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An Infection Model for Examining the Effects of Gender and Diabetic State on Proinflammatory Cytokine Secretion by Phagocytic Cells in Response to Infection with Burkholderia pseudomallei

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AN INFECTION MODEL FOR EXAMINING THE EFFECTS OF GENDER AND

DIABETIC STATE ON PROINFLAMMATORY CYTOKINE SECRETION

BY PHAGOCYTIC CELLS IN RESPONSE TO INFECTION WITH

BURKHOLDERIA PSEUDOMALLEI

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

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Burkholderia pseudomallei is an opportunistic soil pathogen that causes melioidosis, a

life-threatening human disease prevalent in Southeast Asia, northern Australia, the

Middle East, Africa, and South America. The organism also causes disease in plants and

animals. Persons with severe melioidosis usually die of septicemia. Relatively little is

known regarding the virulence mechanisms of *B. pseudomallei*; however, several putative

virulence determinants have been identified. The organism is able to invade and replicate

within phagocytic cells and is particularly pathogenic in males with diabetes mellitus. *B.*

thailandensis is closely related to *B. pseudomallei,* but is not pathogenic. This study

examined various *in vitro* monocyte / macrophage infection models used to study innate

immune responses to *B. pseudomallei*. Several monocyte and macrophage models showed little or no significant differences between proinflammatory cytokines secreted in response to infection with *B. pseudomallei* and *B. thailandensis*. Peripheral blood monocytes from diabetic males produced lower normalized levels of proinflammatory cytokines IL-1α, IL-1β, IL-6, and IL-8 than monocytes from healthy males in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Surprisingly, normalized levels of secreted IL-1β from *B. pseudomallei*-infected monocytes from diabetic females were higher than levels from healthy females. The results revealed a significant interactive effect of gender and diabetic state on peripheral blood monocyte secretion of IL-1β (p = 0.0370) and IL-8 (p = 0.0390), as well as a significant interactive effect of diabetic state and type of infectious agent on peripheral blood monocyte secretion of IL-1α (p=0.0210) and IL-6 (p=0.0204). These results may help explain why diabetic males are unusually susceptible to infection with *B. pseudomallei*.

ACKNOWLEDGMENTS

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Introduction

Burkholderia pseudomallei is an opportunistic soil pathogen that causes melioidosis, a life-threatening, human disease prevalent in Southeast Asia, northern Australia, the Middle East, Africa, and South America. The organism also causes disease in plants and animals.

B. pseudomallei is resistant to many antibiotics (18) and environmental extremes; in fact, it can survive in triple-distilled water for years (72). Melioidosis results in death in almost 100% of non-treated patients. The Center for Disease Control (CDC) has identified *B. pseudomallei* as a Category B select agent that could be used as a biological weapon. There is currently no vaccine available for melioidosis.

Characteristics of Burkholderia pseudomallei

Taxonomy and phylogeny. *Burkholderia* species were previously classified in the genus *Pseudomonas*; however, in 1991, Yabucci et al. proposed that seven species in the *Pseudomonas* RNA homology group II be classified under a new genus, *Burkholderia* (74). *B. pseudomallei* is a gram-negative, motile, aerobic bacillus whose genome is composed of two chromosomes. The larger chromosome (4.07 Mb) contains so-called housekeeping genes required for cellular growth and maintenance, while the smaller chromosome (3.17 Mb) contains accessory genes needed for functions such as adaptation and survival in harsh conditions (69).

The *Burkholderia* genus comprises over thirty species. *B. mallei* is primarily an equine, zoonotic pathogen that causes either chronic infections characterized by nasal and subcutaneous nodules, or acute infections that can result in nodular formation in the lungs,

ulcerated mucous membranes, severe septicemia, and death. *B. cepacia* is a human pathogen that often causes pneumonia in individuals with underlying lung diseases such as cystic fibrosis.

B. thailandensis is closely related to *B. pseudomallei*, but exhibits 10^5-fold less virulence in Syrian hamsters and mice (9). The two bacteria coexist in tropical soils, and were originally classified as the same species. In 1998, Brett et al. proposed that the species *B. thailandensis* be created because of differences in virulence (9), biochemical profiles (73), and 16S rRNA nucleotide sequences (9). Because of its high similarity to *B. pseudomallei*, *B. thailandensis* has been used in comparative virulence studies with *B. pseudomallei* (33, 70).

Other *Burkholderia* species include numerous plant pathogens, as well as nonpathogenic soil dwellers that interact with plants and whose activities include iodinin production and nitrogen fixation (15). Recently, Mao et al. identified a novel *Burkholderia* strain designated MP-1 that produces antifungal compounds active against various filamentous plant fungi (39).

Clinical Manifestations. Melioidosis is a febrile illness that can present in many forms, ranging from acute severe septicemia to chronic, debilitating, localized infections (13). Most patients display purulent abscesses in one or more tissues. The lungs are the most commonly affected organs, but the disease also frequently targets the liver, spleen, skeletal muscle, prostate, kidneys, meninges, skin, eyes, and brain. *B. pseudomallei* has been shown capable of infecting almost every organ in the human body.

Epidemiology. Melioidosis is prevalent in tropical regions within approximately 20˚ N and 20˚ S of the equator (13). The disease is most commonly recognized in the northeast province Ratchathani, Thailand, where *B. pseudomallei* is responsible for 20% of all community-acquired septicemias and causes death in 40% of treated patients (68). It is also a common pathogen in northern Australia, where the overall mortality rate is about 20% (12) and where in 1998, the annual incidence of melioidosis reached 41.7 cases per 100,000 (13).

B. pseudomallei is an environmental saprophyte that occurs widely in the soil and water in areas where the disease is endemic. The majority of melioidosis cases result from direct contact with contaminated soil and water (19). Humans and animals are believed to acquire the pathogen by inhaling dust, ingesting contaminated water, or directly acquiring the organism from soil (19), presumably through skin abrasions.

In many cases, clinical manifestations do not present until many years after initial contact with the organism. Ngauy et al. reported the case of a diabetic World War II veteran who did manual labor as a Japanese prisoner of war for two years in Thailand and other areas in Southeast Asia 62 years before presenting with a cutaneous, nonhealing ulcer that was found to be infected with *B. pseudomallei* (44). The organism was one of the primary etiological agents of death subsequent to contamination of battle wounds in the Vietnam War. Its ability to remain latent in the body for years before causing disease earned *B. pseudomallei* the fitting designation of the 'Vietnam time bomb' (65).

Putative virulence factors

Compared to other gram-negative pathogens, very little is known regarding the virulence mechanisms of *B. pseudomallei*. Several putative virulence factors that have been

identified are discussed below. Most of these are involved in evading host innate immunity; however, extensive research in this area is needed to further elucidate the host-pathogen interactions and molecular mechanisms involved in *B. pseudomallei* pathogenesis. In addition, the mechanisms involved in latency are completely unknown.

Capsular polysaccharide. *Burkholderia pseudomallei* produces an extracellular polysaccharide capsule composed of (-3)-2-*O*-acetyl-6-deoxy-ß-D-*manno*-heptopyranose-(1-). Reckseidler et al. showed that a *B. pseudomallei* strain with a loss-of-function mutation in a gene required for capsular polysaccharide expression had an LD_{50} in Syrian hamsters that was similar to the LD_{50} for *B. thailandensis*, and four orders of magnitude higher than the LD_{50} for wild-type *B. pseudomallei* (48). They later showed that production of the capsular polysaccharide contributed to evasion of death due to complement activation by reducing complement factor C3b deposition and opsonization (49).

Type-III secretion systems. Genes encoding three separate type-III secretion systems have been identified in the *B. pseudomallei* genome. The first two (TTSS1 and TTSS2) are similar to those found in *Ralstonia solanacearum*, a plant pathogen. The third system (TTSS3) is homologous to the type-III secretion system found in *Salmonella typhimurium* (4). Warawa et al. demonstrated that TTSS3 is necessary for full virulence in a hamster model; however, they did not observe any attenuated virulence when they individually mutated several TTS-related effector molecules (67). Stevens et al. showed that *B. pseudomallei* strains with mutations in the *bsa* locus, which encodes proteins involved in a secretion system similar to the *Salmonella* Inv/Spa/Prg and *Shigella*

Inv/Mxi-Spa secretion systems, exhibited reduced phagocyte replication, decreased ability to escape phagocytic vacuoles, and lack of formation of membrane protrusions and actin tails (54).

Flagella. *B. pseudomallei* flagella have been proposed as both potential vaccine targets and putative virulence factors; however, antithetical sets of evidence render the latter clause controversial. DeShazer et al. demonstrated that there was no significant difference in the LD_{50} of wild-type *B. pseudomallei* and aflagellate mutants when tested in Syrian hamsters and diabetic mice models (21). Chua et al. showed that while an aflagellate *B. pseudomallei* mutant was not impaired in its ability to invade and replicate within human epithelial lung cells *in vitro* or kill nematodes in slow-killing assays, the mutant was avirulent compared to wild-type strains in BALB/c mice infected intranasally and intraperitoneally (14). This suggests that flagella may contribute to virulence in an infection route-dependent manner. Boonbumrung et al. used swim and swarm assays to demonstrate that 205 clinical isolates obtained from melioidosis patients at Sappasithiprasong Hospital (in northeast Thailand) showed highly conserved flagella expression; however, they found no correlation between motility and clinical outcomes such as septicemia and death (7).

Lipopolysaccharide. The role of lipopolysaccharide (LPS) in the pathogenesis of melioidosis is unclear. The LPS of gram-negative bacteria is commonly associated with septic shock caused by cytokine overload, and this phenomenon frequently causes death in severe cases of melioidosis; however, the LPS of *B. pseudomallei* is different from the LPS of other gram-negative bacteria. Matsuura et al. speculated that the LPS fatty acid

tails are longer in *B. pseudomallei* than in other gram negative bacteria. They demonstrated that *B. pseudomallei* LPS was less pyrogenic in rabbits, less lethally toxic in mice, less able to activate macrophages, and had stronger mitogenic activity in murine splenocytes than enterobacterial LPS (41). Anuntagool et al. showed that LPS from *B. pseudomallei* and the LPS from *B. thailandensis* are immunologically indistinguishable (3). The LPS from several other gram-negative bacteria is recognized by Toll-like receptor 4 (TLR4), a signaling molecule that associates with macrophage receptor CD14 and facilitates the release of inflammatory cytokines (58). An interaction between *B. pseudomallei* LPS and TLR4 has not been described. C3H/HeJ mice, which are defective in TLR4-mediated signaling and hyporesponsive to infections with most gram-negative bacteria, are highly resistant to infection with *B. pseudomallei* (41).

Cable pili. Cable pili are peritrichous adherence organelles that mediate binding of bacteria to host epithelial cells and mucin. Open reading frames in *B. pseudomallei* encode seven putative type IV pili. Mutations in a gene predicted to encode a type IV A pilus structural protein led to attenuated virulence in a hamster model and reduced adherence to human epilthelial cells (23).

Quorum sensing. Quorum sensing enables bacteria to communicate with other bacteria in a density-dependent manner and to coordinate behavior via signaling molecules. Ulrich et al. demonstrated that *B. pseudomallei* encodes at least three *luxI* and five *luxR* homologues involved in virulence. They used mass spectrometry analysis of culture supernatants to reveal that the organism produced many signaling molecules, including *N*-octanoyl-homoserine lactone, *N*-decanoyl-homoserine lactone, *N*-(3-hydroxyoctanoyl)-

L-homoserine lactone, *N*-(3-hydroxydecanoyl)-L-homoserine lactone, and *N*-(3-oxotetradecanoyl)-L-homoserine lactone. They also demonstrated that *B. pseudomallei* strains with mutations in putative quorum sensing genes had increased times to death and reduced organ colonization in infected BALB/c mice (61).

Actin-based motility. Once *B. pseudomallei* has been internalized by host cells, the organism can escape endocytic vacuoles into the cytoplasm, induce formation of membrane protrusions, and facilitate cell-to-cell spread via actin-based motility. Breitbach et al. demonstrated that the organisms induce actin rearrangement in animal host cells by recruitment and incorporation of α-actinin and the Arp 2/3 complex (8). Stevens et al. identified a previously unknown bacterial factor, BimA, and demonstrated that it was required for *B. pseudomallei* actin-based intracellular motility (55).

Morphotype switching. It has long been known that pure cultures of *B. pseudomallei* exhibit many different colony morphologies. Some researchers have hypothesized that the organisms undergo a process of adaptation in which they alter their surface determinants in various host environments. Recent studies by Peacock et al. have classified seven different isogenic types and demonstrated the organism's ability to undergo morphotype switching *in vivo* during various growth conditions. Mixed morphotypes were present in individual clinical specimens. In addition, some morphotypes were associated with latent infections while others were associated with acute infections (11).

Other putative virulence factors. In addition to the factors described above, *B. pseudomallei* cells are resistant to host defenses such as defensins and dismutase, and

secrete a variety of cell-damaging products (13). *B. pseudomallei* is able to invade many types of eukaryotic cell lines, including epithelial cells, neutrophils, and macrophages. After being engulfed by professional phagocytes, the bacteria are able to escape from the phagosome and replicate in the cytosol (30, 54).

Comparisons to B. thailandensis

Because *B. pseudomallei* and *B. thailandensis* are closely related and differ primarily in their ability to cause disease, many researchers have used comparative bioinformatics to study the genomic and proteomic differences between the two species in an effort to elucidate *B. pseudomallei's* mechanisms of pathogenesis.

Interestingly, *B. thailandensis* contains a nine-gene, arabinose-assimilation operon that is absent in both *B. pseudomallei* and *B. mallei*. Chromosomal studies by Moore et al. indicate that the operon was deleted from *B. pseudomallei* and *B. mallei* genomes. When the group cloned the operon into a laboratory strain of *B. pseudomallei*, the mutant was less virulent than the parent strain in Syrian hamsters. The group also used microarray analysis to demonstrate that in the presence of L-arabinose, several genes in the TTS3 cluster of the mutant's genome were down-regulated, suggesting that an L-arabinose metabolite may play a role in the transcriptional regulation of one or more virulence genes (43). In *E. coli*, the araC gene is adjacent to the L-arabinose operon and is one of the primary proteins involved in repression and induction of the operon (35). In *V. cholerae*, the AraC/XylS family member ToxT has been shown to activate the expression of both cholera toxin and the toxin-coregulated pilus (16).

Reckseidler et al. used subtractive hybridization to compare the genomes of *B. pseudomallei* and its avirulent relative *B. thailandensis* in an effort to identify virulence

determinants of the former. They found several A-T-rich DNA sequences unique to

B. pseudomallei. One of these regions was identified as the capsular polysaccharide gene

cluster discussed previously (48).

Kespichayawattana et al. showed that *B. pseudomallei* was able to more

efficiently invade and adhere to epithelial cells than *B. thailandensis* (33). Recently

Wontrakoongate et al. used two-dimensional gel electrophoresis and mass spectrometry

to compare the proteomic profiles of the two species. They found two proteins expressed

in *B. pseudomallei* that are not expressed in *B. thailandensis* under certain growth

conditions (70). The role of these proteins in virulence will likely be the focus of future

research.

Host immune responses in melioidosis

Innate immunity in humans is mediated by mechanisms that include physical barriers

provided by epithelial cells, phagocytic clearance of foreign entities, and inflammation of

infected tissues. Many of these mechanisms have been studied with regard to *B.*

pseudomallei infection.

Epithelial cell invasion. Tight junctions between epithelial cells in skin and the mucosal

membranes of the respiratory, gastrointestinal, and urogenital tracts provide a protective

barrier between the body and the pathogen-ridden environment. Studies by Jones et al.

demonstrated the ability of *B. pseudomallei* to invade rat alveolar macrophages,

monocyte-like U937 cells, and HeLa cells (30). Utaisincharoen et al. showed that *B.*

pseudomallei stimulated IL-8 production at levels significantly lower than those induced

by *S. typhi* at the cell surface without being internalized (62). They later showed that

invasion of epithelial cells by *B. pseudomallei* required p38 mitogen-activated protein kinase phosphorylation, and confirmed that *B. pseudomallei* could stimulate p38 phosphorylation via surface contact without internalization-mediated interactions with intracellular cytoplasmic components (63). Kespichayawattana et al. demonstrated that *B. psdeudomallei* is more efficient than *B. thailandensis* in adhering to and invading cultured epithelial cells (33), indicating that epithelial-cell manipulation may contribute to *B. pseudomallei* pathogenicity.

Survival and replication in phagocytic cells. Circulating monocytes leave the vasculature and mature into macrophages in various tissues. Neutrophils are not abundant in normal tissues, but are recruited to sites of inflammation. When microorganisms cross the epithelial barrier of a host, they are usually recognized by macrophages and neutrophils bearing cell-surface receptors that can identify microorganisms. These include mannose receptors, scavenger receptors, and CD14, a receptor for LPS found on gram-negative bacteria. Ligation of theses receptors induces phagocytosis of the microorganism into the macrophage or neutrophil, where it becomes enclosed in a phagosome.

The low pH of a mature phagolysosome is lethal for most pathogens. To facilitate pathogen killing, the phagosome fuses with one or more lysosomes containing antimicrobial agents that are lethal to pathogens. In addition, macrophages and neutrophils produce highly reactive nitrogen and oxygen species that can aid in pathogen killing in a process called the respiratory burst. These compounds include nitric oxide (NO), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) (29).

Jones et al. showed that *B. pseudomallei* can survive in U937 macrophages (30), indicating that they are somehow able to avoid these destructive mechanisms. Studies by Stevens et al. showed that a *bsa*-encoded type-III secretion system facilitates escape of *B. pseudomallei* from the endosomal compartment into the cytosol and enhances cell-to-cell spread (54). Utaisincharoen et al. demonstrated that *B. pseudomallei* can interfere with inducible nitric oxide synthase (iNOS) and that macrophages infected with the organism show decreased secretion of TNF-α (64).

Adaptive immune responses to *B. pseudomallei*. Kattheesan et al. stimulated peripheral blood monocytes from patients who had recovered from melioidosis with *B. pseudomallei* antigens and found that lymphocyte proliferation, INF- γ production, and activation of CD4+ and CD8+ cells were higher than in cells from uninfected donors treated in a similar manner. In a different study, Barnes et al. took peripheral blood mononuclear cells from individuals in Papua New Guinea and found that in response to *B. pseudomallei* stimulation, lymphocyte proliferation and IFN-γ production were much higher in cells taken from individuals who were seropositive, but had no clinical history of melioidosis, than in cells taken from individuals who had clinical histories of melioidosis. This indicates that individuals with subclinical melioidosis have a stronger cell-mediated immune response to *B. pseudomallei* antigens, and that such a response might be protective against disease progression (6).

Diabetes mellitus – a risk factor for melioidosis

Predisposing risk factors. After a fourteen-year prospective study in northern Australia, Currie et al. used a whole-population logistic regression model to estimate that the

relative risk factors for melioidosis in males and females with diabetes mellitus were 21.2 and 13.1, respectively. The melioidosis incidence rate for this time period was estimated at 260 cases per 100, 000 diabetics (17). In a separate study in northeast Thailand, Supattamongkol et al. showed that diabetes mellitus was a significant factor associated with bacteremic melioidosis (57).

Other predisposing risk factors for melioidosis include renal failure and alcoholism. Yun-Hwen Gan suggested that that in some cases, compromised immune function may lead to latent or chronic melioidosis, while in other cases, healthy immune responses may lead to acute melioidosis caused by hyperinflammation; however, this hypothesis does not explain why severe (acute) melioidosis is most often seen in type-I diabetics or why diabetic mice are prone to acute disease caused by bacterial infection and characterized by hyperinflammation.

Cytokine profiles in melioidosis

Humans have evolved various mechanisms for discerning foreign cells and potentially pathogenic microbes. Toll-like receptors (TLRs) can recognize various pathogen-associated molecular patterns (PAMPs). Ligation of TLRs activates intracellular signaling cascades that induce expression of genes involved in inflammatory and immune responses (2).

Gram-negative bacteria are opsonized by LPS-binding protein (LBP) and the LPS-LPB complex is recognized by CD14, a membrane-bound receptor on the surface of phagocytic cells. CD14 and MD-2 are thought to stabilize the dimerization and subsequent conformational change of TLR-4, a process necessary for the recruitment of adaptor proteins containing TIR domains. TIR domains are cytoplasmic regions

conserved among TLR and IL-1 receptors that are important in intracellular signaling. Known adaptor proteins include MyD88, TIR-associated protein (TIRAP), TIR-domain-containing adaptor protein-inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM). In TLR-4 signaling, TIRAP recruits MyD88 to the cytoplasmic membrane, where it associates with the receptor via homophilic interactions and recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 via homophilic interactions with their death domains. IRAK-4 phosphorylates IRAK-1, which subsequently associates with TNFR-associated factor 6 (TRAF6), a ubiquitin protein ligase. TRAF6 helps form a complex whose activities lead to the phosphorylation of IKK-B and several MAP kinases. This leads to the activation of the Jun/Fos and NF-κB, transcription factors that regulate the expression of many genes involved in innate immune responses, including proinflammatory cytokines and chemokines (1). In addition to TLR-4, monocytes express other toll-like receptors that may recognize molecular patterns associated with *B. pseudomallei*, including TLR-3, which recognizes peptidoglycan, and TLR-5, which recognizes flagella.

Researchers have identified cytokines as critical immunoregulatory determinants and have characterized two periods of immune response activation: the T-cell-independent pathway (early) and T-cell-dependent pathway (late). The cytokine profile produced during the early (T-cell independent) phase activates macrophages, influences T-lymphocyte differentiation and proliferation in the late phase, and is probably the most salient factor in determining host innate resistance to intracellular pathogens (5).

Friedland et al. measured the plasma levels of IL-8, TNF-α, and IL-6 in melioidosis patients throughout the disease progression until death or recovery. They

found that concentrations of IL-6 and IL-8 were elevated throughout the infection and that elevated plasma IL-6 correlated with 75% mortality in *B. pseudomallei* sepsis. IL-8 was elevated in 50% of the patients who died. TNF- α activity did not relate to disease outcome (24); however, a study by Nuntayanuwat et al. demonstrated that polymorphism in the TNF-α promoter region and corresponding levels of TNF-α production were significantly higher in patients with severe melioidosis compared to control subjects (45).

Ulett et al. showed that in primary infections with *B. pseudomallei*, cytokine responses involve the proinflammatory regulators TNF-α and IL-6 rather than polarized T helper cell patterns (59). They also demonstrated that in susceptible BALB/c mice, cytokine profiles were similar, but less pronounced in response to respective infections with high-virulence and low-virulence strains of *B. pseudomallei* (59). The group then showed that in *B. pseudomallei*-infected BALB/c mice, a model researchers use to study acute melioidosis, liver levels of the proinflammatory cytokines IL-6, TNF- α, and IL-1β mRNA were significantly elevated, while the same mRNA levels in *B. pseudomallei*-infected C57BL/6 mice (used to study chronic melioidosis) were only moderately elevated (60). Lauw et al. found that compared to healthy controls, melioidosis patients had elevated plasma concentrations of IFN-γ, IL-18, IL-12, and IL-15 (36).

Specific aims of this study

The purpose of this study was to examine *in vitro* innate immune responses to *B. pseudomallei* infection with regard to cytokine expression profiles produced by phagocytic cells. There were four specific aims for this study:

Aim 1: U937 infection study: The first aim was to determine whether there are differences in the proinflammatory cytokine expression profiles produced by PMA-differentiated U937 macrophage-like cells in response to infection with *B. pseudomallei* and *B. thailandensis*.

Aim 2: Twenty-four hour, multi-model, inflammatory cytokine study. The second aim was to compare various monoctye / macrophage infection models to determine a) whether there are differences between *B. pseudomallei*- and *B. thailandensis*-induced cytokine profiles in the respective models, and b) at what point post infection maximum activity of proinflammatory cytokines was observed in the various models. Four models were used for this study: 1) Undifferentiated U937 monocyte-like cells (no PMA used), 2) PMA-differentiated U937 macrophage-like cells, 3) Primary peripheral blood monocytes, and 4) Primary peripheral blood monocyte-derived macrophages.

Aim 3: IL-1β Study. The third aim was to characterize IL-1β secretion in primary human peripheral blood monocytes infected with *B. pseudomallei*, *B. thailandensis*, and *E. coli*.

Aim 4: Diabetes mellitus infection study. The fourth aim was to examine the differences between cytokine profiles in *B. pseudomallei*-infected peripheral blood monocytes from healthy subjects and diabetic subjects.

Proinflammatory cytokine secretion was studied by infecting U937 monocyte-like or macrophage-like cells and primary human peripheral blood monocytes or monocyte-derived macrophages from healthy and diabetic donors with *B. pseudomallei*, *B. thailandensis*, *S. aureus*, and *E. coli*. Cytokine production by phagocytic cells from diabetic patients in response to *B. pseudomallei* infection has not been previously described.

Materials and Methods

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

Growth calibration curves. Bacterial cultures were grown overnight in 10 ml of brain heart infusion (BHI) broth in 50-ml conical tubes with loose caps at 37°C without shaking. Five ml of each species were collected (below the pellicle, when applicable), centrifuged at 3645 X *g*, washed, and suspended in supplemented RPMI media. Serial dilutions were made for each species (1:2, 1:4, 1:8, 1:16, 1:160, 1:1600, 1:16,000, 1:1.16 x 10^5, 1:1.16 x 10^6, 1:1.16 x 10^7 1:1.16 x 10^8 1:1.16 x 10^9, and 1:1.16 x 10^{10}). OD readings were recorded for the 1:1, 1:2, 1:4, 1:8, and 1:16 suspensions, and 1-ml samples of the 1:1.16 x 10^7 1:1.16 x 10^8 1:1.16 x 10^9, and 1:1.16 x 10^{10} dilutions were plated in duplicate by membrane filtration on Columbia agar plates and incubated at 37°C. Colony-forming unites (CFU) were counted after 48 hours. Second-order polynomial regression curves were generated using Microsoft Excel® and used to interpolate concentrations of bacteria for all experiments.

PMA preparation. One mg PMA (Sigma) was dissolved in 1 ml DMSO. A 2.5 μl aliquot of this solution was added to 5 ml Hanks Balanced Salt Solution (HBSS) (Hyclone) to produce a 0.5 μg/ml PMA solution. DMSO and HBSS PMA solutions were stored at -20 °C until used.

Aim 1: U937 infection study

Tissue culture. U937 cells were cultured in RPMI media (Hyclone) supplemented with

1% HEPES (Cellgro), 1% sodium pyruvate (ICN Biomedicals), 0.5 g/L glucose, 1.5 g/L

sodium bicarbonate (Cellgro), and 10% bovine growth serum (BGS) (Hyclone) in a 37°C

incubator with 5% CO_2. Each well in a six-well plate was seeded with 1 x 10^6 cells in 1

ml media and incubated overnight with 0.05 μg/ml PMA (100 μl of 0.5 μg/ml PMA-

HBSS solution per well). Media (without PMA) was replaced before cells were infected.

Bacteria. Bacterial strains used were *Burkholderia pseudomallei* ATCC 23343,

Burkholderia thailandensis ATCC 700388, *Staphylococcus aureus* (lab strain identified

using cellular fatty acid profiles, MIDI, Inc.), and *Escherichia coli* 11229. All bacterial

cultures were grown overnight in 10 ml of BHI broth at 37°C without shaking. Five ml

of culture from each strain were collected (below the pellicle, when applicable),

centrifuged at 3645 X *g*, washed, and resuspended in 5 ml of supplemented RPMI media

prior to use in infections. Three optical density (OD) measurements were averaged (at

580 nm for *B. pseudomallei* and *B. thailandensis*, and 600 nm for *S. aureus* and *E. coli*),

and bacteria were diluted with supplemented media as appropriate to achieve the desired

concentration of 6 x 10^6 cfu/ml.

Infection. One ml of the appropriate bacterial suspension was added to U937

macrophage-like cells to achieve a multiplicity of infection (MOI) of 6. After 120

minutes, one ml of media containing 750 μg/ml kanamycin was added to wells (final

kanamycin concentration was 250 μg/ml) in order to inhibit extracellular bacterial growth.

Supernatants were collected 8 hours post infection. Samples were passed through a 0.2

μm, low-protein-binding, Durapore PVDF filters (Millipore) to remove cells, and tested immediately for levels of TNF-α, IL-1β, and IL-6.

Cytokine assays. Levels of TNF-α, IL-1β, and IL-6 were determined using Quantikine ELISA assays (R&D Systems). Manufacturer instructions were followed.

Aim 2: Twenty-four hour, multi-model, inflammatory cytokine study

Tissue culture. *Cell line:* U937 cells were cultured as described above. *Primary human peripheral monocytes:* 50 ml whole blood was collected from two healthy donors in K$_3$EDTA vacutainers (Becton Dickinson) and diluted 1:1 with Hanks Balanced Salt Solution (HBSS) (Hyclone). Approximately 25 ml blood-HBSS solution was carefully layered over 10 ml Lymphocyte Separation Medium (LSM) (Mediatech) and centrifuged at 600xg for 25 minutes. The mononuclear cells were collected between the LSM and plasma layer and resuspended in 13 ml RPMI media supplemented with 1% HEPES, 1% sodium pyruvate, 0.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% BGS. Cells were seeded in 6-well plates with 2 ml media, and incubated at 37°C with 5% CO$_2$. Cells from one donor were incubated for five days with media changes every other day to allow them to differentiate into macrophages. Cells from the other donor were incubated for one day and washed twice before use to remove nonadherent cells. Immediately prior to the infection assays, one well of each plate was treated with 0.5 ml Trypsin (Invitrogen) and the cells were gently detached from the well bottom with a cell scraper (Corning). Monocyte concentrations from trypsin-treated wells were determined with a hemacytometer.

Bacteria. Bacterial strains used were *Burkholderia pseudomallei* ATCC 23343, *Burkholderia thailandensis* ATCC 700388, and *Escherichia coli* 11229. All bacterial cultures were grown as described above. The concentrations of bacteria used to infect peripheral blood monocytes were determined by multiplying the monocyte counts obtained using the hemacytometer by six. Suspension concentrations of 6 x 10^6 cfu/ml were used for the U937 monocytes and macrophages.

Infection. Monocytes were infected (MOI = 6) with 1 ml of appropriate *B. pseudomallei*, *B. thailandensis*, and *E. coli* suspensions. After 90 minutes 200 μg/ml kanamycin was added to wells to inhibit extracellular bacterial growth. Supernatant samples from *B. pseudomallei*, *B. thailandensis*, and mock infections were collected 2, 4, 6, 10, 12, 16, 20, and 24 hours post infection. Supernatant samples from *E. coli* infections were collected 2, 6, 10, 16, 20, and 24 hours post infection. Samples were passed through 0.2 μm, low-protein-binding, Durapore PVDF filters (Millipore) to remove cells, and stored at −80°C until they were tested.

Cytokine assays. Samples were tested for levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α using multiplex cytokine ELISA assays (Q-Plex Human Cytokine Array – Inflammation (Biolegend)). Some data points were excluded from post-assay analysis because their corresponding ELISA CCD detection images displayed excessively bright spots that extended well beyond capture antibody area assignment, indicative of improperly seeded capture antibody, nonspecific detection antibody binding, or some other malfunction.

Aim 3: IL-1β study

Tissue culture. Fifty ml whole blood was collected from five healthy donors and cultured as described above. Cells were incubated for 24 hours and washed twice before infection.

Bacteria. Bacterial strains used were *Burkholderia pseudomallei* ATCC 23343, *Burkholderia thailandensis* ATCC 700388, and *Escherichia coli* 11229. Bacterial cultures were grown (and infection concentrations were determined) as described above

Infection. Monocytes were infected (MOI = 6) with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. After 90 minutes, 200 μg/ml kanamycin was added to wells to inhibit extracellular growth. Supernatants were collected at 10 (n=3) and 11 (n=2) hours post infection. Samples were passed through 0.2 μm, low-protein-binding, Durapore PVDF filters (Millipore) to remove cells and stored at –80°C until tested.

Cytokine assays. Levels of IL-1β were determined with Quantiglo ELISA assays (R&D systems). Manufacturer instructions were followed.

Aim 4: Diabetes mellitus infection study

Tissue culture – diabetes mellitus study. Forty-two ml of whole blood from diabetic males (n=5), healthy males (n=6), diabetic females (n=5), and healthy females (n=6) were collected in EDTA vacutainers (BD). In each of two 50-ml conical tubes, approximately 20 ml of whole blood was diluted with 15 ml of HBSS (Hyclone), layered over 15 ml of LSM (Cellgro), and centrifuged at 600 X *g* for 25 minutes. Approximately 15 ml of

peripheral blood mononuclear cells (PBMCs) were removed, mixed with 6 ml OptiPrep (Accurate Chemical) and overlaid with 7.5 ml and 20 ml of OptiPrep solutions with densities of 1.070 g/ml and 1.086 g/ml, respectively. Cells were centrifuged at 600 X g for 25 minutes, after which the top 20 ml (faction including monocytes) were removed and washed three times. Cells were counted and 1 x 10^5 cells were seeded into each well of 12-well plates with one ml RPMI media supplemented with 1% HEPES (Cellgro), 1% NEAA (Hyclone), 1% sodium pyruvate (ICN Biomedicals), 0.5 g/L glucose, 1.5 g/L sodium bicarbonate (Cellgro), and 10% heat-inactivated BGS (Hyclone). Cells were placed in a 37 °C incubator with 5% CO_2 for 2 – 3 hours before infection.

Bacteria. Bacterial strains used were *Burkholderia pseudomallei* NCTC 13178, *Burkholderia thailandensis* ATCC 700388, and *Escherichia coli* 11229. Bacterial cultures were grown (and infection concentrations were determined) as described above.

Infection. Monocytes were infected (MOI = 6) with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. After 90 minutes 150 μg/ml kanamycin was added to wells to inhibit extracellular bacterial growth. Supernatants were collected 10 hours post infection. Samples were passed through 0.2 μm, low-protein-binding, Durapore PVDF filters (Millipore) to remove cells. Samples were stored at –80°C until tested.

Cytokine assays. Samples were tested for levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α using multiplex cytokine ELISA assays (Q-Plex Human Cytokine Array – Inflammation (Biolegend)).

Statistical analysis. Cytokine concentration data were transformed to the natural logarithmic scale and analyzed using a linear mixed model with gender, diabetic state, infectious agent and all possible interactions of these factors as fixed effects. Subject was included as a random effect in the model. The model was fitted using residual maximum likelihood for the variance components and estimated generalized least squares for the fixed effects. Residuals were computed and plotted versus predicted values to assess goodness-of-fit of the model. Main effects and interactions of the fixed effects were tested using approximate F-tests based on the Kenward-Roger adjustment for small-sample inferences. Specific comparisons of means were carried out using linear contrasts. Least squares means were computed for all combinations of the factor levels.

Results

Aim 1: U937 infection study

The first aim was to determine whether there were differences in the proinflammatory cytokine expression profiles produced by PMA-differentiated U937 macrophages in response to infection with *B. pseudomallei* and *B. thailandensis*. As mentioned previously, the lipopolysaccharide (LPS) of *B. pseudomallei* is immunologically indistinguishable from that of *B. thailandensis*; however, it is possible that the surface determinants that govern host innate immune responses are different for the two species, especially given the fact that *B. pseudomallei* is able to undergo morphotype switching during various growth conditions. To test the hypothesis that proinflammatory cytokine expression profiles produced by PMA-differentiated U937 macrophages differed in response to infection with *B. pseudomallei* and *B. thailandensis*, U937 cells were infected with both species in addition to *E. coli* (gram negative, positive control) and *S. aureus* (gram-positive, negative control). Concentrations of TNF-α, IL-1β, and IL-6 were determined using ELISA analysis.

The concentration of TNF-α was significantly higher (t-test assuming unequal variances, $p = 0.0135$) in cultures infected with *B. pseudomallei* than in those infected with *B. thailandensis* (Figure 2A); however, the results of this study do not support the hypothesis that there are differences in secretion levels of either IL-1β (Figure 2B) or IL-6 (Figure 2C) between cells infected with *B. pseudomallei* and cells infected with *B. thailandensis*. Levels of all cytokines were negligible in response to infection with *S. aureus*. In addition, cells infected with *E. coli* produced significantly higher levels of IL-6 than any other bacterium tested ($p<0.05$).

Aim 2: Twenty-four hour, multi-model, inflammatory cytokine study

The second aim was to compare various monoctye / macrophage infection models to determine a) whether there are differences between *B. pseudomallei-* and *B. thailandensis*-induced cytokine profiles in the respective models, and b) at what point post infection maximum activity of proinflammatory cytokines was observed in the various models. Four models were used for this study: 1) Undifferentiated U937 monocyte-like cells (no PMA used), 2) PMA-differentiated U937 macrophage-like cells, 3) Primary peripheral blood monocytes, and 4) Primary peripheral blood monocyte-derived macrophages (peripheral blood monocytes that had been allowed to differentiate into macrophages for 5 days).

Because U937 cells are a leukemia cell line, they may have different genotypic and phenotypic characteristics from peripheral blood monocytes and macrophages. Peripheral blood monocytes from healthy donors were cultured for various periods, and their cytokine responses to *B. pseudomallei*, *B. thailandensis*, and *E. coli* were compared to those of the U937 cell line.

The cytokine profiles differed for each model used. *Undifferentiated U937 cells*: The levels of all assayed cytokines produced by undifferentiated U937 cells in response to infection with *Burkholderia* species were negligible (Figure 3). Levels of most cytokines were negligible in response to infection with *E. coli* with the exception of IL-8, which was slightly elevated (compared to uninfected cells) throughout the infection and was significantly elevated at 24 hours (Figure 3 and data not shown).

PMA-differentiated U937 cells: PMA-differentiated U937 cells produced elevated levels (compared to uninfected cells) of TNF-α and IL-10 in response to

infection with *Burkholderia* species (Figure 4). Cells infected with *B. pseudomallei* produced similar levels of TNF-α compared to cells infected with *B. thailandensis* (Figure 4a) early in the infection (2-6 hours); however, cells infected with *B. pseudomallei* produced more TNF-α than cells infected with *B. thailandensis* (Figure 4a) late in the infection (8 - 24 hours). Cells infected with *B. pseudomallei* produced more IL-10 than cells infected with *B. thailandensis* later in the infection (10 – 24 hours) (Figure 4D). PMA-differentiated U937 cells infected by various agents did not produce significantly elevated levels of TNF-β, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, and IFN-γ, with the exception of cells infected with *E. coli*, which produced elevated significant levels of IL-8. (Figure 4 and data not shown).

Primary human peripheral blood monocytes: Primary human peripheral blood monocytes produced elevated levels of TNF-α and TNF-β (Figures 4A and 4F, respectively) and decreased levels of IL-1α, IL-6, and IL-10 (Figures 4B, 4D, and 4E, respectively) in response to *B. pseudomallei* and *B. thailandensis* infections. These differences occurred late in infection (16-24 hours). The cells produced substantially lower levels of TNF-α than the peripheral blood monocyte-derived macrophages (Figures 4A and 5A, respectively). Cells infected with *B. thailandensis* produced significantly elevated levels of IL-1β throughout the infection, while cells infected with *B. pseudomallei* produced baseline levels of IL-1β throughout the infection. Levels of most cytokines of interest peaked at about 10 hours post infection. Levels of IL-8 were above detection limits in all infections (data not shown). Production of IL-2, IL-4, IL-5, IL-13, and IFN-γ was negligible for all infections (data not shown).

Primary human peripheral blood monocyte-derived macrophages: Primary monocyte-derived macrophages produced elevated levels of TNF-α, IL-1α, and IL-1β (Figures 5A, 5B, and 5C, respectively) throughout the infections with *B. pseudomallei* and *B. thailandensis*. The cells produced decreased levels of IL-6 and increased levels of TNF-β late in the infections with *B. pseudomallei* and *B. thailandensis*. (16-24 hours post infection). There were no significant differences between the levels of cytokines produced by cells infected with *B. pseudomallei* and *B. thailandensis*. Cells infected with *E. coli* produced slightly elevated levels of TNF-α and significantly elevated levels of IL-6 throughout infection. Levels of IL-8 were above detection limits in all infections (data not shown). The cells did not produce significantly elevated levels of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ (Figure 5 and data not shown).

Aim 3: IL-1β study

The third aim was to characterize IL-1β secretion in peripheral blood monocytes infected with *B. pseudomallei*. The results from the 24-hour inflammation study indicated that peripheral blood monocytes might respond differently to infections with *B. pseudomallei* and *B. thailandensis* with regard to IL-1β production; however, when the experiment was repeated with peripheral monocytes from several subjects, the differences between IL-1β production in response to *B. pseudomallei* and *B. thailandensis* were not significantly different for either 10-hour (Figure 7) or 11-hour (data not shown) infection periods. In addition, there were no significant differences between *B. pseudomallei-* and *E. coli-* induced IL-1β profiles, or between *B. thailandensis-* and *E. coli-*induced IL-1β profiles for either infection period (Figure 7 and data not shown).

Aim 4: Diabetes mellitus infection study

The fourth aim was to examine the differences between cytokine profiles in *B. pseudomallei*-infected peripheral blood monocytes from healthy subjects and diabetic subjects. As discussed above, persons with diabetes mellitus, particularly men older than 40 years, are especially susceptible to infection with *B. pseudomallei*. The results from previous studies indicate that diabetic susceptibility to bacterial infections is due to defects in innate, rather than adaptive immunity. In this study, the ability of primary peripheral monocytes from diabetic and nondiabetic controls to secrete various proinflammatory cytokines and chemokines upon infection was evaluated. Fresh peripheral blood monocytes were used in order to minimize the effects that artificial cell culture would have on the intracellular milieu.   Peripheral monocytes were infected with *B. pseudomallei*, *B. thailandensis*, *E. coli* (positive control) and sterile media (negative control). Sample supernatants were tested for levels of cytokines. Statistical analyses were performed to examine the effects of 1) infecting agent, 2) diabetic state, and 3) gender, on cytokine secretion by peripheral monocytes. Many of the cytokines examined (IL-2, IL-4, IL-5, IL-13, TNF-β, and IFN-γ) have not been shown to be secreted by monocytes, and therefore serve as negative controls. All cytokine concentrations were normalized against corresponding concentrations in uninfected cultures.

There was no significant difference between normalized levels of TNF-α secreted by monocytes with regard to agent, gender, or diabetic state (Figure 8A). Normalized levels of secreted IL-1α, IL-1β, IL-6, and IL-8 were significantly higher for monocytes from healthy males than they were for monocytes from diabetic males (Figures 9, 10, 11, and 12, respectively) for all three infectious agents. Normalized levels of secreted IL-1β

were significantly higher for monocytes from diabetic females than they were in monocytes from healthy females (Figure 10) in response to infection with *B. pseudomallei*. Normalized levels of IL-13 were significantly higher in samples from diabetic females than healthy controls (Figure 13); however, IL-13 has not conclusively been shown to be produced by macrophages and the levels produced were very low compared to other cytokines, so it is likely that the statistical difference observed isn't biologically meaningful. Interestingly, there was no significant difference between normalized levels of TNF-α secreted by monocytes with regard to agent, gender, or diabetic state (Figure 8A). Statistical analysis of this data revealed a significant interactive effect of gender and diabetic state on peripheral blood monocyte secretion of IL-1 β (p = 0.0370) and IL-8 (p = 0.0390). In addition, the analysis revealed a significant interactive effect of diabetic state and infectious agent for IL-1α (p=0.0210) and IL-6 (p=0.0204).

The concentrations of TNF-β, IL-2, IL-4, IL-5, and IFN-γ were not significantly elevated in response to infection with any agent in this study (Figure 14 and data not shown). In addition, the concentrations of IL-10 and IL-13 (Figures 12 and 13, respectively) were not significantly elevated in any of the experiments.

Discussion

Aim 1: U937 infection study

Although the production of TNF-α by U937 macrophage-like cells infected with *B. pseudomallei* was significantly higher (t-test assuming unequal variances, p = 0.0135) than by those infected with *B. thailandensis* (Figure 2A), the cells showed no difference in IL-1β (Figure 2B) or IL-6 (Figure 2C) production in response to the two species. These results indicate a possible difference in the ability of *B. pseudomallei* and *B. thailandensis* to induce TNF-α production *in vitro*; however it is important to keep in mind that U937 cells are from a leukemia cell line that likely has different genotypic and phenotypic characteristics from human cells *in vivo*, so caution should be used in the extrapolation of these results to an *in vivo* situation. The infection of cells with *S. aureus* served as a negative control since gram-positive bacteria haven't been shown to induce significant levels of these inflammatory cytokines.

Aim 2: Twenty-four hour, multi-model, inflammatory cytokine study

The second aim examined the kinetics and profiles of cytokine production by various cell types. The cytokine profiles differed for each model used. The fact that undifferentiated U937 monocyte-like cells produced insignificant cytokine levels indicate that they are a poor model for studying innate immune responses to *Burkholderia* species.

Consistent with the Aim 1 study, PMA-differentiated U937 cells infected with *B. pseudomallei* produced elevated levels of TNF-α compared to cells infected with *B. thailandensis* late in the infection; however, these results should be interpreted cautiously

since cells infected with *B. pseudomallei* also produced more IL-10 (which has been shown to inhibit production of proinflammatory cytokines, including TNF-α) than those infected with *B. thailandensis*. In addition, the same phenomenon was not observed when primary human cells were infected. The results could be due to a unique phenotype of the U937 cell line.

The observation that IL-6 was not significantly elevated in response to infections is consistent with the results of the Aim 1 study, in which secreted levels of IL-6 were negligible (Figure 2C). The fact that PMA-stimulated U937 cells produced cytokine profiles that were significantly different from primary human monocytes and monocyte-derived macrophages in response to infection indicates that these cells are probably not a good model for studying cytokine responses to *Burkholderia* infection.

Peripheral blood monocytes produced substantially lower levels of TNF-α than the peripheral blood monocyte-derived macrophages (Figures 4A and 5A, respectively). It is not clear why IL-1β production by monocytes in response to infection with *B. pseudomallei* was near baseline levels, while that of cells infected with *B. thailandensis* was significantly elevated throughout the infection. While it is interesting that the levels of TNF-α produced by monocytes infected with *B. pseudomallei* and *B. thailandensis* increased and corresponding levels of IL-1α, IL-6, and IL-10 decreased 16 hours post infection, it should be noted that these changes were accompanied by mildly elevated levels of TNF-β, which has not been shown to be produced by monocytes or macrophages. This indicates that there may have been low levels of contaminating lymphocytes in these cell preparations, and it is impossible to determine the full extent of their effects. It is interesting, however, that the cells infected with *E. coli* didn't follow

the same trend as cells infected with *Burkhoderia* species, suggesting that *B. pseudomallei* and *B. thailandensis* elicit different immune responses than other common gram-negative bacteria. Levels of most cytokines of interest peaked at about 10 hours post infection.

Primary monocyte-derived macrophages produced elevated levels of TNF-α, IL-1 α, and IL-1β (Figures 5A, 5B, and 5C, respectively) throughout the infection with *B. pseudomallei* and *B. thailandensis*; however there were no significant difference between profiles produced in response to the two infectious agents. As with the monocyte infections, the cytokine profile patterns produced by monocyte-derived macrophages infected with *Burkholderia* species were markedly different than the profiles produced by cells infected with *E. coli*. Interestingly, monocyte-derived macrophages produced decreased levels of IL-6 and increased levels of TNF-β late in the infections with *B. pseudomallei* and *B. thailandensis*. Increased levels of TNF-β late in infection again suggest that there were low levels of contaminating lymphocytes in these cell preparations.

Results from these studies indicated that infection of peripheral monocytes elucidated the most pronounced differences between infections with *B. pseudomallei* and *B. thailandensis*. Peripheral monocytes were used for the remaining studies since they are likely to undergo fewer modifications as a result of artificial culturing than monocyte-derived macrophages. In addition, the optimal time post-infection for cytokine assays was about 10 hrs; therefore, this infection period was used in subsequent experiments.

Aim 3: IL-1β study

The results from the 24-hour inflammation study indicated that peripheral blood monocytes might respond differently to infections with *B. pseudomallei* and *B. thailandensis* with regard to IL-1β production; however, when the experiment was repeated with peripheral monocytes from several subjects, the differences between IL-1β production in response to *B. pseudomallei* and *B. thailandensis* were not significantly different for either 10-hour (Figure 7) or 11-hour (data not shown) infection periods. The results from the 24-hour time course study indicating different responses to the two species could possibly be explained by morphotype switching in response to cells from various donors.

Aim 4: Diabetes mellitus infection study

The interactive effect of gender and diabetic state on peripheral monocyte cytokine production in response to infection with *B. pseudomallei* has not been previously reported. The current study indicates that IL-1β and IL-8 production by infected monocytes can be influenced by a combination of gender and diabetic state. This is particularly interesting with regard to *B. pseudomallei* infection, since melioidosis occurs more often and is more severe in diabetic males.

Type I diabetes mellitus may be due to an autoimmune disorder in which the beta cells in the Islets of Langerhans of the pancreas are attacked and killed. Beta cells normally produce insulin, which is critical in regulating blood glucose levels.

Researchers have endeavored to determine why patients with diabetes mellitus are more susceptible to bacterial infections than healthy persons. There is increasing evidence that patients with diabetes mellitus have defects in their innate immunity.

Marhoffer et al. demonstrated that neutrophils from patients with diabetes have decreased

phagocytic function (40) and Kjersem et al. showed that neutrophils from patients with

type I diabetes have impaired particle uptake after short-term hyperglycemica where

glucose concentrations reflected normal physiological concentrations (34).

Studies using peripheral blood mononuclear cells from diabetic patients indicate

that these cells have decreased chemotactic and phagocytic activity in response to gram-

negative bacterial stimulation (27); however, other studies demonstrated higher baseline

levels of proinflammatory cytokines, particularly IL-1β in macrophages from diabetic

mice stimulated with lipopolysaccharide (46). In addition, Salvi et al. demonstrated that

fresh monocytes from patients with diabetes mellitus produced higher levels of secreted

IL-1β and PGE_2 after overnight stimulation with *Porphyromonas gingivalis* and *E. coli*

(50). The clinical relevance of these findings is unclear. Interestingly, no *in vivo* defects

in adaptive immunity have been described for diabetes mellitus patients (26).

The results of the current study indicate that *B. pseudomallei*-infected monocytes

from diabetic males have decreased ability to secrete IL-1α, IL-1β, IL-6, and IL-8 in

comparison to monocytes from healthy, nondiabetic males. While these results may

appear to be contradictory to other studies reporting increased cytokine secretion in

response to LPS stimulation (particularly those by Salvi et al.), there are key differences

in the experiments that may account for these disparities. This study used live bacteria to

infect monocytes rather than isolated LPS to *stimulate* monocytes. *Burkholderia*

pseudomallei is an intracellular pathogen whose effects on host cell signaling are just

beginning to be elucidated, and whose associations with toll-like receptors are almost

completely uncharacterized. The differences in conclusions drawn from this study and

those of Salvi et al. can possibly be explained by the differences in cytokine signaling pathways induced by live, intracellular, abnormal-LPS-bearing bacteria versus isolated LPS from common gram-negative species.

Lipid A is generally considered to be the pharmacologically active component of LPS. While lipid A is highly conserved among gram-negative bacteria, its structure is not invariant. Researchers have demonstrated that variations in lipid A structure can have significant pathogenic implications. For example, *Yersinia pestis* produces two different lipid A variants depending on the temperature at which the organism is grown: the molecule is hexa-acylated in *Y. pestis* grown at 21 - 27 °C (flea temperatures), and tetra-acylated in organisms grown at 37 °C (mammalian temperature) (32). Tetra-acylated lipid A has been shown to have poor TLR-4-stimulating activity (37). In an elegant experiment, Montminy et al. modified *Y. pestis* to produce a potent TLR-4-simulating LPS with a lipid A structure similar to lipid A from *Y. pestis* grown at 21 °C. Remarkably, the mutant was completely avirulent in mice, depite the presence of all other known virulence determinants associated with *Y. pestis* pathogensis (42). Other researchers have shown that mutations in TLR4 signaling are associated with increased susceptibility to infection in humans (66). In addition, Lien et al demonstrated that fatty acid composition of LPS can affect receptor binding affinity (37). It is plausible that *B. pseudomallei* LPS induces poor TLR4 signaling and that this property contributes to its ability to evade host immune responses, particularly in those individuals with underlying modulated immune responses such as diabetics.

Some researchers have speculated that persons with type-I diabetes are more susceptible to melioidosis because of the effects of insulin. Woods et al. hypothesized

that insulin deficiencies directly affected the growth rate of *B. pseudomallei* and demonstrated that the organism grew significantly more slowly in minimal media containing recombinant human insulin than in control cultures containing no insulin (71). In addition, the group showed an increase in the organism's growth rate in serum from diabetic rats over serum from control rats and that *B. pseudomallei* possesses a high-affinity binding site for insulin (71). These results suggest a role for insulin in susceptibility to *B. pseudomallei* infection; however, another research group has contested such a role. Simpson et al. did a retrospective study in which they determined that fewer than 10% of 382 patients in Thailand with diabetes mellitus who had previously been diagnosed with melioidosis were insulin dependent; however, information regarding insulin dependence for 140 of those patients was not available. The group conjectured that since no records were available, it was "unlikely" that their glycemia was being actively managed, and by extension, that those diabetic patients did not have insulin deficiencies (52). They claimed that insulin was widely available in the community, but did not provide any evidence that the claim or their suppositions were valid. In a separate set of experiments, the group demonstrated that *B. pseudomallei* growth was inhibited by m-cresol at concentrations found in common pharmaceutical insulin preparations, but not by insulin alone. They concluded that the results of the studies performed by Woods et al. may have been confounded by the effect of the insulin preservatives used. Because some of the experiments performed by Woods et al. did not involve pharmaceutical insulin preparations containing m-cresol, the effect of insulin on *B. pseudomallei* growth remains unclear.

It is possible that insulin affects melioidosis progression through mechanisms not related to bacterial growth. As mentioned above, insulin is produced by the beta cells within islets of Langerhans. Insulin reduces blood glucose by circulating in the blood and binding receptor tyrosine kinase (RTK) receptors on muscle cells and adipocytes. Insulin-receptor ligation activates signal transduction through a phosphoinositide pathway that results in the activation of protein kinase B (PKB), which is able to induce the fusion of GLUT4 glucose transformer-containing vesicles with the plasma membrane. As the number of GLUT4 on the cell surface increases, glucose influx into the cell increases, thereby lowering blood sugar. As insulin is removed, the GLUT4 molecules are internalized by endocytosis, and subsequently, glucose transport from the blood into the cell is reduced (38). In persons with type one diabetes mellitus, this process is blunted or absent. It is possible that the transcription and proteomic processes characteristic of defective insulin signaling renders diabetics more susceptible to *B. pseudomallei* morbidity by interfering with or otherwise altering molecular processes involved in immune activation.

The discovery that monocytes from male and female diabetic subjects produce different cytokine profiles in response to infection with *Burkholderia* species is consistent with studies indicating that males are more susceptible to diabetes (17) and studies showing that some cytokines can be up- or down-regulated in response to the presence of different sex hormones. Carruba et al. showed that PMA-differentiated macrophage-like U937 cells treated with estrogen secreted increased levels of TNF-α and IL-10 (10), and Schneider et al. demonstrated that androgens such as testosterone directly affected cytokine production in various tissue macrophages (51). In addition, studies by de Groot

et al. showed that gender has an effect on cytokine profiles in young pigs (20) and studies by Stapleton et al. demonstrated that gender affects macrophage cytokine production after trauma (53). Differences in cytokine secretion between genders are possibly due to hormone-dependent effects; however, the mechanisms by which this phenomenon occurs are still being elucidated. Synergistic effects of androgen and insulin signaling pathways may affect macrophage cytokine production and other functions.

It is possible that the intracellular, intercellular, and/or the organ or tissue milieu of diabetic males provides a particularly friendly environment for *B. pseudomallei* organisms to set up a persistent infection. Evidence indicating that monocytes from diabetic males have a blunted IL-8 response (Figure 12) coordinates nicely with data suggesting that diabetics have decreased neutrophil infiltration at sites of infection (31), since IL-8 is the primary chemokine responsible for neutrophil recruitment.

An interesting result of this study is that diabetic males secrete lower levels of IL-1β in response to *B. pseudoamallei* infection. IL-1β is cleaved by interleukin-1 converting enzyme (ICE or Caspase 1) to a 17-kD mature form (22). IL-1β ligates its cognate receptor on other immune cells and activates NF-κB signaling and subsequent inflammatory responses. It has been shown that inhibiting caspase1 prevents apoptosis. Whether Caspase 1 activity as ICE is related to its ability to induce apoptosis is not clear. An interesting possibility is that *B. pseudomallei* may be able to survive in phagocytes and other cells by inhibiting apoptosis.

The significant interactive effect of diabetic state and type of infective organism on cytokine production by primary peripheral monocytes in response to infection with *Burkholderia* species provides evidence that immune systems of persons with diabetes

mellitus may respond differently to *B. pseudomallei* infections than to infections with other agents. Many researchers believe that the devastating effects of septic shock are due to an imbalance in activating and inhibitory cytokines rather than merely a drastic increase in cytokine production (47).

We propose the following model for melioidosis progression in diabetic males: After the host is exposed to *B. pseudomallei*, the organisms invade and replicate within epithelial and phagocytic cells. During this period, the organisms may avoid critical host immune responses because a) the host has blunted or imbalanced inflammatory responses that prevent proper recruitment of neutrophils and lymphocytes b) the organisms are able to prevent clearance by host defense mechanisms by escaping phagocytic vesicles using type-three secretion system and by preventing lethal processes like the respiratory burst in phagocytic cells. It is possible that after a period of time, a separate event or condition triggers a renewed response to the organism that results in the septic shock that is characteristic of acute melioidosis. The fact that nearly all persons in endemic areas are seropositive for *B. pseudomallei* indicates that the organism's virulence factors are generally insufficient to cause disease, and that host susceptibility plays an important role in *B. pseudomallei* pathogenesis. Defective host cytokine responses could account for both the chronic and acute forms of melioidosis.

In conclusion, peripheral blood monocytes provide a meaningful model for examining host innate immune responses to infection with *B. pseudomallei*. These studies indicate that gender and diabetic state influence immunoregulatory macrophage activities that are critical in determining whether the host's immune responses to infection will be successful.

Table 1 Cytokines, Producer Cells, and Known Functions.*

Cytokine	Producer cell(s)	Function
IL-1α	Macrophages Epithelial cells	Fever T-cell activation Macrophage activation
IL-1β	Macrophages Epithelial cells	Fever T-cell activation Macrophage activation
IL-2	T cells	T-cell proliferation
IL-4	T cells Mast cells	B-cell activation IgE switch Differentiation into Th2 cells
IL-5	T cells Mast cells	Eosinophil growth Differentiation
IL-6	T cells Macrophages Endothelial cells	T- and B-cell growth/differentiation Acute phase protein production Fever
IL-8	Monocytes/macrophages Fibroblasts Keratinocytes Endothelial cells	Mobilizes, activates, and degranulates neutrophils Angiogenesis
IL-10	T cells Macrophages EBV-transformed cells	Potent suppressant of macrophage functions
IL-13	T cells Macrophages Endothelial cells	B-cell growth/differentiation Inhibits macrophage inflammatory cytokine production and Th1 cells Induces allergy/asthma
TNF-α	Macrophages NK cells T cells	Local inflammation Endothelial activation
TNF-β	T cells B cells	Killing Endothelial activation
IFN-γ	T cells NK cells	Macrophage activation Increased MHC molecules and antigen processing components Ig class switching Supresses Th2

*Adapted from Janeway et al. Immunobiology: the immune system in health and disease. 6th edition.Garland Science Publishing, 2005. Pp. 80 and 747-49. Cytokines secreted by monocytes / macrophages are shown in green.
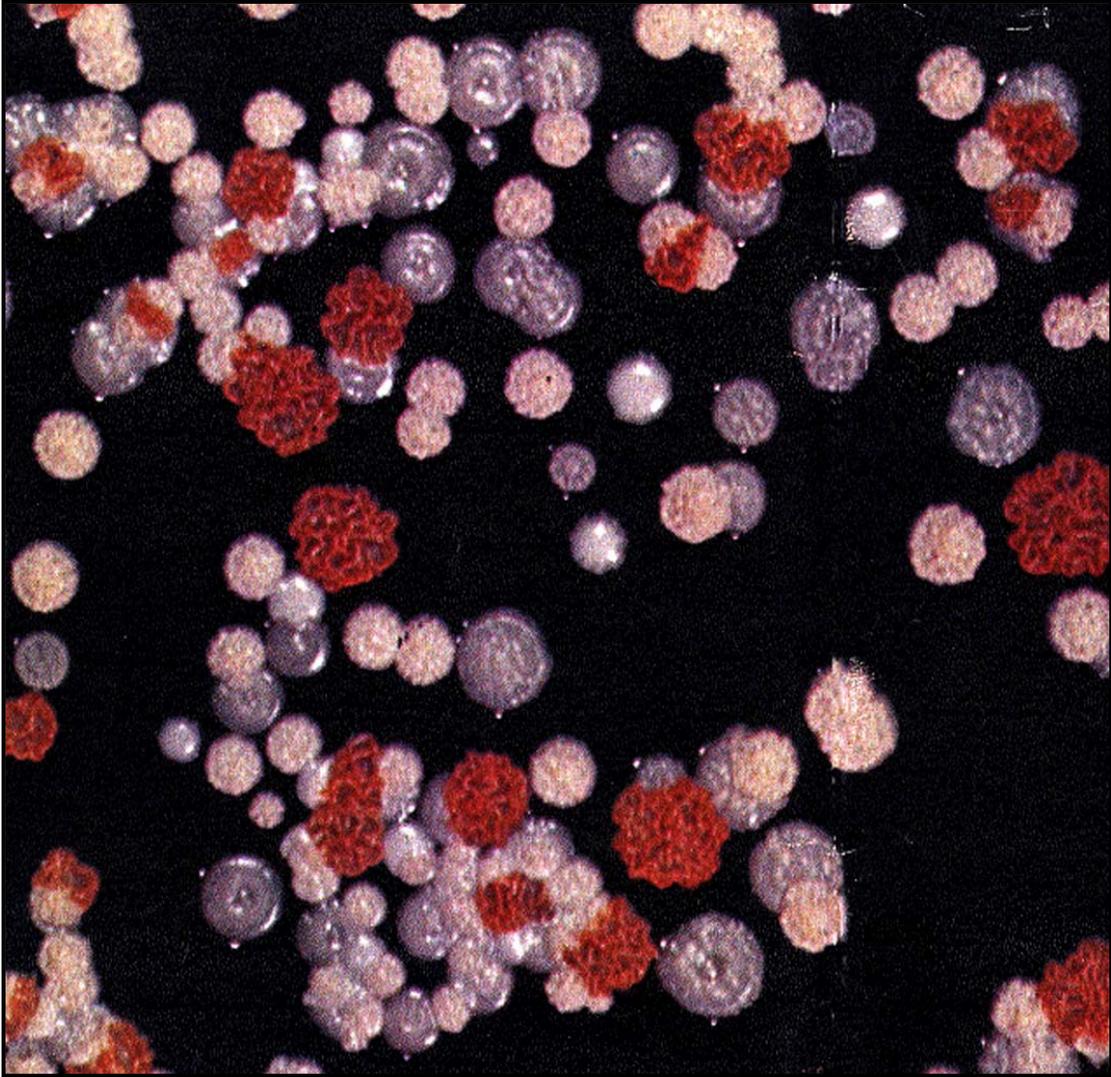
Figure 1. *B. pseudomallei* demonstrates variable colony morphologies in pure culture.
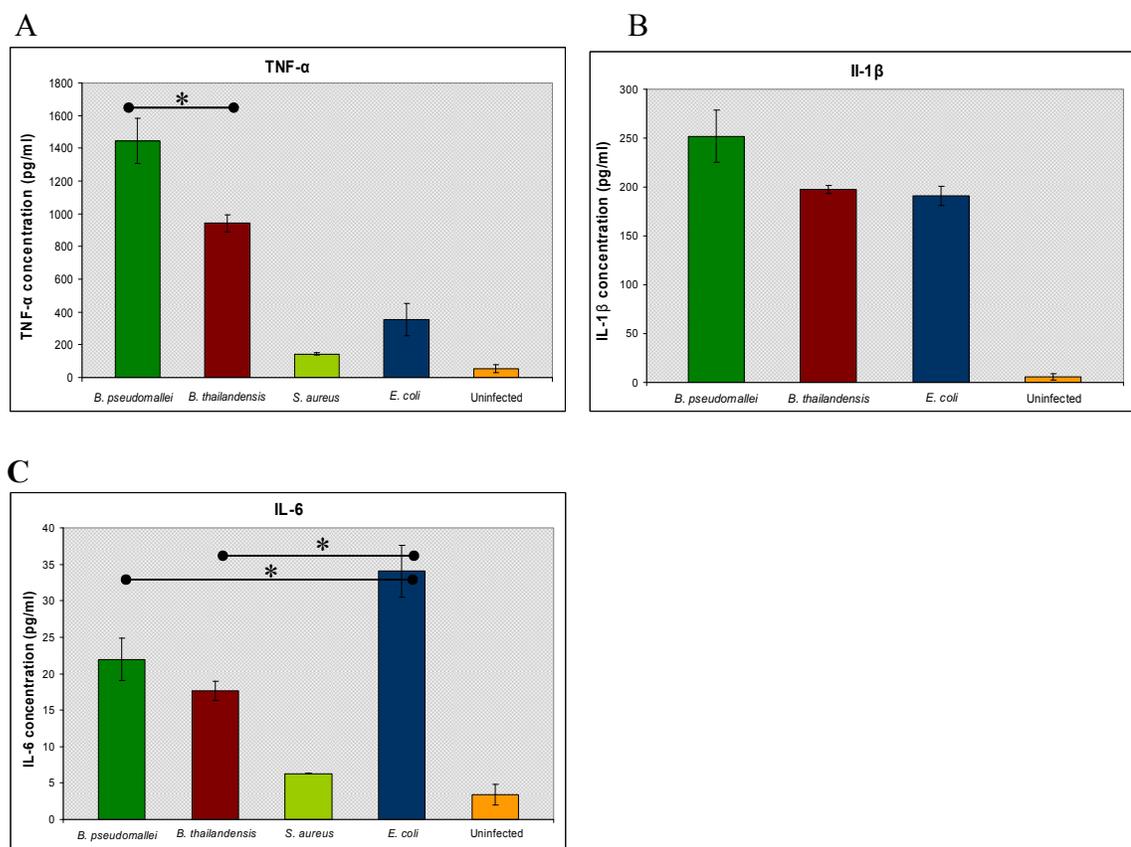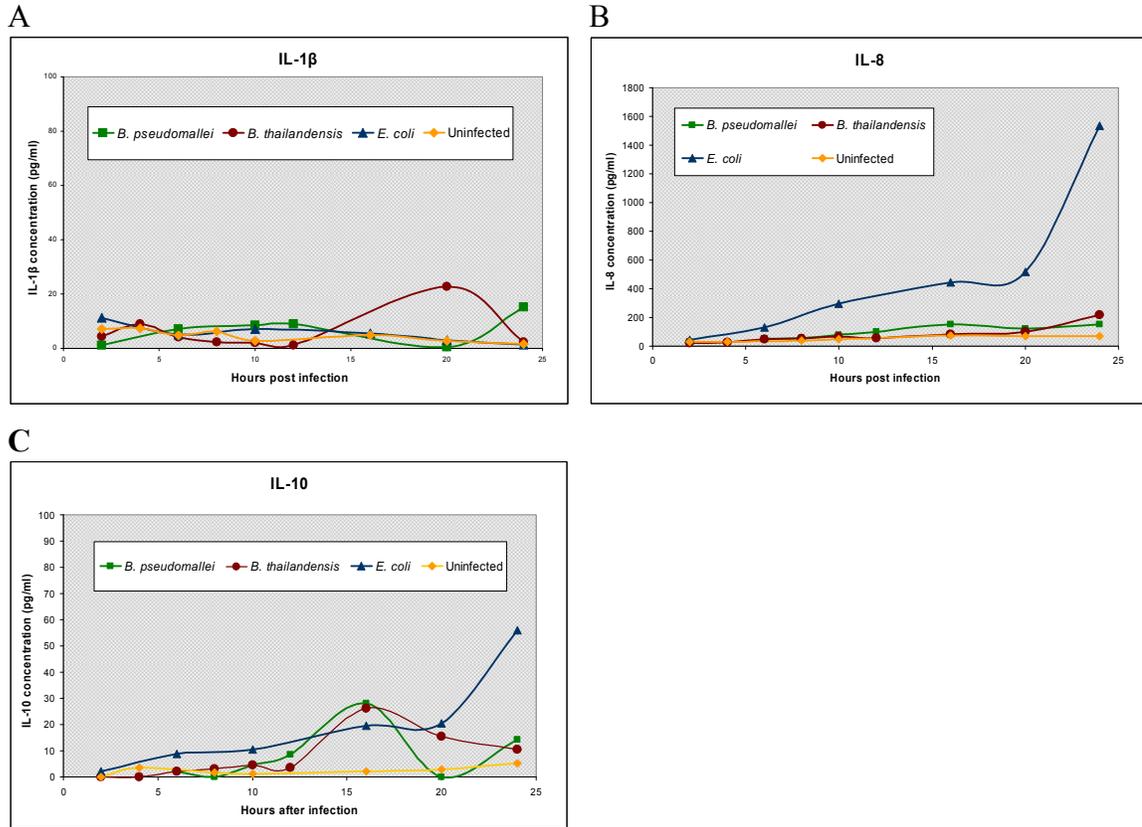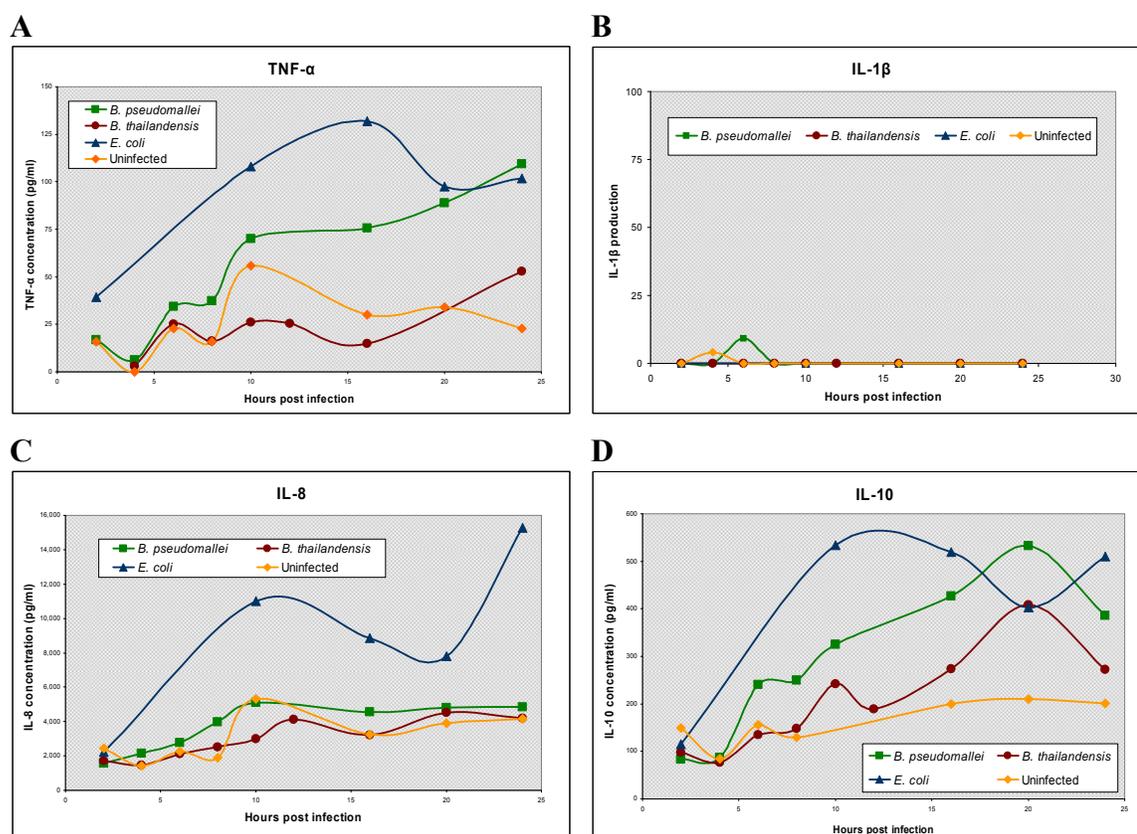
A

B

C

Figure 2. Cytokine production by PMA-differentiated U937 macrophage-like cells infected with *B. pseudomallei*, *B. thailandensis*, *S. aureus*, or *E. coli*.
One million cells were infected with an MOI of 6. Supernatants were collected 8 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least three experiments. Standard errors are shown.

* indicates significant difference in cytokine production ($p < 0.05$).

A

B

C

Figure 3 Kinetics of cytokine production by undifferentiated U937 monocyte-like cells infected with *B. pseudomallei*, *B. thailandensis*, or *E. coli*.
One million cells were infected with an MOI of 6. Supernatant samples were collected 2, 4, 6, 9, 10, 12, 16, 20, and 24 hours post infection and assayed for cytokine levels using ELISA.
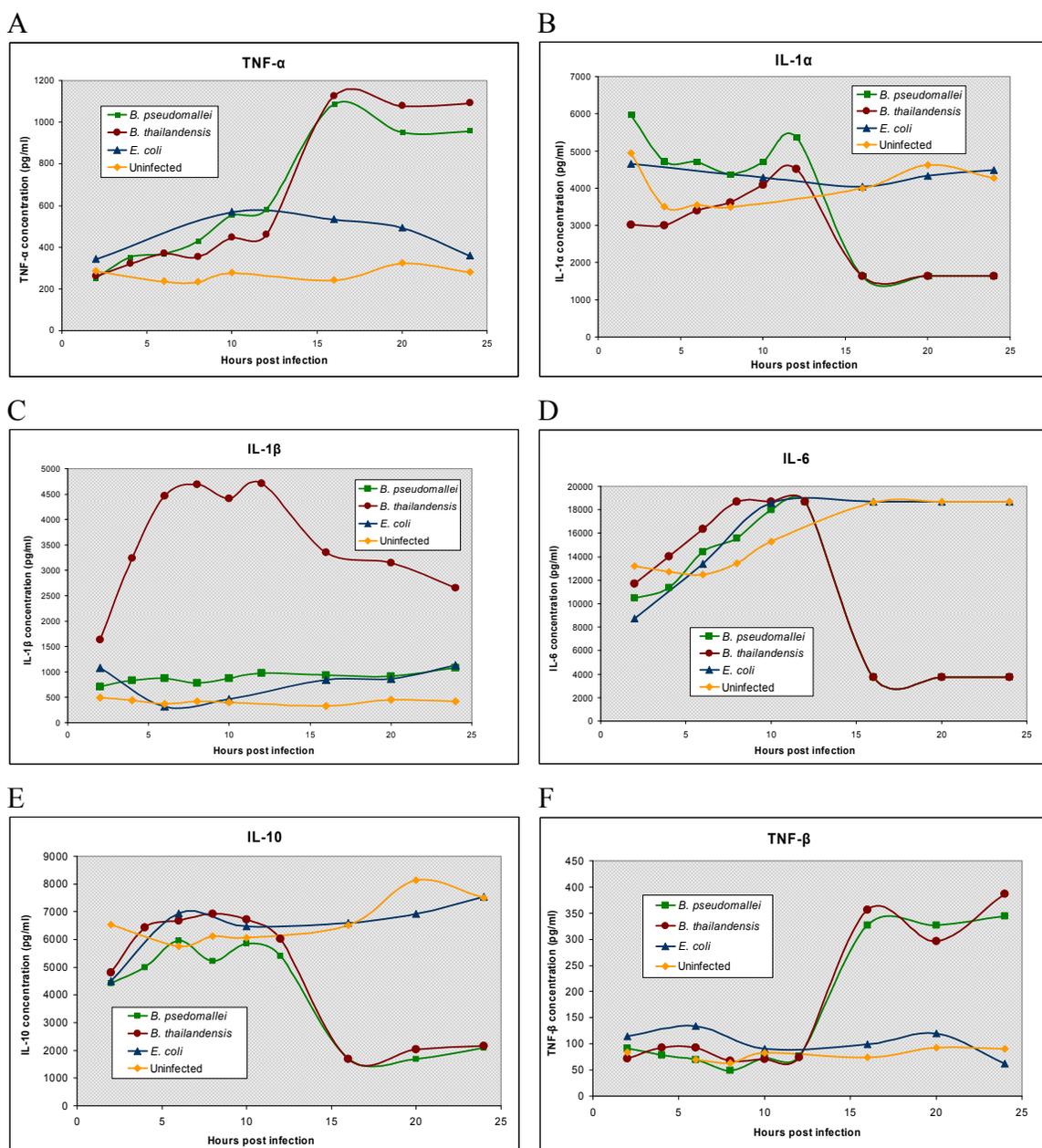
Figure 4. Kinetics of cytokine production by PMA-differentiated U937 macrophage-like cells infected with *B. pseudomallei*, *B. thailandensis*, or *E. coli*.
One million cells were infected with an MOI of 6. Supernatant samples were collected 2, 4, 6, 9, 10, 12, 16, 20, and 24 hours post infection and assayed for cytokine levels using ELISA.
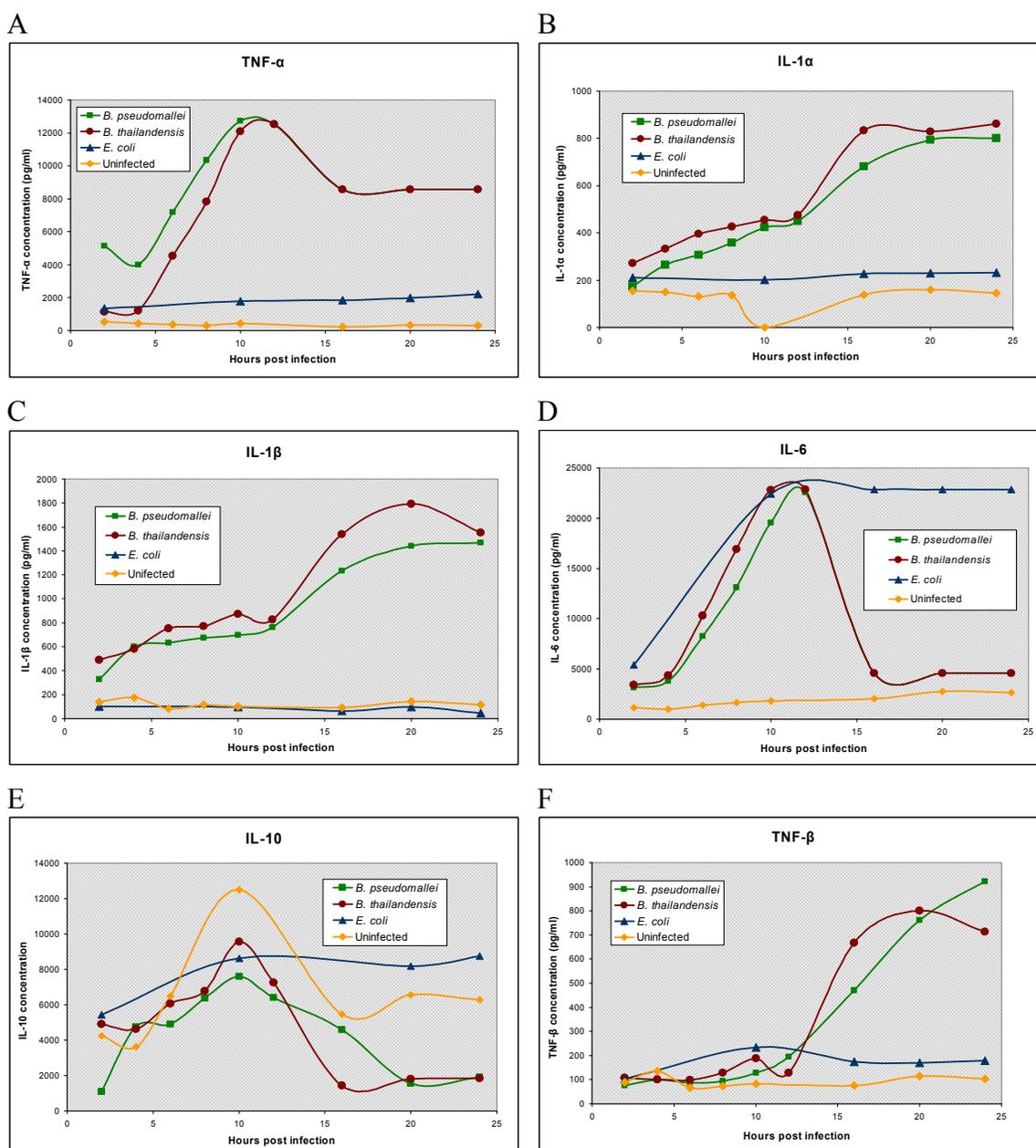
Figure 5. Kinetics of cytokine production by primary peripheral blood monocytes infected with *B. pseudomallei*, *B. thailandensis*, or *E. coli*.

Primary human peripheral blood monocytes from healthy donors were infected with an MOI of 6. Supernatant samples were collected 2, 4, 6, 9, 10, 12, 16, 20, and 24 hours post infection and assayed for cytokine levels using ELISA.
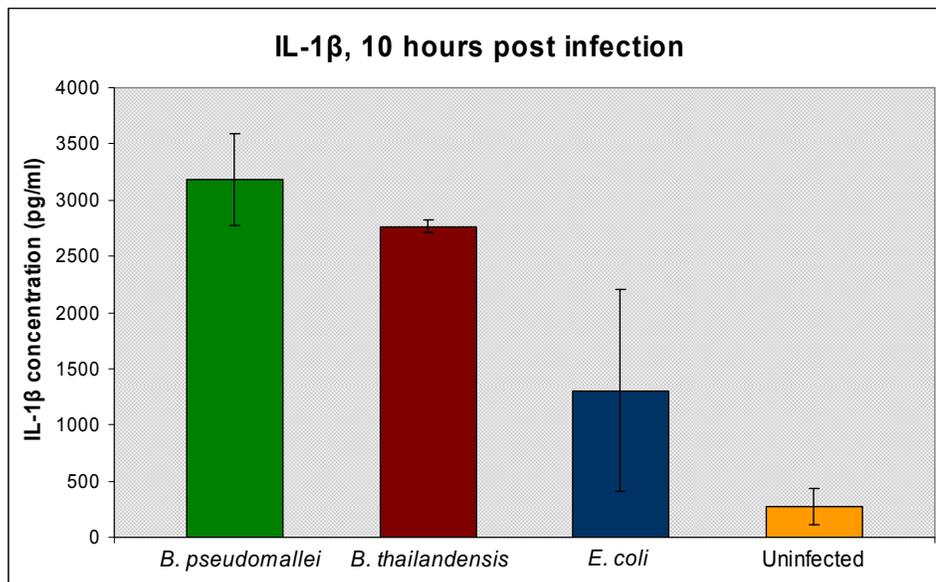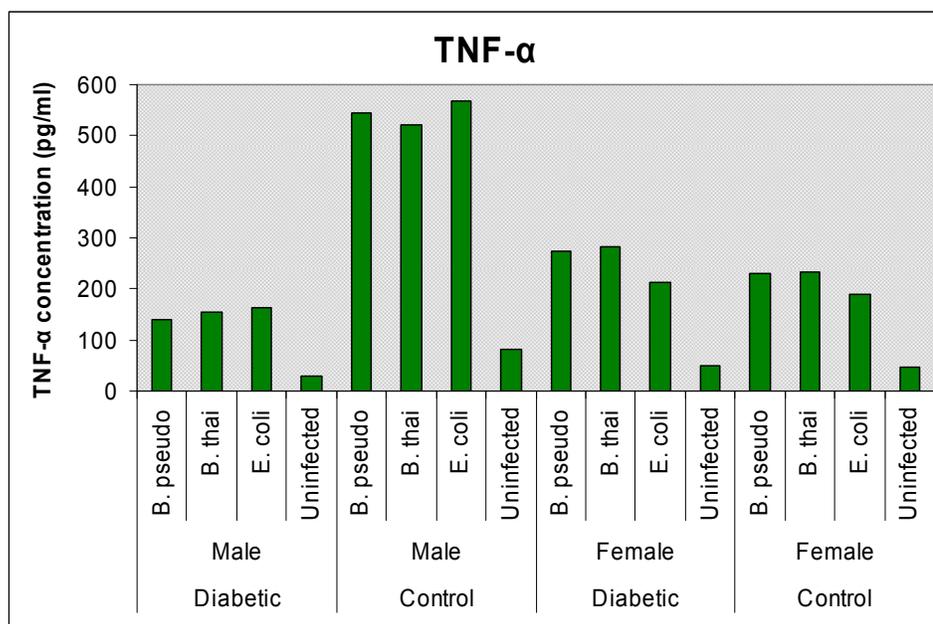
Figure 6. Kinetics of cytokine production by primary peripheral blood monocyte-derived macrophages infected with *B. pseudomallei*, *B. thailandensis*, or *E. coli*.
Primary human peripheral blood monocyte-derived macrophages from healthy donors were infected with an MOI of 6. Supernatant samples were collected 2, 4, 6, 9, 10, 12, 16, 20, and 24 hours post infection and assayed for cytokine levels using ELISA.

Figure 7. IL-1β production by primary peripheral blood monocytes infected with *B. pseudomallei*, *B. thailandensis*, or *E. coli*.
Primary human peripheral blood monocytes from healthy donors were infected with an MOI of 6. Supernatants were collected at 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least three experiments. Standard errors are shown.
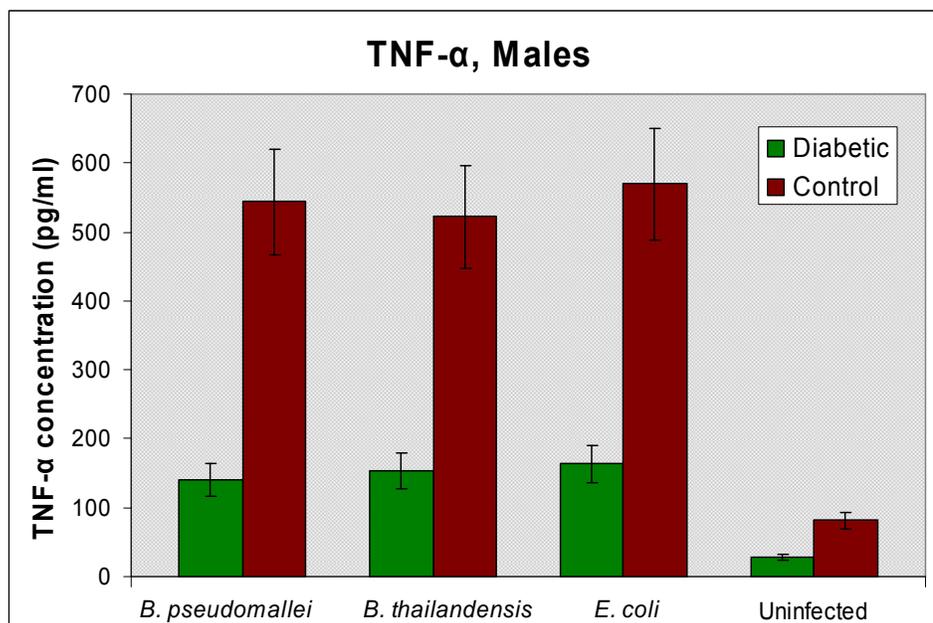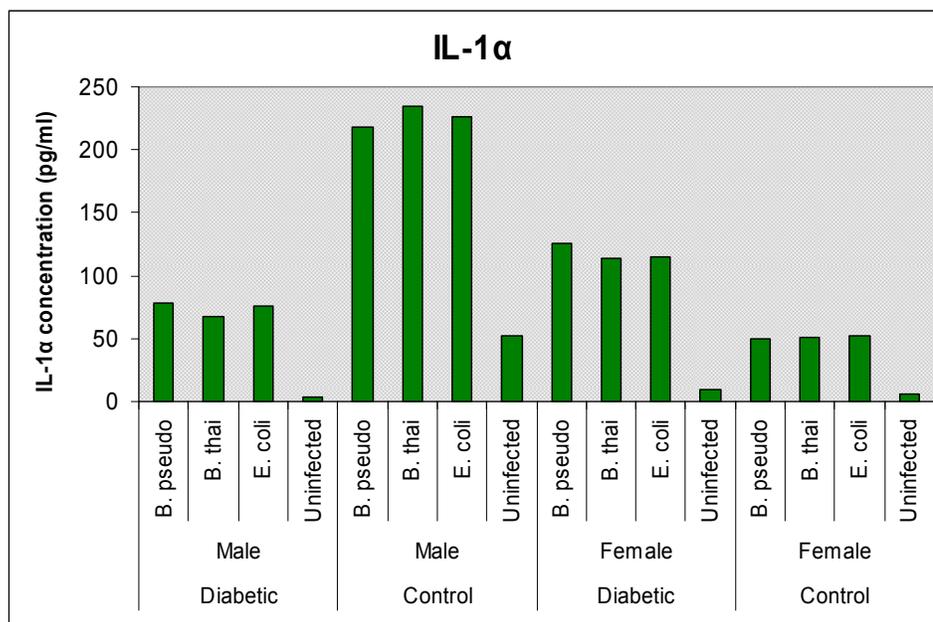
A

B

Figure 8. TNF-α production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown.
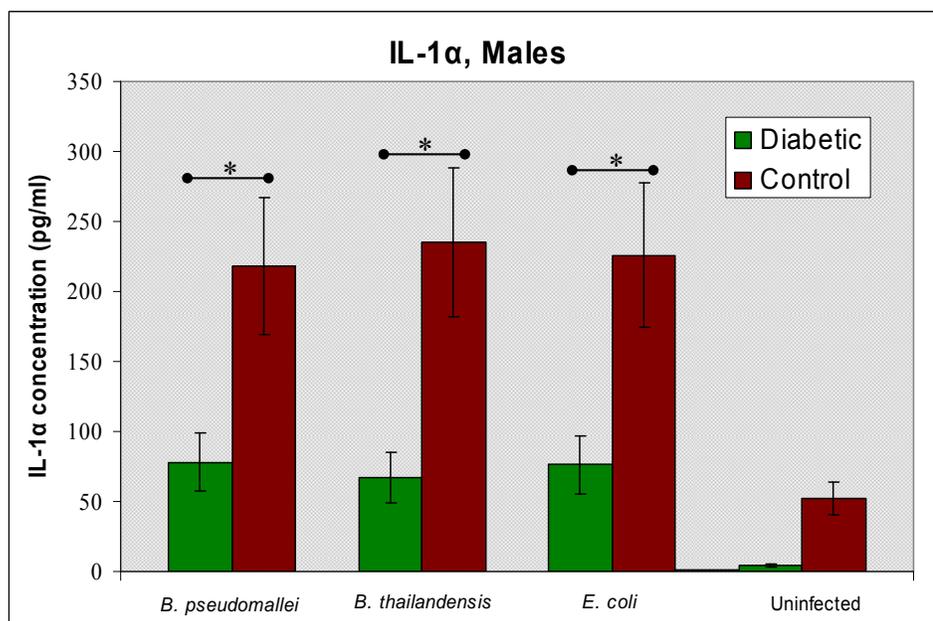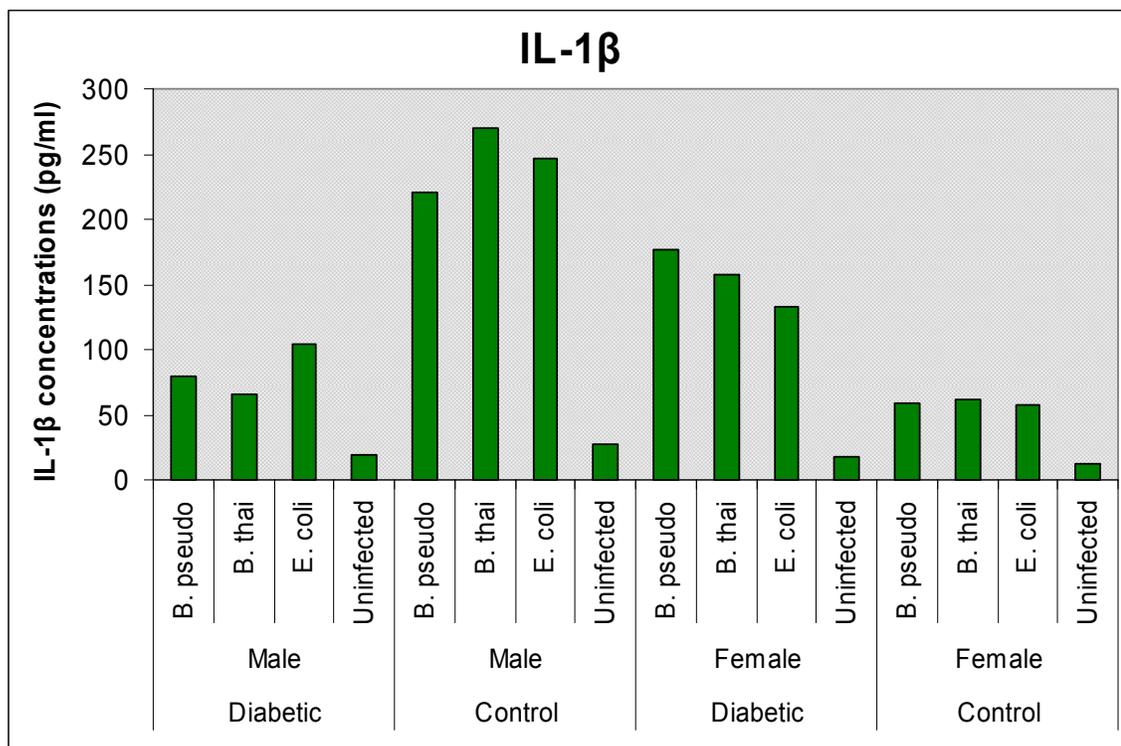
A

B

Figure 9. IL-1α production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown.
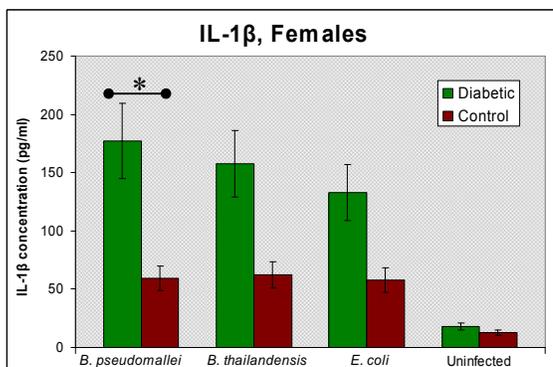
* indicates significant difference in cytokine production ($p < 0.05$).

A

B

C

Figure 10. IL-1β production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown.

* indicates significant difference in cytokine production ($p < 0.05$).

A

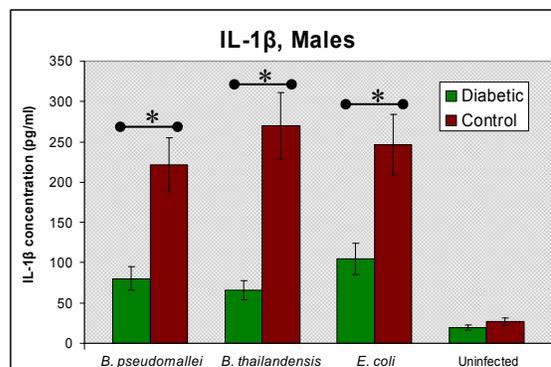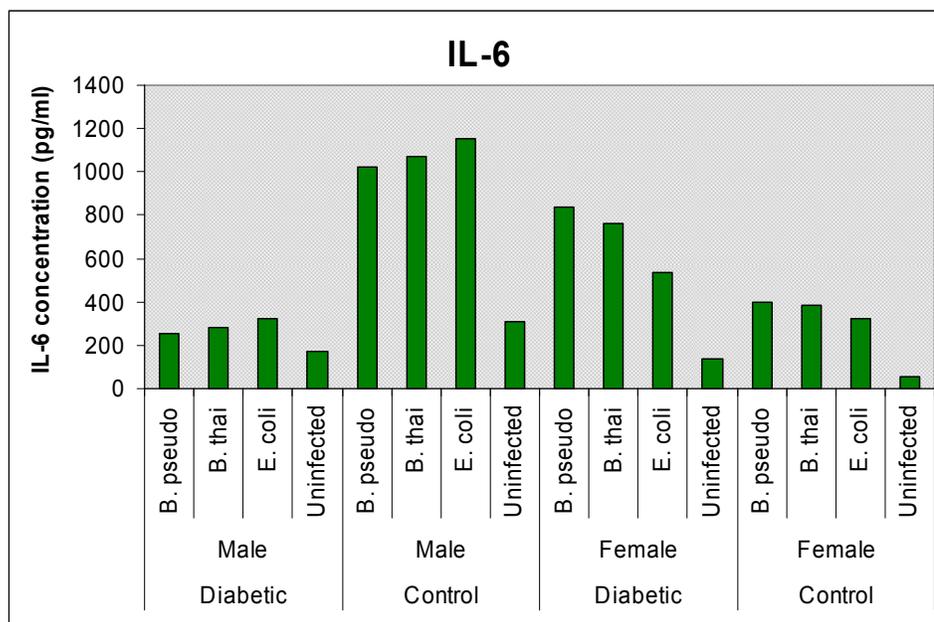

B

Figure 11. IL-6 production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown.

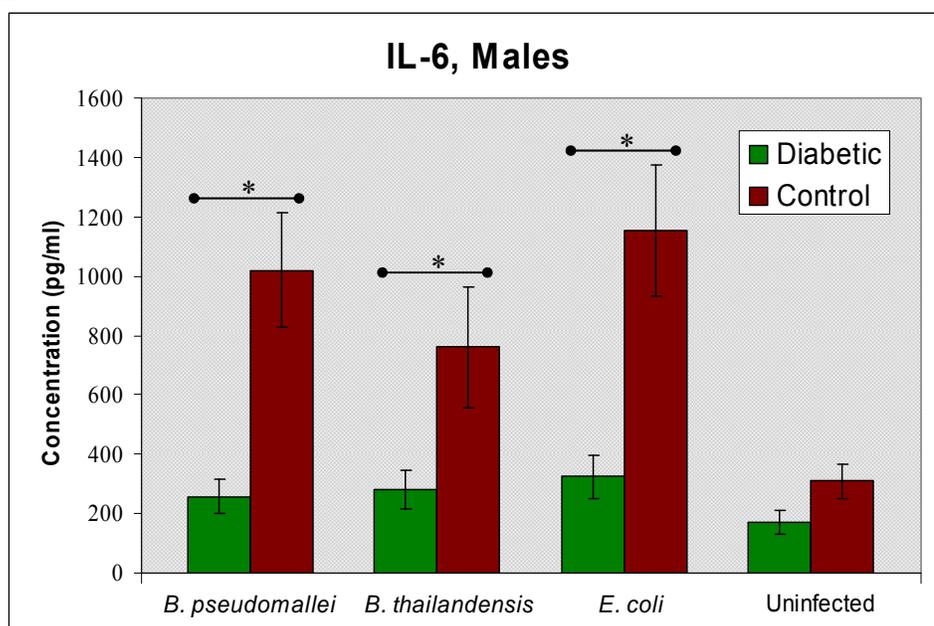* indicates significant difference in cytokine production ($p < 0.05$).

A

B

Figure 12. IL-8 production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown.

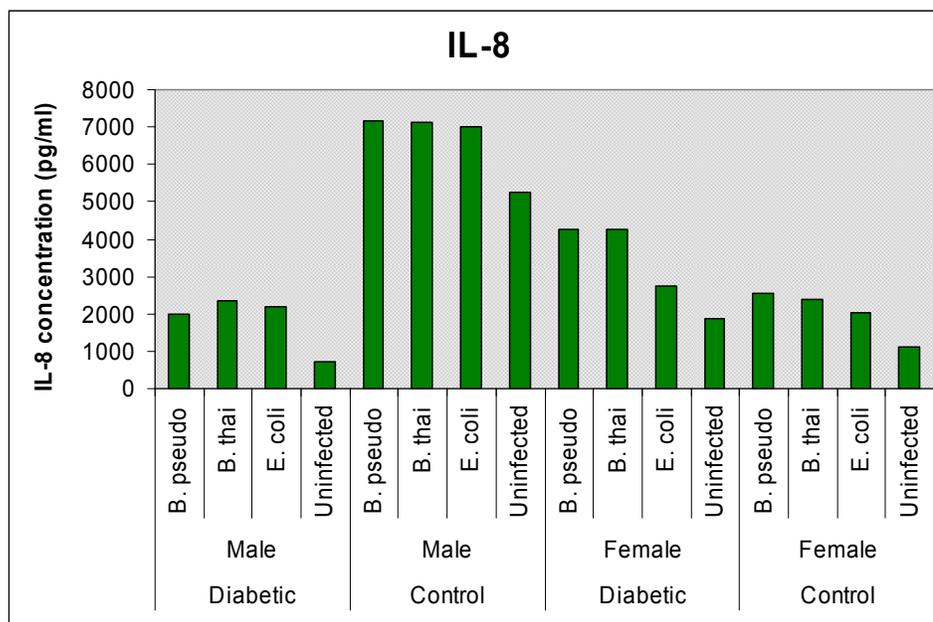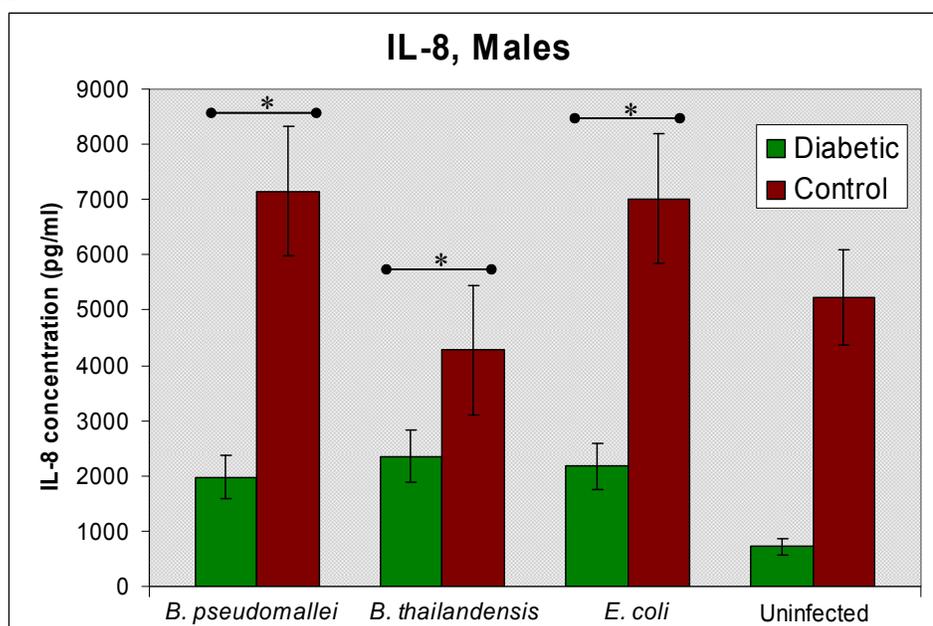* indicates significant difference in cytokine production ($p < 0.05$).
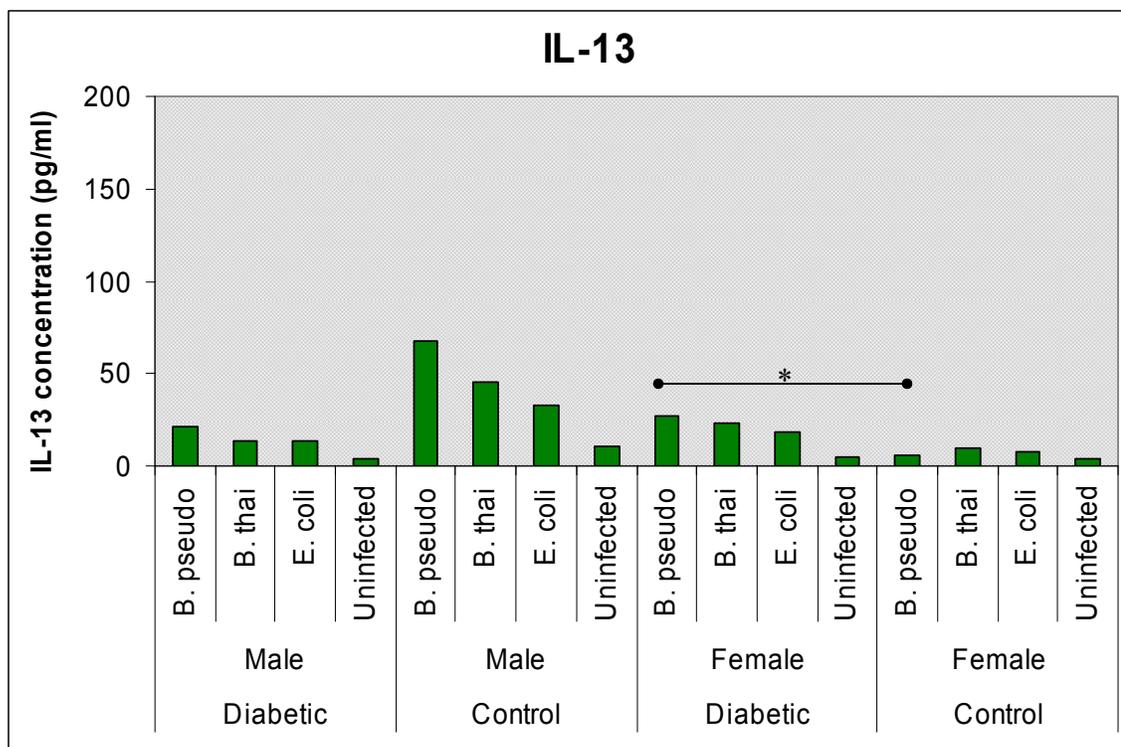
Figure 13. IL-13 production by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments.

* indicates significant difference in cytokine production ($p < 0.05$).
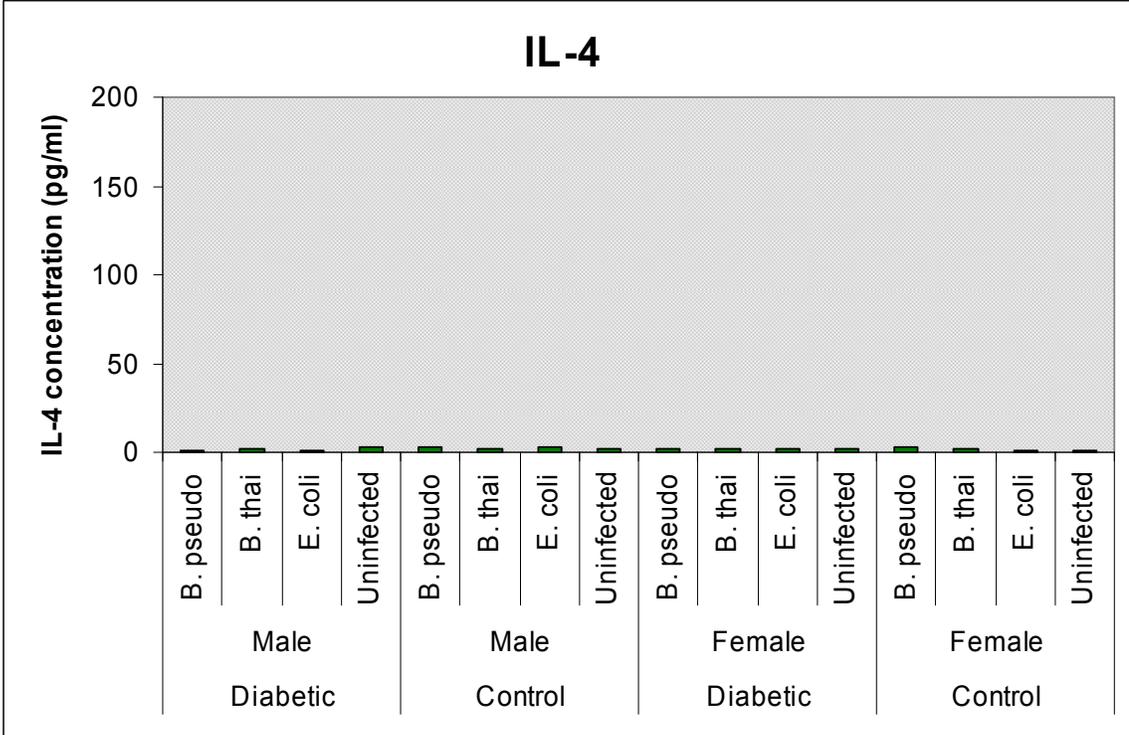
Figure 14. Production of IL-4 by primary monocytes from diabetic and control subjects in response to infection with *B. pseudomallei*, *B. thailandensis*, and *E. coli*. Primary human peripheral blood monocytes from diabetic or healthy donors were infected with an MOI of 6. Supernatants were collected 10 hours post infection and assayed for cytokine levels using ELISA. Each point represents the mean of at least five experiments. Standard errors are shown. Similar results were obtained for IL-2, IL-5, IL-10, TNF-β, and IFN-γ.

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