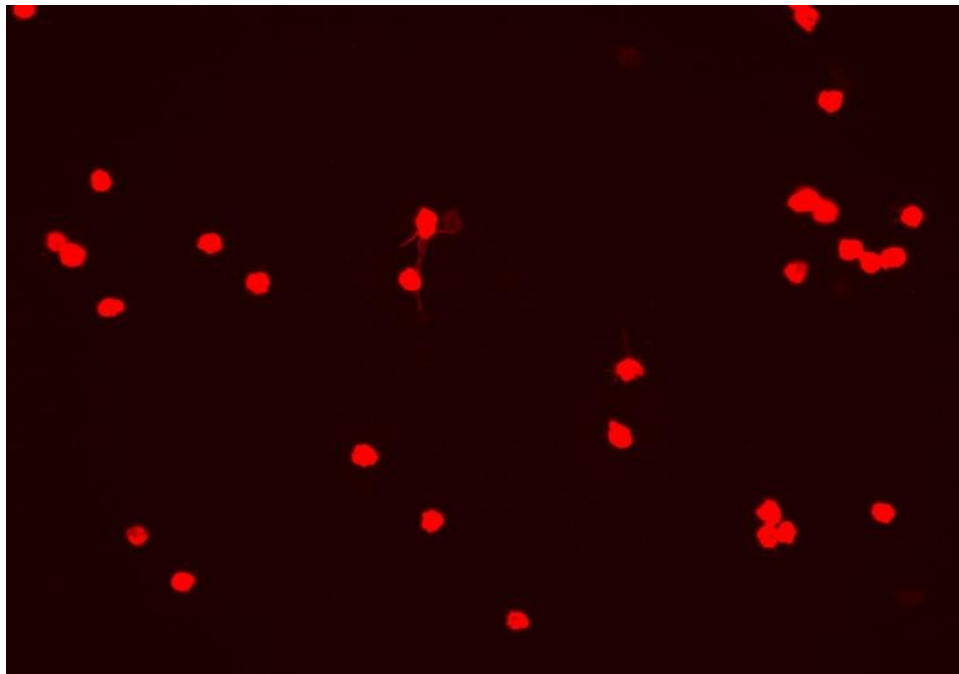
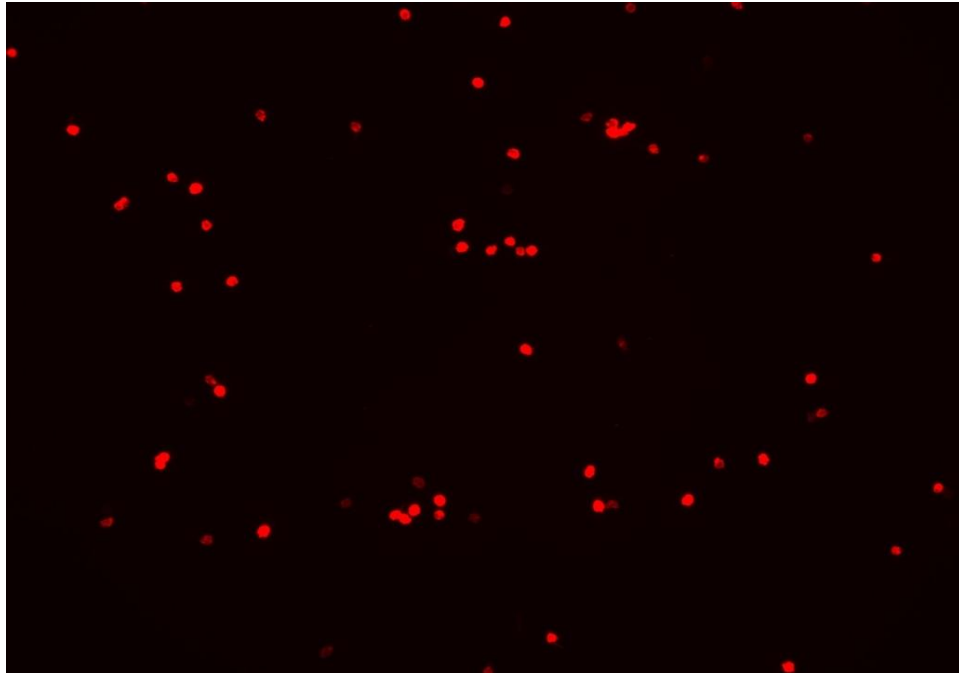


Figure 2. Macrophages isolated from human whole blood. Using OptiPrep density gradients, monocytes were separated from human whole blood and then allowed to grow and differentiate into macrophages for 24 hours in supplemented RPMI with GM-CSF. Macrophages were stained with PE-labeled anti-CD14 antibody and viewed under fluorescence microscopy at 10x (A) and 20x (B).

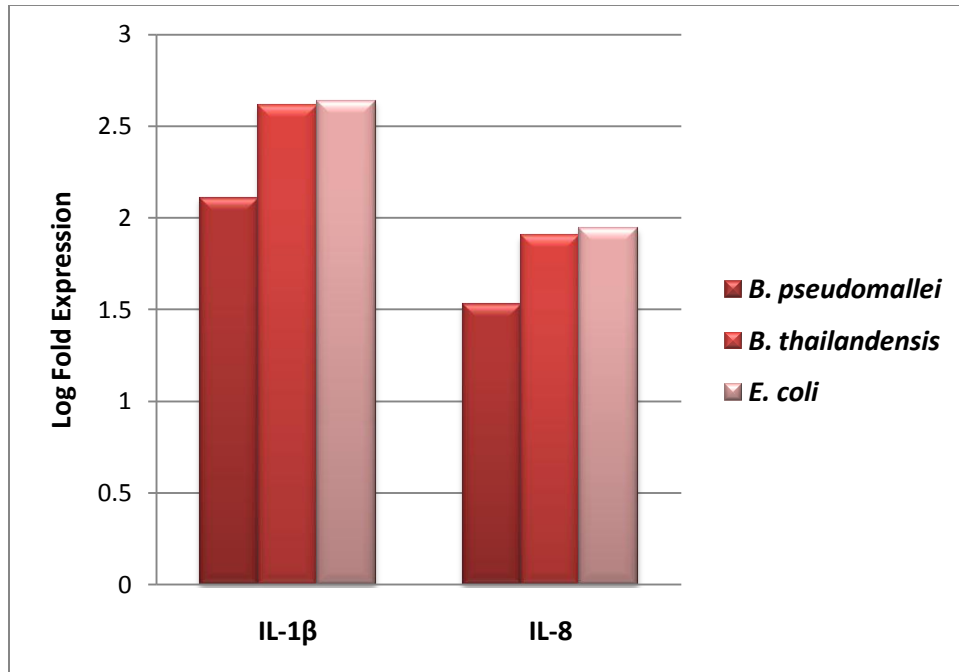


Figure 3. Log fold increase in expression of IL-1 β and IL-8 in human macrophages infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Cells were infected at an MOI of 10 and mRNA was extracted at 6 hours post-infection. Total mRNA was assayed using qPCR.

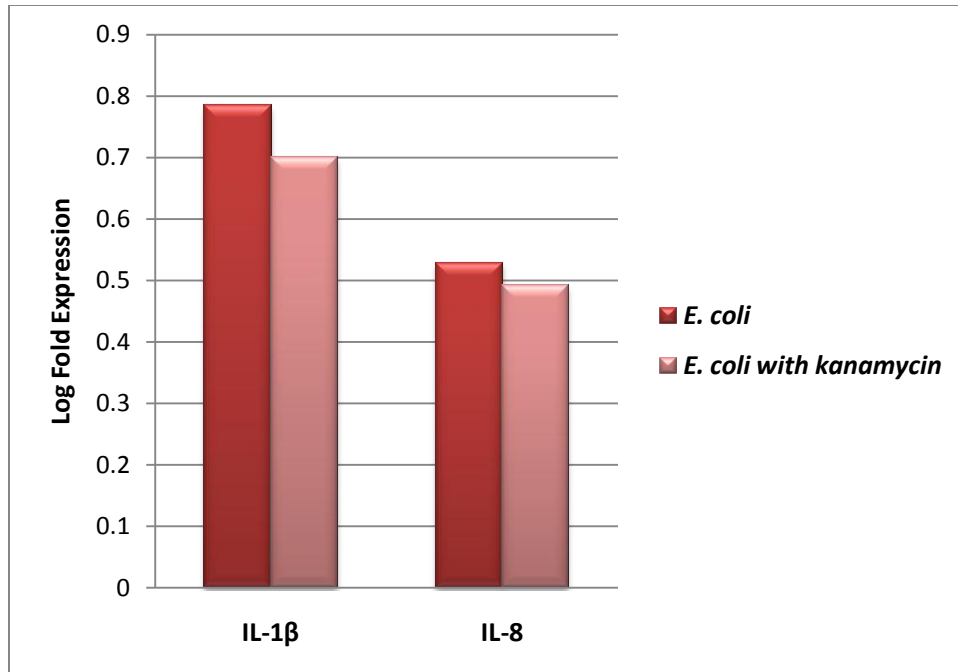
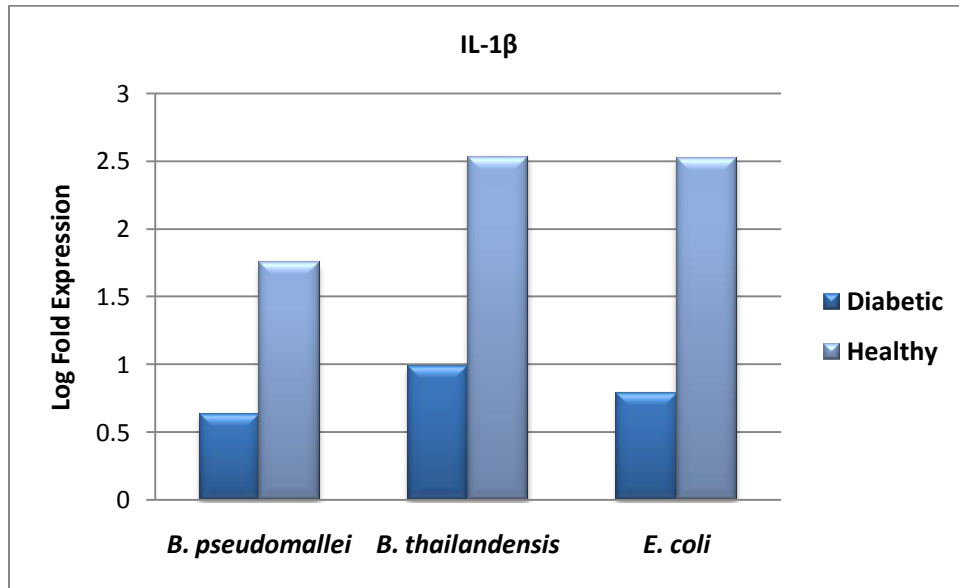


Figure 4. Effect of kanamycin on IL-1 β and IL-8 mRNA production. Kanamycin was added 90 minutes p.i. Macrophages from a healthy male were infected with *E. coli*. mRNA was collected 6 hr p.i. and qPCR was performed. The use of this antibiotic did not significantly affect IL-1 β or IL-8 mRNA production.

A.



B.

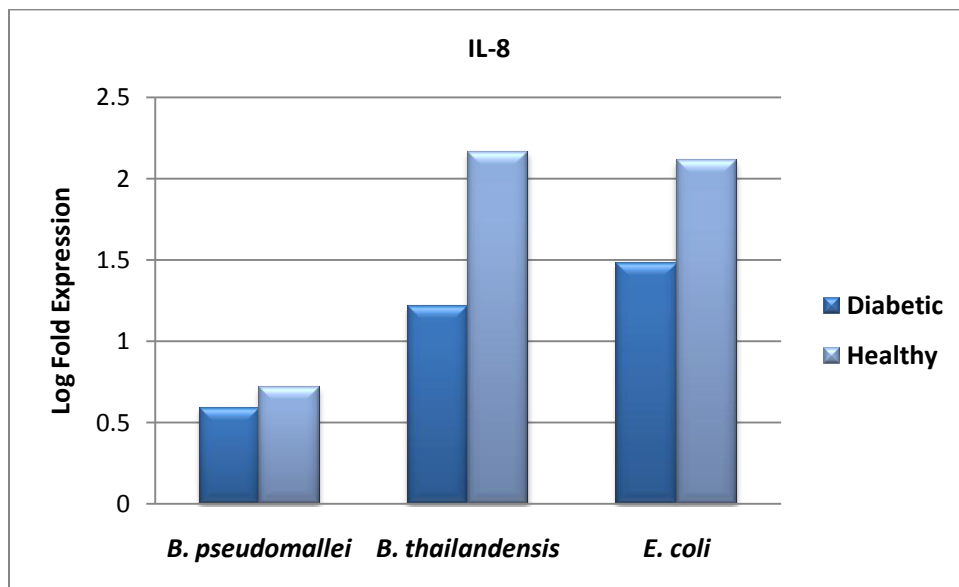
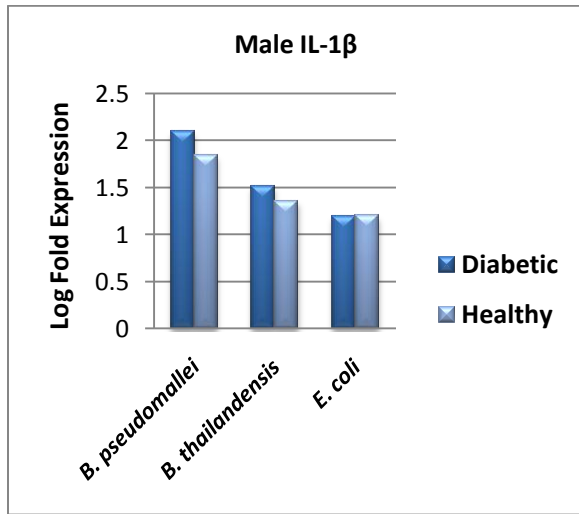
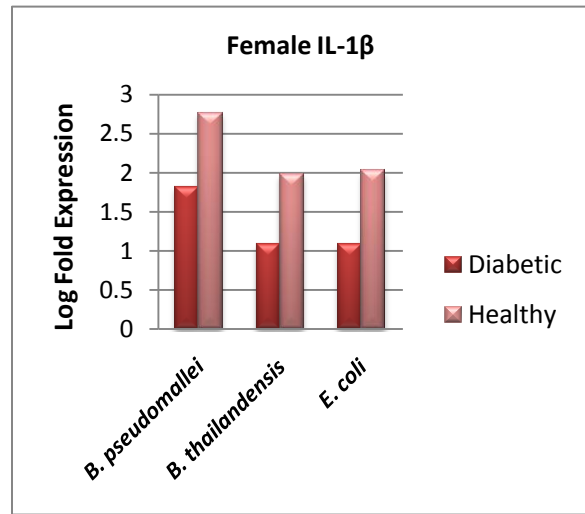


Figure 5. IL-1 β (A) and IL-8 (B) mRNA production by macrophages from a diabetic male and an age-matched healthy control in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Monocytes collected from a male diabetic and an age-matched control were allowed to differentiate to macrophages and were then infected at an MOI of 10. mRNA was collected 6 hr p.i. and qPCR was performed.

A.



B.



C.

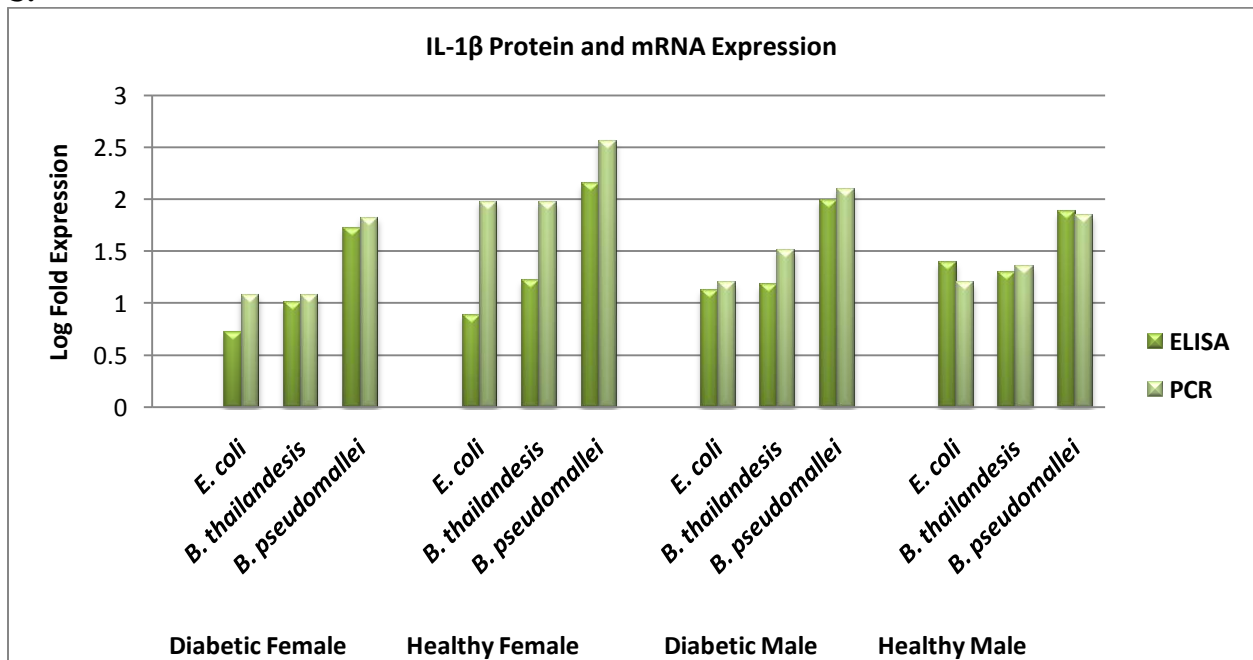
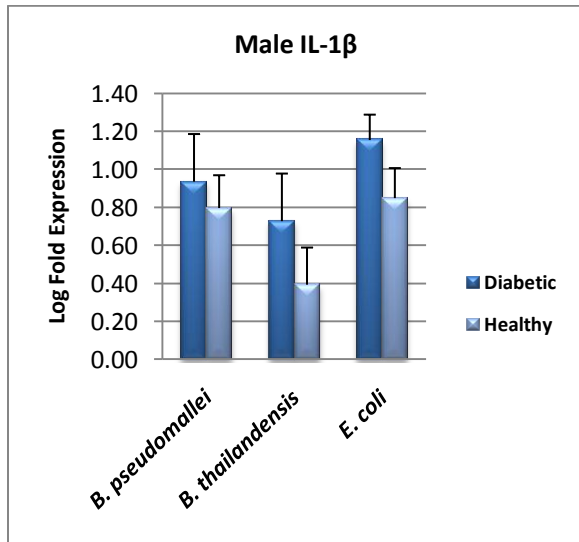
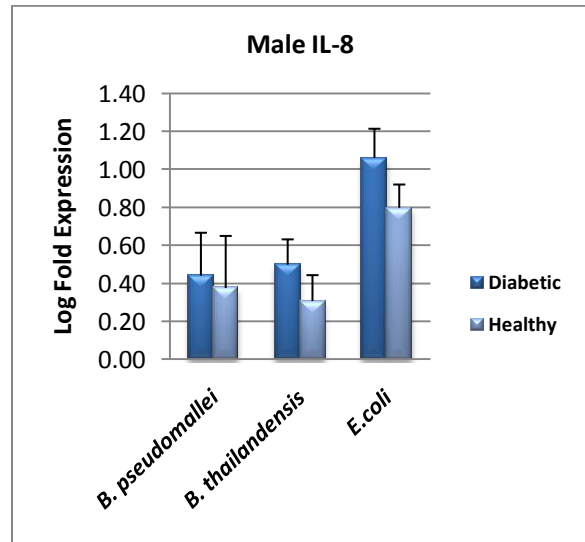


Figure 6. Expression of IL-1 β by infected macrophages from both male (A) and female (B) diabetics and healthy age- and gender-matched controls. Monocytes were isolated from whole blood, differentiated into macrophages and infected at an MOI of 10. mRNA and supernatants were collected 6 hr p.i. (C). Supernatants were assayed for IL-1 β levels using ELISA and IL-1 β mRNA was assayed using qPCR. IL-1 β mRNA production and protein production correlated significantly ($p < 0.01$).

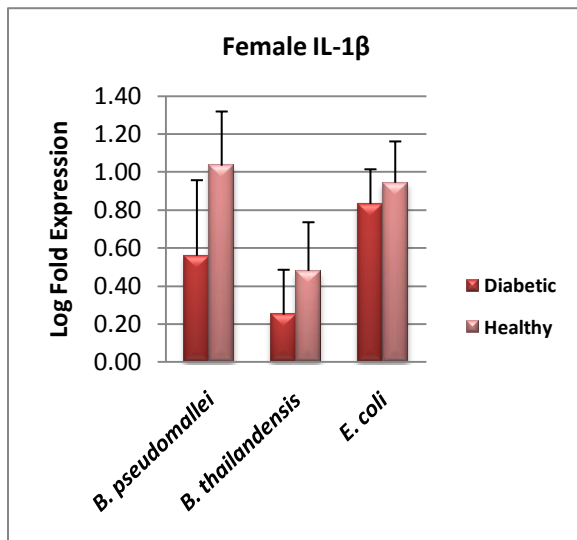
A.



B.



C.



D.

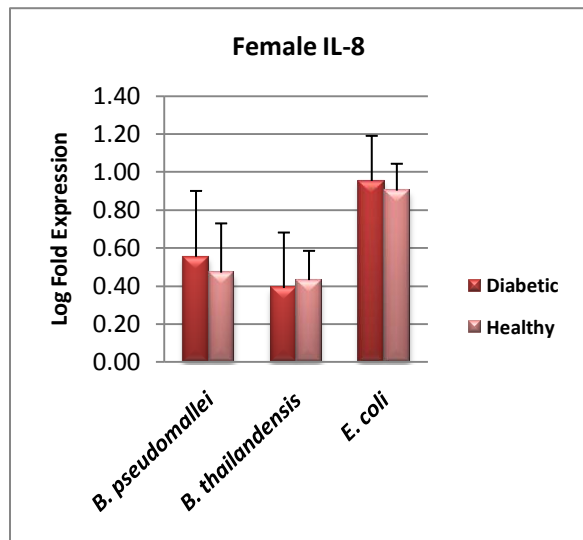
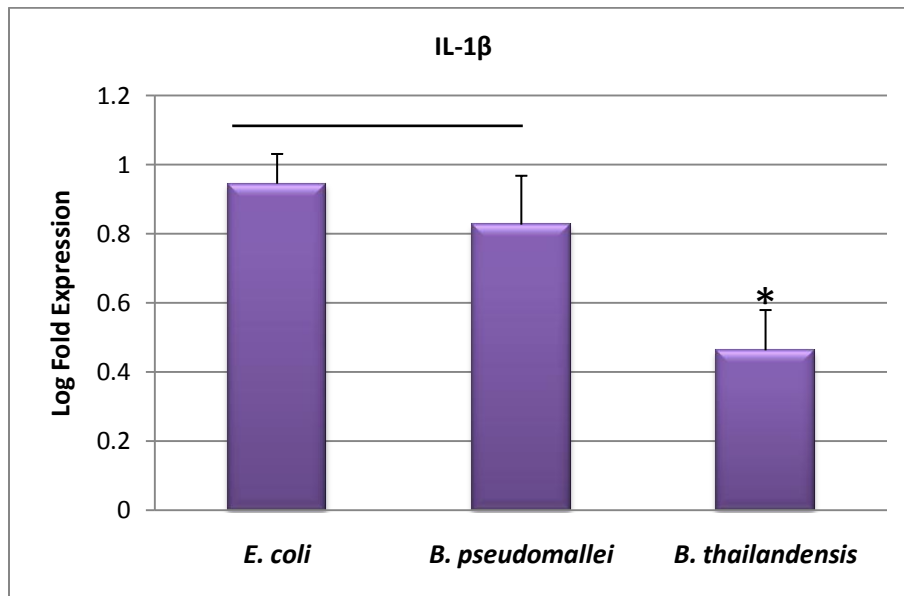


Figure 7. Mean log fold increases in IL-1 β and IL-8 mRNA by macrophages from both male and female, diabetic and age-matched healthy controls, infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Monocytes were isolated from whole blood and differentiated into macrophages. Macrophages were then infected at an MOI of 10. mRNA was extracted at 6 hr p.i. and qPCR was performed. Each point represents a mean of 10 experiments. Standard errors are shown.

A.



B.

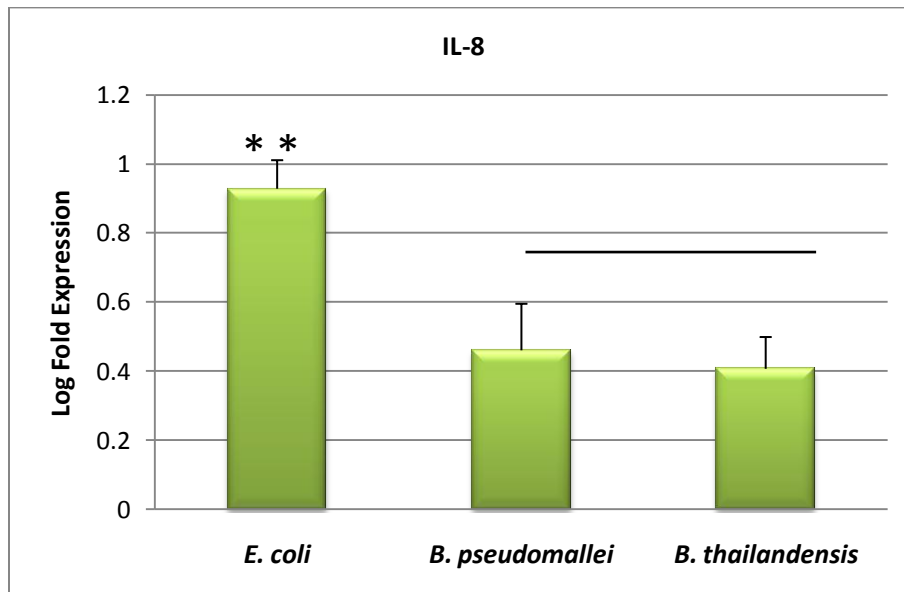


Figure 8. Mean log fold increase in IL-1 β (A) and IL-8 (B) production by macrophages when infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli*, averaged across gender and diabetic state. mRNA was extracted at 6 hr p.i. and qPCR was performed. Macrophages responded differently to infection with each organism. Each value represents a mean of 40 experiments. Standard errors are shown.

*indicates significant difference in cytokine production ($p < 0.001$)

**indicates significant difference in cytokine production ($p < 0.0001$)

Bar indicates no significant difference

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