INTERACTION OF

BURKHOLDERIA PSEUDOMALLEI

WITH CELLS OF THE IMMUNE SYSTEM

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LIST OF ABBREVIATIONS

Abbreviations	
APCs	Antigen Presenting Cells
bp	Base pair
B. pseudomallei	*Burkholderia pseudomallei*
Bsa	*Burkholderia* Secretion Apparatus
B. thailandensis	*Burkholderia thailandensis*
cDNA	Complementary DNA
CFU	Colony Forming Unit
CMI	Cell-mediated Immune
CsA	Cyclosporin A
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzymed-linked Immunosorbent Assay
FliC	Flagellin
IFN-γ	Inteferon-gamma
IL	Interleukin
KO	Knock Out
LPS	Lipopolysaccharide
Min	Minute
MOI	Multiplicity of Infection
OD	Optical Density

PAMPs	Pathogen-associated Molecular Patterns
PAM	PAM_3CysSK_4
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PI	Pathogenicity Island
PRR	Pattern Recognition Receptors
RNA	Ribonucleic Acid
RT	Reverse Transcription
TCR	T Cell Receptor
TLR	Toll-like Receptors
TSA	Tryptic Soy Agar
TTSS	Type III Secretion Systems
WT	Wild Type

ABSTRACT

Burkholderia pseudomallei is the causative agent of melioidosis. Currently no vaccines are available and not much is known about the mechanisms of adaptive immunity to the bacterium. In this project, *in vitro* systems were employed to study interactions of the bacteria with host T cells. *B. pseudomallei* is able to invade and replicate intracellularly in T cells and dendritic cells. We found that bacteria do not induce rapid cell death in infected T cells as in dendritic cells. In fact, bacterial infection modulates T cell function by enhancing IL-2 production upon the engagement of the T cell receptor. Further studies reveal bacterial flagellin as a potent costimulatory molecule of T cell activation. However, flagellin is not the major contributor to T cell costimulation during live bacterial infection as determined by flagellin null mutant bacteria. The direct coactivation of T cells by *B. pseudomallei* could contribute to severe inflammation seen in acutely-infected patients.

CHAPTER 1

INTRODUCTION

Melioidosis

Burkholderia pseudomallei, discovered by Whitmore and Krishnaswani in 1912, is a free-living Gram-negative bacterium found in the soil and surface water (Whitmore and Krishnaswami, 1999). It is the causative agent for melioidosis, a disease that is predominantly endemic in Southeast Asia, particularly Thailand as well as northern Australia (Dance, 1991, Thomas, 1981, Rode and Webling, 1981, Nachiangmai *et al*., 1985, Charoenwong *et al*., 1992). Most cases occur during the rainy season when the bacterium is found most frequently in surface water and soil shortly after rainfall (Leelarasamee and Bovornkitti, 1989, Ashdown, 1979). Infection can occur through inhalation of aerosolized infectious particles, ingestion or contact of damaged skin surfaces or wounds with infectious agents found in contaminated soil or water (Currie *et al*., 2000). Melioidosis is also endemic in Singapore, where the bacterium has been previously isolated from soil and drain water (Thin *et al*., 1971). Being associated with high mortality rates, it is a disease of concern in the country. An average of 59 cases were reported each year with an average annual fatality rate of 27.4 % during the period 1990 to 2003 (Ministry of Health, Singapore Communicable Diseases Surveillance in Singapore 2003, Singapore, 2004). Moreover, *B. pseudomallei* is classified as a category B bioterrorism agent by the Centers for Disease Control and Prevention (CDC) (www.bt.cdc.gov/agent/agentlist.asp).

Melioidosis is a disease with diverse clinical manifestations. It can be categorized into acute, subacute and chronic infections (Currie *et al*., 2000). The disease is often associated with pneumonia-like symptoms such as fever and cough (Poe *et al*., 1971). The most severe form is acute septicaemic melioidosis, usually associated with bacterial

dissemination to distant sites leading to abscesses in organs such as the liver and spleen as well as other organs (Puthucheary *et al*., 1981 and 1992, Vatcharapreechasukul *et al*., 1992). Latent infections, whereby initial exposure to the pathogen is followed by a prolonged incubation period of even up to 62 years in one patient, have been documented (Mays and Ricketts, 1975, Chodimella *et al*., 1997, Ngauy *et al*., 2005). Recurrence of disease after apparently successful antibiotic treatment is also commonly seen (Chaowagul *et al*., 1993, White, 2003). Disease relapse could likely be due to reactivation of a persistent endogenous bacterial source such as within abscesses in the liver or spleen (Mays and Ricketts, 1975, Kanai and Dejsirilert, 1988, Desmarchelier *et al*., 1993). The majority of relapse cases is due to reactivation of the original infecting strain, though infection with different strains was demonstrated in some patients (Desmarchelier *et al*., 1993, Vadivelu *et al*., 1998). Asymptomatic conditions or subclinical infections also occur where healthy individuals have positive serological tests indicating prior exposure to the pathogen (Kanaphun *et al*., 1993, Currie *et al*., 2000). Risk factors predisposing to melioidosis include diabetes, alcoholism and chronic lung or renal disease (Leelarasamee and Bovornkitti, 1989, Brett and Woods, 2000, Currie *et al*., 2000).

Treatment and diagnosis

B. pseudomallei is known to develop resistance to many commonly used antibiotics, including quinolones and macrolides as well as third generation cephalosporins, penicillins and aminoglycosides (Dance *et al*., 1989, White, 2003, Cheng and Currie, 2005). Currently, initial treatment employs intravenous administration of ceftazidime and carbapenem antibiotics (imipenem and meropenem). The oral antibiotic TMP-SMX, with or without doxycycline and chloramphenicol is used for the prolonged

eradication phase (White *et al*., 1989, Sookpranee *et al*., 1992, Smith *et al*., 1996, Jenney *et al*., 2001, Chetchotisakd *et al*., 2001). However, relapse is still seen in some patients likely due to reactivation of persistent endogenous bacterial source. Antibiotic therapy would also be less efficient once the bacteria gain residence within intracellular compartments (Kanai and Dejsirilert., 1998, Jones *et al*., 1996, Chaowagul *et al*., 1993).

With protean clinical manifestations that often mimic that of other diseases, melioidosis needs to be diagnosed early to ensure prompt treatment. The standard diagnosis is the isolation of *B. pseudomallei* from bodily fluids of patients using culture-based methods. This requires the use of selective media such as the commonly used Ashdown medium (tryptase soy agar, glycerol, crystal violet, neutral red and gentamycin), now modified with colistin (Ashdown, 1979, Dance *et al*., 1989). The use of *B. pseudomallei* selective agar (BPSA) has also been recently described (Howard and Inglis, 2003). However, diagnosis using conventional media-based blood culture is time consuming. By the time culture positivity is indicated, bacteriamia would have occurred resulting in mortality. Alternative methods such as isolator lysis centrifugation provide a shorter time to positivity but sensitivity is compromised (Simpson *et al*., 1999). Apparently a reliable means to identify *B. pseudomallei*, the Vitek automated system has also been tested (Lowe *et al*., 2002). To reduce time to diagnosis, techniques such as antigen detection, antibody detection and molecular techniques have been employed. A monoclononal antibody latex agglutination test against a 200-kDa protein that could agglutinate *B. pseudomallei* positive blood culture fluid is currently widely used in Thailand (Anuntagool *et al*., 2000). Indirect Haemagglutination Assay (IHA) to detect antibodies remains the most widely used test. However, IHA poses a problem in endemic

regions where subclinical infections are commonly encountered with around 80 % of people who are healthy and seropostive by the age of 4 years (Khupulsup and Petchclai, 1986, Kanaphun *et al*., 1993). IHA is also associated with poor sensitivity and specificity (Sirisinha *et al*., 2000). Others have developed molecular methods such as PCR techniques to detect 16S RNA and 23S rRNA using specific-primers (Brook *et al*., 1997, Dharakul *et al*., 1996, Kunakorn *et al*., 2000, Rattanathongkom *et al*., 1997, Sirisinha *et al*., 2000). Reasonable sensitivity and specificity is also seen with Enzyme-Linked ImmunoSorbent Assays (ELISA) to detect *B. pseudomallei* proteins or monoclonal antibodies against cell wall components (Wongratanacheewin *et al*., 1993, Dharakul *et al*., 1999, Pongsunk *et al*., 1999).

Pathogenesis of *Burkholderia pseudomallei*

Over the past decade, *B. pseudomallei* is increasingly catching the attention of scientists whose work are beginning to shed light on the molecular pathogenesis of this bacterium. *B. pseudomallei* can invade and replicate in both non-phagocytic cells such as epithelial cells as well as phagocytic cells including macrophages and neutrophils (Kespichayawattana *et al*., 2004, Pruksachartvuthi *et al*., 1990, Jones *et al*., 1996, Harley *et al*., 1998). The bacterium is apparently capable of escaping phagosome-lysosome fusion after ingestion by phagocytes (Harley *et al*., 1998). It can also evade macrophage-mediated killing by interfering with inducible nitric oxide synthase (iNOS) and Tumour Necrosis Factor alpha (TNF-α) production (Utaisincharoen *et al*., 2000 and 2001). Once within the host cell, *B. pseudomallei* can also induce death of host cells through a caspase-1-dependent mechanism (Sun *et al*., 2005).

Many virulence factors of *B. pseudomallei* have been recognized. Firstly, *B. pseudomallei* possess three Type III Secretion Systems (TTSS) gene clusters. The TTSS 3, also known as Burkholderia secretion apparatus (*bsa*), has been shown to be required for full virulence in a hamster infection model (Warawa and Woods, 2005). This gene cluster encodes for a secretion apparatus and several secreted effectors. It exhibits homology to the TTSS found on the SP-1 pathogenicity island from *Salmonella enterica* that is needed for cellular invasion (Attree and Attree, 2001, Stevens *et al*., 2002). Type III-encoded translocator proteins associates with the host cell membrane and type III effector proteins are being translocated into the host cell cytosol (Cornelis *et al*., 2000). One *B. pseudomallei* type III effector, BopE, is homologous to *Salmonella enterica* SopE/ SopE2 and found to facilitate bacterial invasion of epithelial cells (Stevens *et al*., 2003). *BipD* gene encodes a component of the *B. pseudomallei* translocation apparatus and BipD mutants show attenuated virulence with impaired ability to replicate in the liver and spleen of infected BALB/c mice (Stevens *et al*., 2004). *B. pseudomallei* BipB was shown to induce apoptosis and formation of multi-nucleated giant cells of infected cells (Suparak *et al*., 2005).

B. pseudomallei capsular polysaccharide and flagella have also been associated with virulence (Reckseidler *et al*., 2001, Chua *et al*., 2003). In the presence of normal human serum, phagocytosis was more efficient for the acapsular mutant (Atkins *et al*., 2002). This correlates to a recent study demonstrating reduced deposition of complement factor C3b on bacterial surface, hence promoting their survival in the host circulation (Reckseidler *et al*., 2005). The capsule might also act as a barrier to prevent recognition of the complement-oposonized bacteria by complement receptor expressed on phagocytes

(Reckseidler *et al*., 2001). This data is in line with a previous study that showed *B. pseudomallei* resistance to complement-mediated lysis (Egan and Gordon, 1996). Although afflagellate mutant was still capable of invading and replicating in lung epithelial cells, it was found to be less virulent in an intraperitoneal and intranasal mouse model of infection, indicated by higher LD50 values compared to the wild type (Chua *et al*., 2003). Other putative virulence factors include *B. pseudomallei* Type IV pili involved in bacterial adherence, endotoxin lipopolysaccharide (LPS) that is less capable of activating mouse macrophages compared to *E. coli* LPS as well as *B. pseudomallei* quorum-sensing system-controlled virulence factors and processes (Essex-Lopresti *et al*., 2005, Utaisincharoen *et al*., 2000, Ulrich *et al*., 2004, Chan and Chua, 2005).

Host immunity to bacteria

During the initial encounter with pathogens, innate immune cells such as monocytes, neutrophils and dendritic cells (DCs) recognize pathogen-associated molecular patterns (PAMPs) expressed on the bacteria through pattern recognition receptors (PRR) such as Toll-like receptors (TLR) and scavenger receptors. They then become activated to kill the pathogen. *B. pseudomallei* have several candidate TLR ligands such as LPS (TLR4), peptidoglycan (TLR2), flagellin (TLR5) and CpG DNA (TLR9). To date, little is known about the role of TLRs in *B. pseudomallei* infection. TLRs are important initiators of innate immunity as well as integral links between innate and adaptive immunity. For instance, DCs require TLR signals to undergo maturation into cells capable of presenting captured antigens to T cells (Kaisho and Akira, 2000, Takeda *et al*., 2003, Pearce *et al*., 2006). T cell activation requires both antigenic signals

as well as costimulatory signals provided by professional antigen presenting cells such as DCs (Jenkins *et al*., 1990, Johnson and Jenkins, 1993).

The pro-inflammatory cytokine Inteferon-gamma (IFN-γ) is important for early host resistance against *B. pseudomallei* infection (Santanirand *et al*., 1999). NK cells and T cells have been found to be major sources of early IFN-γ (Haque *et al*., 2006). A recent study showed that *B. pseudomallei* could suppress IFN γ -responses in infected mouse macrophages by activating suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) (Ekchariyawat *et al*., 2005). In comparison with BALB/c mice, C57BL/6 mice are more resistant to *B. pseudomallei* infection. Bacterial counts in C57BL/6 mice were decreased 12 h after infection compared to BALB/c mice, which suggests that lower susceptibility of C57BL/6 mice could be due to their ability to develop innate immune responses during the early phase of *B. pseudomallei* infection (Hoppe *et al*., 1999, Liu *et al*., 2002). BALB/c mice were shown to succumb to septicemic infection with organ inflammation due to overwhelming high bacterial loads (Leakey *et al*., 1998, Hoppe *et al*., 1999, Liu *et al*., 2002). A transient hyperproduction of IFN-γ was seen in BALB/c mice, which correlated to disease severity (Liu *et al*., 2002). Hence, uncontrolled proinflammatory responses could also result in disease pathology. Clinical support for hyperinflammation in melioidosis patients includes elevated serum concentrations of pro-inflammatory cytokines such as IL-6 and IFN-γ in patients with septicemic melioidosis (Simpson *et al*., 2000, Brown *et al*., 1991).

The elicitation of adaptive immune responses is believed to be important during infection with intracellular pathogens such as *B. pseudomallei* (Raupach *et al*., 2001, Healey *et al*., 2005). Recent clinical studies by Keethesan and coworkers (2002) have

shown that lymphocytes taken from patients who have recovered from melioidosis show enhanced proliferation and IFN-γ production in response to *B. pseudomallei* antigens. This demonstrates the development of cell-mediated (CMI) immune responses in infected patients. Their data also suggest the importance of CMI for protection against disease progression in seropositive but healthy individuals and that patients could succumb to infection as a result of inadequate CMI responses (Ketheesan *et al*., 2002, Barnes *et al*., 2004). *B. pseudomallei* could prime both antigen-specific $CD4^+$ and $CD8^+$ T cells, reflected by enhanced IFN-γ production in response to heat killed bacteria by these cells taken from infected mice (Haque *et al*., 2006). In particular, $CD4^+$ T cells are important for late host resistance to *B. pseudomallei* in mouse infection models (Haque *et al*., 2006). Therefore, T cell-mediated adaptive immune responses and their production of cytokines are believed to be important for control of *B. pseudomallei* infection (Healey *et al*., 2005, Haque *et al*., 2006).

Project aims

Knowledge of the pathogenesis of *B. pseudomallei* is essential to facilitate therapeutic developments. Our objective is to gain insights into the pathogenesis of the bacteria by employing the use of *in vitro* culture systems to study the interactions of *B. pseudomallei* and immune cells. T cells play an important role in cell-mediated responses while dendritic cells, being professional antigen presenting cells necessary for priming naïve T cells, are important initiators of adaptive immunity. One of the most direct means to subvert host immune defenses involves inducing death or disrupting the functions of such immune cells in the infected host. Interestingly, previous work in the laboratory suggests that T cells seem to be able to control bacterial intracellular replication at early

stages of infection. DNA laddering, a characteristic feature of apoptosis, was not observed in T cells at 24 hours after infection compared to other cell types. One of the aims is to examine the ability of *B. pseudomallei* to invade and replicate intracellularly in T lymphocyte and dendritic cell lines. We also want to determine whether viability of the infected cells was affected as a result of *B. pseudomallei* infection. Currently little is known if *B. pseudomallei* could directly modulate T cell function despite the pathogen being known to interfere with cellular functions of phagocytic cells such as macrophages. Another aim is thus to examine whether *B. pseudomallei* could affect cytokine production by T cell lines in response to T cell receptor (TCR) stimulation.

The third aim of the project is to identify bacterial components either associated with live *B. pseudomallei* or their secreted products to modulate T cell activation. To do this, we used a flagellin-deficient mutant and a TTSS 3 mutant. The mechanism of this modulation will also be examined.

CHAPTER 2

IN VITRO INTERACTION BETWEEN *BURKHOLDERIA PSEUDOMALLEI* AND HOST T CELLS AND DENDRITIC CELLS

INTRODUCTION

Bacterial internalization and their ability to survive and replicate within host cells represents one facet of the pathogenic strategy of *B. pseudomallei*. Such a means of escape from host immune defenses could contribute to conditions of chronic infections and relapse after recovery from the clinical disease, where the dormant organism could become triggered from latency leading to acute disease especially in an immunocompromised individual (Pruksachartvuthi *et al*., 1990, Chaowagul *et al*., 1993).

The bacterium can invade phagocytic cell lines such as mouse macrophage and human monocytic cell lines as well as non-phagocytic cells such as human lung epithelial A549 cell line (Harley *et al*., 1998, Jones *et al*., 1996, Utaisincharoen *et al*., 2004). Pathogenic mechanisms of the bacteria include escape from membrane-bound phagosomes into cytosol and evasion of macrophage killing by interference with inducible nitric oxide synthase (iNOS) production (Harley *et al*., 1998, Utaisincharoen *et al*., 2001). Once within the host cell, *B .pseudomallei* can also cause host cell death through a caspase-1-dependent mechanism seen in macrophages (Kespichayawattana *et al*., 2000, Sun *et al*., 2005).

Induction of pathogenic mechanisms within the very cells, such as antigen presenting cells (APCs) and T cells, which play an important role in driving host innate or adaptive immune responses, could well represent one of the most direct ways to subvert host immune defenses. Dendritic cells (DCs) are professional APCs that determine the primary activation of naïve T cells, thereby playing a pivotal role in initiating adaptive immunity. T cells control the adaptive immune response by acting as effector cells through cytotoxic activity and the production of lymphokines that further

help to activate innate cells like the macrophages (Schuurhuis *et al*., 2006, Alam and Gorska, 2003). Hence, by invading and causing harm to these cells, the bacterium can potentially disrupt both arms of host immunity. To date, little is also known about the pathogenic interaction between *B. pseudomallei* and host T cells. From previous work in the laboratory, it was found that *B. pseudomallei* also induces rapid cell death in dendritic cells (Sun *et al*., 2005).

Therefore, we are interested in studying the interaction between the bacterium and host T cells and dendritic cells. We first examine the ability of the virulent *B. pseudomallei* to invade and replicate within cultured T lymphocyte and dendritic cell lines, as well as determine how their cell viability is affected by bacterial infection.

MATERIALS AND METHODS

Culture and maintenance of cell lines

Jurkat T cell clone E6-1(ATCC No. TIB-152) was purchased from American Type Culture Collection (ATCC, Manessa, VA). The mouse CD8 T cell hybridoma B3Z that recognizes SIINFEKL in association with H-2K^b was a kind gift from Dr. Ronald Germain (NIH, Bethesda, MD). DC2.4 cell line (*H-2*^{b}) was a gift from Dr. Wong Siew Heng (Dept. of Microbology, NUS). All cells were maintained using RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10 % Fetal Calf Serum (FCS, Hyclone Laboratories, Logan, UT), 200 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (complete RPMI). Cells were passaged at a ratio of 1:10 every 3 to 4 days. At least 3 hours before bacterial infection, cell culture medium was changed to 10 % FCS in the absence of antibiotics.

Bacterial strains

KHW is a virulent strain of *B. pseudomallei* isolated from a local patient who died from melioidosis (Liu *et al*., 2002). To prepare mid-log phase bacteria, 2 ml of Luria Bertani (LB) medium was inoculated with 100 µl of overnight culture and allowed to grow for 3 hours with constant shaking in 37 ºC incubator. All experiments involving the handling of live *B. pseudomallei* bacteria were carried out in a Class IIA biosafety cabinet level 2 with BSL3 safety practices in the $BSL2^+$ pathogen-specific laboratory.

Infection of cell lines with *B. pseudomallei* and intracellular bacteria replication

Cells were seeded in 12-well plates in antibiotic-free RPMI medium at a density of 1 x 10^6 cells/ml. Cells were infected with *B. pseudomallei* strain KHW at multiplicity of infection (MOI) of 5:1 or 30:1. Cells were incubated with bacteria at 37°C with 5 % CO_2. Two hours after infection, cells were centrifuged at 300 g for 5 minutes and supernatant was discarded. Cells were washed twice with Phosphate-buffered saline (PBS) and resuspended in 1 ml of fresh medium containing 250 µg/ml of kanamycin to suppress the growth of extracellular bacteria. Four, 8 or 24 hours after infection, cells were lysed with 0.1 % Triton-X-100. Serial dilutions of the lysate were plated on Tryptic Soy Agar (TSA) plates containing 5 µg/ml of gentamycin. Bacterial colonies on the plates were counted after 24 hours.

Infection and XTT assay

Cells were seeded in a 96-well plate antibiotic-free medium at a density of 0.2 x 10^6 cells/ml. Cells were infected with live bacteria at the desired MOI as described above. Two hours after infection, 250 µg/ml of kanamycin was added to suppress the growth of extracellular bacteria. Two hours after addition of kanamycin, 100 µl of XTT yellow

tetrazolium salt reagent (Roche Molecular Biochemicals, Germany) was added to the cells. Cells were incubated with XTT for 3 hours, during which viable cells will convert XTT to a red formazan product released into the medium with an absorbance OD490 nm that was measured by a spectrophotometer. In this case, the total hours of infection before OD490 nm is recorded are 7. For the 11 hour time point, XTT was added 4 hours after addition of kanamycin followed by 3 hours incubation with XTT before the OD was measured. XTT accumulation was determined at various time points up to 30 hours after infection.

Infection and LDH assay

Cells were seeded in a 96-well plate antibiotic-free medium with 2 % FCS at a density of 0.2 x 10^6 cells/ml. Cells were infected with live bacteria at the desired MOI as described above. Two hours after infection, 250 μg/ml of kanamycin was added to suppress the growth of extracellular bacteria. Four, 8 or 24 hours after infection, cell supernatant was collected. Lactate dehydrogenase (LDH) activity in the supernatant measured with Cytotoxicity Detection Kit (Roche Diagnostics, IN) according to the manufacturer's instructions. Maximum release was achieved by lysing cells with 1 % Triton X-100. LDH activity in supernatant of uninfected cells was taken as spontaneous release. Percentage cytotoxicity was calculated according to this formula: % cytotoxicity = (Test LDH release – spontaneous release) / (Maximum release - spontaneous release).

RESULTS

B. pseudomallei strain KHW invasion of and intracellular replication in T and DC cell lines

To examine the interaction of *B. pseudomallei* with T cells, the intracellular replication of the bacteria in a mouse $CD8^+$ T cell line, B3Z T cell, was measured. Four hours after infection of B3Z T cells at MOI of 30 bacteria per cell, the intracellular bacteria count was 1.68×10^5 cfu per ml, equivalent to 1.12 % of the inoculum. After 24 hours of infection, the number of intracellular bacteria was found to increase approximately 20 fold, to 3.35×10^6 cfu per ml, indicating that the bacteria were able to replicate within the B3Z T cells (Fig 2.1A).

Internalization of *B. pseudomallei* into human Jurkat $CD4^+$ T cells is apparently less efficient compared to B3Z T cells. At 4 hours after inoculation, an intracellular count of only 1.10×10^3 cfu per ml, correlating to 0.01 % of the inoculum, was detected in Jurkat T cells. However, after 24 hours of infection, the intracellular bacteria count was 5.28×10^5 cfu per ml (Fig 2.1B). This increase in intracellular bacteria count detected at the later time point suggests that the bacteria, once internalized into the Jurkat T cell, can still survive and replicate intracellularly. The bacteria could require a longer period of time to adhere and enter the cells since Jurkat cells tend to clump together, thereby reducing the cell surface area through which the bacteria could be internalized.

B. pseudomallei was also able to invade the DC2.4 cells, a phagocytic dendritic cell (DC) line. Four hours after infection of DC2.4 cells at MOI of 5 bacteria per cell, the intracellular bacteria count was 4.00×10^5 cfu per ml, corresponding to 10.2 % of the inoculum. At 8 hours after bacterial inoculation, the intracellular number increased

approximately 2 fold, to 8.73 x 10^5 cfu per ml. Being a phagocytic cell line, it is not surprising to observe a high rate of bacteria uptake into the DCs at early hours after infection. However, after 24 hours of infection, the intracellular bacteria count decreased to 3.58 x 10^5 cfu per ml (Fig 2.1C). Under the light microscope, DC2.4 cells appeared to be lysed by the bacteria at this time point, which could account for low intracellular bacteria count detected after 24 hours of infection.

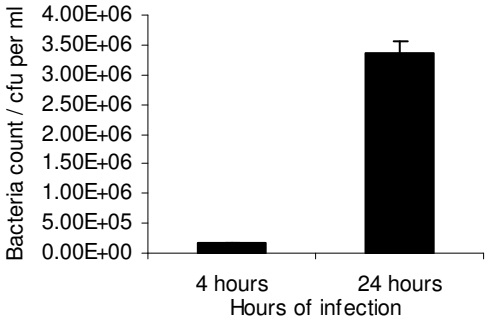
Cell Viability of B3Z T cell, Jurkat T cell and DC2.4 cell line infected with *B. pseudomallei* strain KHW

Results from the LDH cytotoxicity assay indicated that DC2.4 cells have undergone significant cell lysis (25.5 % cytotoxicity) at 24 hours of infection (Fig 2.2). Thus, I compared the cell viability of B3Z T cells and Jurkat T cells with DC2.4 cells. When B3Z T cells were infected at MOI 5:1 and 30:1, cell viability of infected cells remained above 90 % and 85 % respectively from 7 to 20 hours after infection. B3Z T cells exhibited a significant decrease in cell viability to 28.2 % at approximately 22 hours after infection at MOI 30:1. At MOI 5:1, they exhibited only a gradual decrease in cell viability from 22 hours of infection onwards (Fig 2.3A). Of the three cell lines tested, Jurkat T cells were more resistant to bacteria-induced cell death. At both MOI 5:1, cell viability of infected Jurkat T cells remained close to 100 % from 5 to 28 hours after infection. At MOI 30:1, cell viability of infected cells remained above 97 % from 5 to 24 hours after infection, and decreased to 48.5 % at 28 hours after infection (Fig 2.3B). Interestingly, at the earlier time point of 5 hours after infection, Jurkat cells infected at MOI 30:1 exhibited higher cell viability of 191.8 % relative to the uninfected T cells, indicated by the higher amounts of XTT conversion to formazan product by the infected cells compared to the uninfected cells (Fig 2.3B). This suggests that *B. pseudomallei*

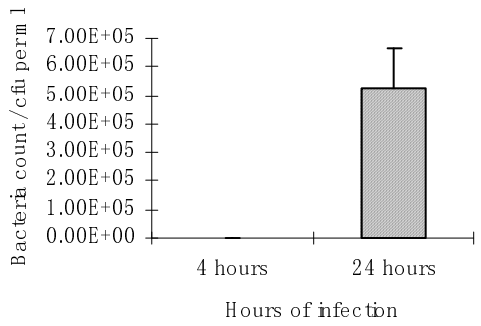
could lead to T cell proliferation during early hours of infection, instead of causing early cell death as in dendritic cells. This result could not be a false positive as there was no significant conversion of XTT reagent to formazan product by *B. pseudomallei* cultured alone, in the absence of T cells (data not shown).

In comparison to the T cell lines tested, *B. pseudomallei* infection causes rapid cell death in DC2.4 cell line. At both MOI 5:1 and 30:1, cell viability of DC2.4 cells was 81.8 % and 73.4 % respectively at 7 hours after bacterial infection and continued to decrease over time to only 15.1 % and 9.3 % respectively at 15 hours after infection (Fig 2.3C). The above results show that non-phagocytic T cells are less susceptible to a bacteria-induced cell death as compared to the phagocytic DCs, which could be correlated to the higher extent of bacteria internalization seen in the DCs than in the T cells.

(A)



(B)



(C)

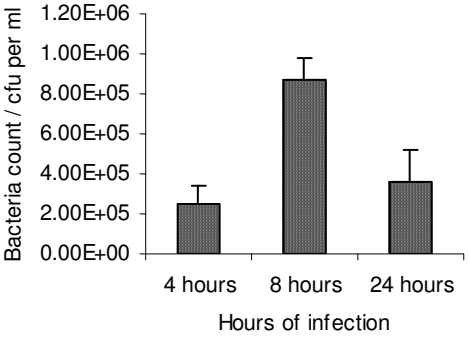


Figure 2.1 Invasion and replication of *B. pseudomallei* in cell lines

1 x 10^6 B3Z T cells (A), Jurkat T cells (B) and DC2.4 (C) cells were infected with *B. pseudomallei* strain KHW at an MOI of approximately 30:1 (B3Z and Jurkat T cells) and 5:1 (DC2.4 cells). 4, 8 and 24 hours after infection, the intracellular bacterial cfu count was determined. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

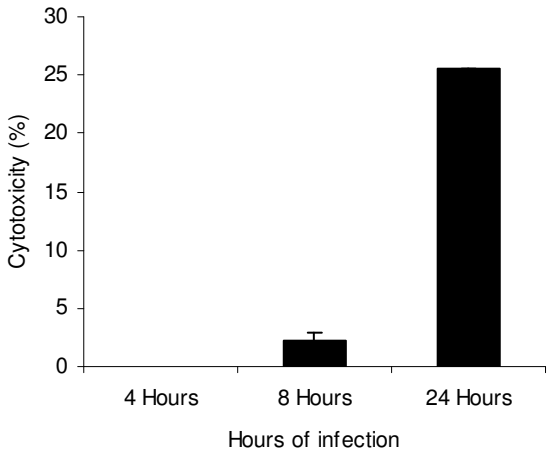


Figure 2.2 Percent cytotoxicity of DC2.4 cells after infection with *B. pseudomallei*

0.2 x 10^6 DC2.4 cells were infected with *B. pseudomallei* strain KHW at an MOI of approximately 5:1. At various time points after bacterial infection, the amount of LDH in the cell supernatant was measured. LDH activity of the uninfected cells was taken as the spontaneous release and % cytotoxicity of infected cells was calculated as described in Materials and Methods. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

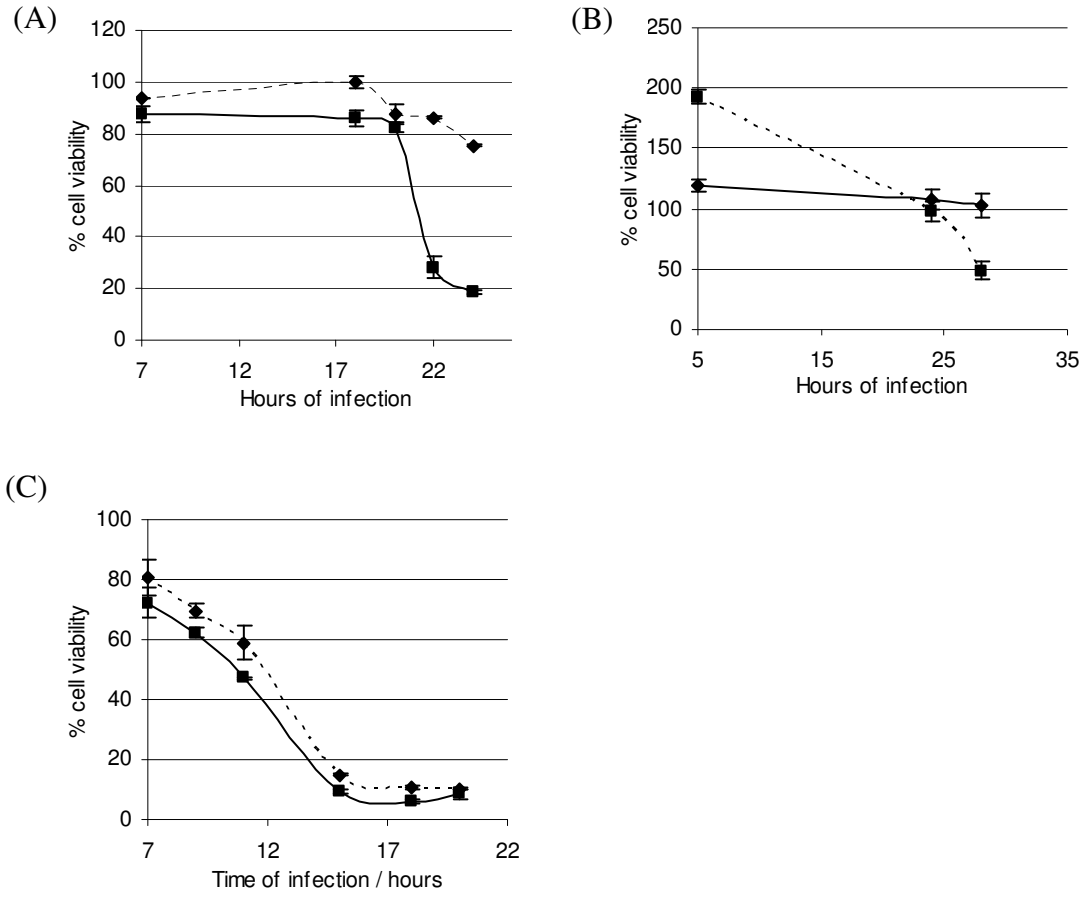


Figure 2.3 Percent cell viability of B3Z T cells, Jurkat T cells and DC2.4 cells after infection with *B. pseudomallei*

0.2 x 10^6 B3Z T cells (A), Jurkat T cells (B) and DC2.4 cells (C) were infected with *B. pseudomallei* strain KHW at an MOI of approximately 5:1 (dashed line) and 30:1 (solid line). At various time points after bacterial infection, the amount of XTT conversion by the cells was determined by measuring OD490 nm of the red formazan product. Percent cell viability of the bacteria-infected cells was calculated relative to the uninfected cells as a control with 100% cell viability. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

DISCUSSION

It is evident that *B. pseudomallei* can invade, survive and multiply within both phagocytic cells including macrophages and monocytes as well as non-phagocytic cell lines such as human lung epithelial cells. This could account for the occurrence of latent infections and disease relapse from reactivation of the bacterium persisting within the host (Harley *et al*., 1998, Jones *et al*., 1996). Results of our study have shown that the above list can be expanded to include murine phagocytic dendritic cells and also non-phagocytic T cells of mouse or human origin. To date, there have been no published reports on intracellular replication of *B. pseudomallei* in T cell lines.

The interaction of *B. pseudomallei* with mouse and human T cells provides a means to study how the bacterium can directly disrupt the adaptive arm of host immunity. In our study, we found that internalization and intracellular replication of virulent *B. pseudomallei* KHW strain could occur in human Jurkat T cells and mouse B3Z T cells, both being non-phagocytic cells. T cells are central players that control the adaptive immune responses. Therefore, the entry and persistence of the pathogen within these cells could potentially affect their functions by inhibiting cellular signaling pathways or directly decreasing their viability. *B. pseudomallei* infection was found to result in significant decrease in B3Z T cell and Jurkat T cell viability at around 22 and 28 hours after infection. When the bacteria eventually cause death of the infected T cells, the host could then be unable to mount an adequate adaptive immune response. During an intracellular bacterial infection, antibody responses provide protection before the bacteria become internalized and reside within the host cells. In this case, the eradication of the pathogen would largely rely on the antigen-specific effector T cells that can cause

cytotoxicity of infected cells or release cytokines that help to further activate the infected macrophages. $CD4^+$ helper T cells secrete cytokines required for activation of macrophages and for antibody responses (Raupach and Kaufmann, 2001, Alam and Gorska, 2003). Macrophages are important phagocytic antigen-presenting cells (APCs) of the innate immune system, which are capable of engulfing bacteria and killing through phagosomal acidification and production of reactive oxygen intermediates (Kaufmann, 1993). Hence, by causing T cell death, *B. pseudomallei* can also indirectly affect host innate immunity.

B. pseudomallei internalization into Jurkat T cells was observed to be less efficient compared to B3Z T cells, with close to 0 % of the inoculum detected in the former at 4 hours after infection. However, the numbers of intracellular bacteria increased at later time points after infection, indicating that the bacterium was still capable of entering and replicating within the human T cells. Internalization and intracellular replication of *B. pseudomallei* was also observed in dendritic cells (DCs). 10.2 % of the bacterial inoculum was detected in these cells at 4 hours after infection, corresponding to the highest rate of bacteria uptake in comparison to the other two T cell lines tested. This observation is not unforeseen since DCs are professional APCs that are phagocytic in nature. In this context, host APCs retain their ability to phagocytose extracellular pathogenic organisms, and bacterial antigens are expected to become processed and presented to naïve T cells. However, although the bacteria could be phagocytosed by DCs, this facet of the innate immune response is still botched by the bacteria-induced rapid cell death seen in the infected DCs. Cell viability of infected DCs started to decline rapidly from as early as 7 hours after infection.

DCs are professional APCs which capture the bacteria for digestion within the phagosomes. The released immunogenic antigens are processed to peptides being complexed with MHC molecules and then presented to naïve T cells. The latter requires antigen recognition by T cell receptor and a second coactivation signal provided by surface costimulatory ligands e.g. CD80/ CD86 found on the DCs to achieve full activation (Jenkins and Johnson, 1993). Hence, DC-T cell interaction is important for controlling the development of T cell responses in protective immunity. A recent study reported that immunization with DCs that were pulsed with heat-killed *B. pseudomallei* strain K96243 generated strong protective immune responses in mice (Elvin *et al*., 2006). By causing early death of the host DCs, *B. pseudomallei* could destroy this pivotal link between the innate and adaptive arm of immunity. Rapid cell death of DCs caused by *B. pseudomallei* is supported by previous data demonstrating bacteria-induced early cell death in human DCs (Sun *et al*., 2005).

By entering and residing in DCs as well as T cells, the bacterium could hide itself from the host humoral response. At the same time, the intracellular pathogen can induce death of these cells, hence destroying the essential cellular players that control the development of adaptive immunity.

CHAPTER 3

IN VITRO STUDIES ON FUNCTIONAL EFFECTS OF *BURKHOLDERIA PSEUDOMALLEI* ON T CELLS

INTRODUCTION

During initial stages of bacterial infection, innate immune responses are important to control the pathogen. Innate immune cells such as macrophages, natural killer (NK) cells and neutrophils recognize pathogen-associated molecular patterns (PAMPs) expressed on the bacteria through pattern recognition receptors (PRR) such as Toll-like receptors (TLR), scavenger receptors and mannan-binding lectins to become activated to kill. However, *B. pseudomallei* have developed various mechanisms to evade host innate immune responses. Studies have shown that the bacterium can interfere with phagocytic cell functions and resist killing within these phagocytes (Dorman *et al*., 1998, Egan and Gordon, 1996, Renella *et al*., 2006). Such pathogenic interference could result in persistence of the bacteria within the host. At such a juncture when innate immunity is breached, CMI responses could be important to control the infection.

In intracellular bacterial infections caused by *Salmonella* and *mycobacterial* species, T cells are also known to play a pivotal role in CMI responses. Being a facultative intracellular pathogen, *B. pseudomallei* can similarly invade and reside within host cells. T cell-mediated responses are particularly essential since antibodies specific to the bacterium have little protective effect once the pathogen remains intracellular (Ho *et al*., 1997, Raupach and Kaufmann, 2001). It is important for the infected host cells to interact with antigen-specific T cells, to trigger T cell proliferation, clonal expansion and differentiation into effector T cells (Alam and Gorska, 2003). The latter includes cytotoxic $CD8^+$ T cells that can induce lysis of infected host cells and $CD4^+$ helper T cells that secrete cytokines, which are soluble intercellular messenger molecules, each of them having specific effects on the target cells. For example, IFN-γ is important for

macrophage activation while IL-2 and IL-4 promote B cell proliferation and differentiation (Andersen *et al*., 2006). T cells thus play an important role in orchestrating appropriate host CMI responses.

There are recent reports supporting the importance of CMI responses for protection against progression in melioidosis. One study reported that T lymphocytes taken from patients who recovered from melioidosis showed enhanced proliferation and IFN-γ production in response to *B. pseudomallei* antigens (Ketheesan *et al*., 2002). The pro-inflammatory cytokine IFN-γ is important for early host resistance against *B. pseudomallei* infection (Santanirand *et al*., 1999). In addition, asymptomatic individuals who are seropositive demonstrate stronger CMI responses as reflected by enhanced proliferation of their lymphocytes in response to *B. pseudomallei* antigens when compared to individuals who had clinical symptoms of infection (Barnes *et al*., 2004). These reports provide evidence suggesting that a strong CMI response could be important for protection against disease progression, where those who fail to mount an adequate CMI response could succumb to clinical infection. A recent study has also shown that T cells contribute to host resistance during later stages of *B. pseudomallei* infection, whereby $CD4^+$ T cells could be primarily involved in protection since $CD4^+$ T cell-depleted mice showed shorter mean survival time after infection (Haque *et al*., 2006). *B. pseudomallei* could also prime both antigen-specific $CD4^+$ and $CD8^+$ T cells, reflected from enhanced IFN-γ production in response to heat killed bacteria by these cells taken from infected mice (Haque *et al*., 2006).

Although some information is available on how *B. pseudomallei* can compromise innate immunity, for example by affecting phagocytic cell function, there have not been

similar reports on T cell responses. In the case of *Yersinia* infection, T cell responses are critical to the host survival. However, *Yersinia* have the ability to directly suppress T cell activation through the virulence factor YopH, a tyrosine phosphatase, which binds to and inhibits signaling intermediates crucial for T cell antigen receptor (TCR) signaling such as linker for activation of T cells (LAT) and SH2-domain-containing leukocyte protein of 76 kD (SLP-76) (Alonso *et al*., 2004, Gerke *et al*., 2005). This is an example of a mechanism by which pathogens could alter T cell-mediated immune responses by crippling T cell signaling.

To date, not much is known about the interaction of *B. pseudomallei* and T cell-mediated immunity. We have shown in the previous chapter that the bacterium can invade and replicate within human Jurkat and mouse B3Z T cell lines. The bacterium did not induce rapid cell death in T cells as seen in dendritic cells. Hence, we are interested in studying how *B. pseudomallei* infection could affect host T cell function before eventually causing death of the infected cells. To do this, we test if human Jurkat $CD4^+$ T cells and mouse B3Z $CD8^+$ T cells that are infected with virulent *B. pseudomallei* strain *KHW* show any altered response to T cell receptor (TCR) stimulation by measuring Interleukin-2 (IL-2) production by the infected cells upon engaging their TCR.

To correlate the observed effects of *B. pseudomallei* infection on IL-2 production by T cells to the presence of certain bacterial virulence factors, cells were also tested with *bsaQ* and *fliC* mutants. The *bsaQ* mutant does not have the *bsaQ* gene that encodes a conserved structural component of the TTSS and the *fliC* mutant is an isogenic *fliC* gene deletion mutant of *B. pseudomallei* strain *KHW*. Effects of the mutants on IL-2 production of infected T cells were compared to that of wild type.

MATERIALS AND METHODS

Culture and maintenance of cell lines

Jurkat T cell clone E6-1 and B3Z T cell line were cultured and maintained as described in Chapter 2 Materials and Methods section.

Bacterial strains

KHW is a virulent strain of *B. pseudomallei* isolated from a local patient who died from melioidosis (Liu *et al*., 2002). *bsaQ*, a *B. pseudomallei* mutant with loss of *bsaQ* gene was constructed by insertional mutagenesis from strain *KHW* (Sun *et al*., 2005). *KHWFliCKO* is an isogenic FliC/ flagellin deletion mutant of *B. pseudomallei* by targeted gene replacement via homologous recombination (Chua *et al*., 2003). WT *E. coli* bacteria used was *E. coli* strain *M15*. *E. coliFliCKO* was a kind gift from Dr. Nancy Kleckner (Department of Molecular and Cellular Biology, Harvard University, Cambridge) (Bates *et al*., 2005). *B. thailendensis* (ATCC No. 700388), an avirulent species closely related to *B. pseudomallei*, was purchased from ATCC. To prepare mid-log phase bacteria, 2 ml of Luria Bertani (LB) medium was inoculated with 100 µl of overnight culture and allowed to grow for 3 hours with constant shaking in 37ºC incubator. *KHWFliCKO* was cultured in LB medium containing 50 µg/ml of kanamycin since the mutant carried a kanamycin resistant cassette inserted into the gene encoding for *fliC* protein, a monomer making up bacterial flagella.

Infection of T cells with live bacteria strains

Cells were seeded in 12-well plates in antibiotic-free medium at a density of 2 x 10^6 cells/ ml at least 3 hours before infection. Cells were inoculated with *B. pseudomallei KHW*, *bsaQ*, *KHWFliCKO*, *B. thailandensis*, *E. coli* strain *M15* or *E. coliFliCKO* at MOI

of approximately 30 colony forming unit (cfu) per cell. Bacteria for inoculation was prepared from mid log phase bacteria culture by resuspending a bacteria pellet approximated to 60 cfu in 100 µl of fresh LB, which was added to the cells. Plates were subsequently centrifuged at 600 g for 10 minutes prior to incubation at 37°C with 5% CO_2 for 2 hours.

Costimulation of bacteria-infected T cells with TCR stimulus

Two hours after infection, cells were centrifuged at 300 g for 5 minutes and supernatant was discarded. Cells were washed twice with PBS and resuspended in 1 ml of RPMI containing 40 µg/ml of tetracycline to suppress the growth of extracellular bacteria (100 µg/ml streptomycin was also used for *E. coli* infection). 0.2 x 10^6 cells were transferred to 96-well plates previously coated with 2.5 µg/ml of anti-CD3 monoclonal antibody (mAb) (BD Pharmingen, San Diego, CA) per well. Infected cells were incubated with surface-bound anti-CD3 mAb, in medium containing 40 µg/ml of tetracycline, at 37°C with 5% CO_2 for 22 hours (Jurkat T cells) or 20 hours (B3Z T cells). Cell supernatant was collected and IL-2 levels were determined using ELISA.

Costimulation of T cells with bacteria culture supernatant and TCR stimulus

Mid-log phase bacteria were prepared by inoculating 100 µl of overnight bacteria culture in 2 ml LB and allowed to grow for 3 hours with constant shaking in 37ºC incubator. *KHWFliCKO* was cultured in LB medium containing 50 µg/ml of kanamycin. The log phase subculture was centrifuged at 4000 g for 10 min to pellet down the bacteria. Supernatant was removed and passed through a 0.22 micron membrane syringe filter to remove any remaining bacteria. Jurkat T cells were seeded in 12-well plates in antibiotic-free medium at a density of 2 x 10^6 cells/ml. 200 µl of supernatant was added

to the cells prior to incubation at 37°C with 5% CO_2. Two hours later, cells were centrifuged and washed with PBS followed by costimulation with anti-CD3 mAbs according to the protocol described above. Cell supernatant was collected and IL-2 levels were assayed. Proteinase K-treated supernatant was prepared by incubating 200 µl of filtered bacteria supernatant 1 µg/ml pf proteinase K for 1 hour at 37°C, followed by incubation for 45 min at 80°C to inactivate proteinase K. Heat-treated supernatant was prepared by boiling 200 µl of supernatant in a boiling water bath for 10 minutes.

Costimulation of T cells with heat-killed bacteria and TCR stimulus

Heat-killed bacteria were prepared by heating mid log phase bacteria resuspended in PBS in a 90°C for 1 hour. Jurkat T cells were seeded in 12-well plates in antibiotic-free medium at a density of 2 x 10^6 cells/ml. Heat-killed bacteria were added to the cells at a bacteria-cell ratio of 5:1, 30:1 or 100:1 prior to incubation at 37°C with 5% CO_2. Two hours later, cells were centrifuged and washed with PBS followed by costimulation with anti-CD3 mAbs according to the protocol described above. Cell supernatant was collected and IL-2 levels were determined using ELISA.

Protein precipitation using trichloroacetic acid (TCA)

5-10 ml of mid-log phase bacteria culture were centrifuged at 4000 g for 5 min. Supernatant was filtered through a 0.22 micron syringe filter. In a 1.5 ml microfuge tube, 1 volume of 100 % TCA was added to 4 volumes of supernatant followed by spinning at 14,000 rpm for 5 min. Supernatant was carefully removed leaving the protein pellet intact. 1 ml of cold acetone was added to the pellet and samples were centrifuged at 14,000 rpm for 5 min. Two more acetone washes were performed and the protein pellet was dried by placing tube in 95°C heat block for 5-10 min to drive off the acetone.

Immunodetecion of flagellin by Western Blotting

Protein pellet prepared by TCA precipitation was dissolved in sodium dodecyl sulfate-polyacrylamide (SDS) loading dye containing βmercaptoethanol, boiled for 10 minutes in a boiling water bath and run on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel with 4 % and 10 % polyacrylamide stacking and resolving gel respectively followed by electroblotting onto nitrocellulose membrane. Western blot hybridization was performed using 20 mM Tris-HCl (pH 7.5) with 500 mM NaCl and 0.05 % Tween (TBS-T) containing 5 % bovine serum albumin (Sigma) as the blocking reagent. 1:100 dilution of mouse polyclonal anti-flagellin antibodies was used as the primary antibody. Mouse serum containing anti-flagellin antibodies was obtained from intra-peritoneal immunization of mice with recombinant FliC protein prepared by Ng H. L in the laboratory. After 1 hour incubation with primary antibody, the membrane was washed three times with TBS-T and incubated with 1:10000 dilution of horseradish peroxidase-conjugated goat anti-mouse Immunoglobulin G (Amersham, UK) for 1 hour. The membrane was washed 3 times and immersed in Enhanced chemiluminescence (ECL) detection reagent (Amersham) for the detection of bound HRP-conjugated secondary antibody by autoradiography.

Antigenic stimulation of bacteria-infected B3Z T cells with OVA peptide

DC2.4 cells were seeded at a density of 5 x 10^4 cells/ml in a 96-well plate in complete medium. Cells were fixed for 20 minutes with 1 % formaldehyde. Cells were washed 2 times with PBS and pulsed with 200 pg/ml of SIINFEKL OVA peptide for 1 hour. B3Z T cells were infected with bacteria for 2 hours at various MOIs according to the procedure described above and 5 x 10^4 cells were transferred to 96-well containing

pulsed DC2.4 cells. 18 hours later, cell supernatant was collected and IL-2 levels were determined by ELISA.

Cytokine ELISA

At various time points indicated, cell supernatant was harvested and production of IL-2 cytokine by infected Jurkat and B3Z T cells upon TCR engagement was measured by ELISA according to manufacturer's protocol (BD Pharmingen).

RESULTS

Enhanced IL-2 production by Jurkat T cells infected with live *B. pseudomallei*

Jurkat T cell clone E6-1 is a human leukemic T cell line known to produce large amounts of IL-2 cytokine after stimulation (Weiss *et al*., 1984). Furthermore, much *in vitro* work to elucidate important T cell signaling pathways, for example TCR-mediated signaling, have been largely based on using Jurkat T cell line as a study model (Abraham and Weiss, 2004). Hence, Jurkat T cells were chosen as the cell line to study the effects of *B. pseudomallei* infection on T cell cytokine production upon TCR engagement.

Jurkat T cells that were infected with *B. pseudomallei* strain *KHW* at MOI 1:1, MOI 5:1 and MOI 30:1 for two hours, followed by exposure to TCR stimulus supplied in the form of surface-coated anti-CD3 mAbs, produced 1.7, 2.2 and 4.0 fold greater IL-2 amounts respectively as compared to uninfected cells (Fig 3.1A). Our laboratory has previously shown the inability of *bsaQ* mutant to induce lysis of THP-1 cells (Sun G. W. *et al*, 2005). Jurkat T cells that were infected with *bsaQ* at MOI 30:1 similarly exhibited 4.1 fold increase in IL-2 production compared to uninfected cells upon TCR engagement (Fig 3.1B). Cells that were incubated with *B. thailandensis* at MOI 30:1, a closely related avirulent species of *Burkholderia*, were found to produce 7.0 fold higher IL-2 levels than uninfected cells (Fig 3.1B). In all cases, cells were incubated with bacteria for 2 hours followed by 22 hour incubation with anti-CD3 mAb, resulting in a total 24-hour period of infection, after which time, cell supernatant was harvested and assayed for IL-2. Throughout this period, cells were still viable as supported by previous data showing that cell viability of infected Jurkat T cells decreased only at 28 hours after infection. It is thus

evident from the results that bacterial interaction with host T cells seems to provide a coactivation signal that is manifested in their increased IL-2 cytokine production.

Enhanced IL-2 production by Jurkat T cells interacting with live *B. pseudomallei* in the absence of direct cell-bacteria contact

B. pseudomallei were previously found to invade Jurkat T cells with poor efficiency where only 0.01 % of the inoculum was found in host cells after 4 hours of infection. We hypothesize that bacterial modulation of host T cell cytokine production may not be solely dependent on bacterial entry into cells. Jurkat T cells were incubated for 2 hours with live *B. pseudomallei* strain *KHW* added into a transwell with a 0.4 micron membrane pore size to prevent cell-bacteria contact and subsequently exposed to surface-bound anti-CD3 mAb for 22 hours. The cells were found to produce 2.0 fold higher amounts of IL-2 compared to uninfected cells (Fig 3.2), indicating that the bacteria could be secreting a soluble costimulatory factor that can pass through the transwell membrane to contact the cells. To ensure that bacteria in the transwell had not passed through the membrane to contact the cells in the bottom well, the latter were observed with the light microscope under high magnification to check for the absence of bacteria swimming among the cells in the medium. Infection of T cells with live *B. pseudomallei* in the absence of a transwell separation, where bacteria can contact the cells, resulted in a 3.3 fold enhancement of IL-2 production (Fig 3.2).

To further test the hypothesis that *B. pseudomallei* strain *KHW* could be secreting a costimulatory factor, cells were treated with bacterial culture supernatant harvested from log phase bacterial culture for 2 hours, followed by exposure to anti-CD3 mAb. Cells treated with bacterial supernatant similarly showed a 1.9 fold increase in IL-2 production compared to untreated cells (Fig 3.3). Bacterial culture supernatant was

filtered before addition to the cells to ensure the absence of live bacteria that would otherwise directly contribute to T cell costimulation. Incubation of cells with live *B. pseudomallei* resulted in a 4.5 fold increase in IL-2 production (Fig 3.3). In the positive control, cells were costimulated with soluble anti-CD28 mAb and found to exhibit a similar fold enhancement (4.6 fold) in IL-2 production as when the cells were costimulated by live bacteria (Fig 3.3). It is known that CD28-mediated T cell costimulation is an important coactivation signal provided by CD80/ CD86 ligands on antigen-presenting cells to enable full activation of naïve T cells (Jenkins and Johnson, 1993).

Infection with live bacteria resulted in greater enhancement of IL-2 production as compared to incubation with bacteria in a transwell set up or with bacterial supernatant. This suggests that bacterial coactivation of host T cells is potentially more potent in the event when bacteria are allowed to contact the cells while at the same time, a stimulatory bacterial factor is also released by the bacteria onto the cells.

Bacterial culture supernatant pre-treated with proteinase K showed a significantly reduced enhancement (1.2 fold) of IL-2 production by Jurkat T cells compared to untreated bacterial supernatant that resulted in 1.7 fold increase in IL-2 production (Fig 3.4). However, heat-treated bacterial supernatant was able to enhance IL-2 production to a similar extent as untreated bacterial supernatant (Fig 3.4). This suggests that the costimulatory component found in *B. pseudomallei* supernatant could be a protein. In addition, heat denaturation could not disrupt the availability of this protein's epitope for recognition by costimulatory receptors on the T cells.

Absence of costimulatory effect of heat-killed *B. pseudomallei* on IL-2 production by Jurkat T cells

When Jurkat cells were incubated with heat-killed *B. pseudomallei* strain *KHW* at bacteria-cell ratios of 5:1 and 30:1, there was no enhancement IL-2 production from the low basal levels of IL-2 produced by untreated cells. Incubation of T cells with heat-killed bacteria alone did not result in any IL-2 production (Fig 3.5). This shows that costimulatory properties of *B. pseudomallei* could either be due to the presence of heat-labile bacterial components or is essentially dependent on the bacteria being alive and hence able to secrete costimulatory components or interact with host T cells.

Comparison of IL-2 production by Jurkat T cells infected with live WT *B. pseudomallei* strain *KHW* or *KHWFliCKO* live bacteria and culture supernatant

B. pseudomallei is motile due to the presence of flagella, filamentous appendages required for bacterial chemotaxis and motility, which are also virulence determinants of the pathogen (Chua *et al*., 2003). Flagella are composed of a structural repeating protein monomer called flagellin (FliC). FliC is a known ligand for TLR5 that has been shown to have direct costimulatory effects on human $CD4^+$ T cell proliferation and cytokine production (Lino, 1977, Caron *et al*., 2005). To determine if *B. pseudomallei* modulation of host T cell IL-2 production can be correlated to the presence of flagella on the bacteria, we tested the T cell costimulatory effects of *KHWFliCKO.* We observed that wild type (WT) *B. pseudomallei* strain *KHW* had greater costimulatory effects on Jurkat T cell cytokine production than *KHWFliCKO*. Cells that were infected with WT *KHW* produced 9.2 fold higher amounts of IL-2 upon TCR engagement than uninfected cells. On the other hand, infection with *KHWFliCKO* resulted in a 4.9 fold increase in IL-2 production upon TCR stimulation (Fig 3.6). Since *KHWFliCKO* mutant was aflagellate and non-

motile in semisolid agar, upon inoculation of WT or mutant bacteria, the plate was centrifuged at 600g for 10 minutes to facilitate bacteria-cell contact within the well (Chua *et al*., 2003). In an unpublished data in our laboratory, infection with KHWFliCKO together with the centrifugation procedure resulted in similar percent cell death as WT KHW in human macrophages. Hence, this procedure has been shown to be able effectively facilitate bacteria-host cell contact. Therefore, the partial reduction in IL-2 response of cells to costimulation by *KHWFliCKO* bacteria could not be a result of the reduced ability of the non-motile mutant bacteria to contact or invade the cells. The above results suggest that costimulatory effects of live *B. pseudomallei* is only partially due to the presence of FliC protein since *fliC* deletion mutant was still able to costimulate T cells to augment their cytokine production, though to a lower extent when compared to costimulation by WT *KHW*. This indicates the presence of other costimulatory bacterial components in *B. pseudomallei* that are responsible for augmenting T cell cytokine production.

The flagellae of many motile bacteria e.g. *Salmonella*, are comprised of the basal body, the hook, and the filament that is composed mainly of the protein FliC (Macnab, 2004). The secretion of flagellin monomer for the assembly of flagellae in *Salmonella* is similar to the secretion of virulence factors and requires an export machinery across both inner and outer bacterial cell membranes (Macnab, 1999, Galan and Collmer *et al*., 1999). Flagellin is normally found in abundance in *S. typhimurium* supernatants and is easily detected by immunoblot. In order to determine if flagellin was a T cell costimulatory component released by the bacteria, supernatants from the WT *B. pseudomallei* strain *KHW* or *KHWFliCKO* bacteria culture used for the above cell infection were also

harvested, filtered and separately incubated with the cells for 2 hours, followed by subsequent exposure to anti-CD3 mAb. WT *KHW* supernatant resulted in a 4.9 fold increase in IL-2 production compared to untreated cells. However, no significant increase in IL-2 production was detected when cells were treated with *KHWFliCKO* supernatant (Fig 3.6). This indicates that flagellin could be the costimulatory factor in the WT bacterial supernatant. To confirm the presence of flagellin in the WT supernatant, protein was precipitated from filtered WT *KHW* and *KHWFliCKO* culture supernatant and subjected to immunodetection by Western blotting using mouse polyclonal anti-FliC antibodies. Flagellin was detected in WT *KHW* supernatant but not at all in *KHWFliCKO* supernatant (Fig 3.7). Altogether, the results indicate that, in contrast to that of live bacteria, the costimulatory effects of bacterial supernatant could be predominantly attributed to FliC protein secreted by the bacteria into the culture, the absence of which could almost completely abolish the costimulatory activity of *B. pseudomallei* culture supernatant.

The experiment was repeated with WT *E. coli* and *fliC* deletion *E. coli* mutant bacteria and bacterial supernatants. Jurkat T cells that were infected with WT *E. coli* produced 3.4 fold higher amounts of IL-2 upon TCR engagement than uninfected cells. However, infection with *E. coliFliCKO* did not result in any enhancement of IL-2 production compared to uninfected cells (Fig. 3.8). Likewise, WT *E. coli* supernatant resulted in a 2.5 fold increase in IL-2 production while *E. coliFliCKO* supernatant did not result in an increase in IL-2 production compared to untreated cells (Fig. 3.8). This indicates that similar to *B. pseudomallei*, flagellin could be the costimulatory factor present in *E .coli* supernatant. However, *E. coliFliCKO* bacteria, unlike *KHWFliCKO*,

did not appear to exhibit any costimulatory properties. This suggests that flagellin could likely be the main costimulatory component associated with live *E. coli*, while costimulatory components other than flagellin are involved in the case of live *B. pseudomallei.*

Enhanced IL-2 production by purified human CD4$^+$ T cells infected with live *B. pseudomallei*

Previous observations of *B. pseudomallei* modulation of IL-2 production were seen in Jurkat T cells, a transformed leukemic T cell line. To determine the physiological relevance of T cell costimulatory effects of *B. pseudomallei* previously observed in Jurkat cells, we tested the effects of live *B. pseudomallei* infection on IL-2 production by primary human CD4$^+$ T cells, purified from human peripheral blood mononuclear cells (PBMCs). When these cells were infected for 2 hours with *B. pseudomallei KHW* at MOI 30:1 and exposed to anti-CD3 mAb, they produced higher levels of IL-2 detected in cell supernatant after 24 hours, as compared to uninfected CD4$^+$ T cells. Infection with *KHWFliCKO* bacteria resulted in low IL-2 levels, similar to that of uninfected T cells (Fig 3.9). In comparison to Jurkat T cell lines, primary human T cells require a longer period of incubation of 30 hours or more with anti-CD3 mAb to produce detectable IL-2 levels (Fig 4.7). In this study, human CD4$^+$ T cells were infected for 2 hours and incubated with TCR stimulus for only 22 hours, hence only very low amounts of IL-2 could be detected in the event of costimulation. The total time period of bacterial infection was limited to 24 hours, since the cytotoxic effects of live *B. pseudomallei* on human T cells have not been tested and long hours of infection could affect cell viability. Nonetheless, it is evident from the above results that costimulatory effects of WT *B.*

pseudomallei strain *KHW* is also seen for human $CD4^+$ T cells, supporting the physiological relevance of the previous *in vitro* studies using Jurkat T cell lines.

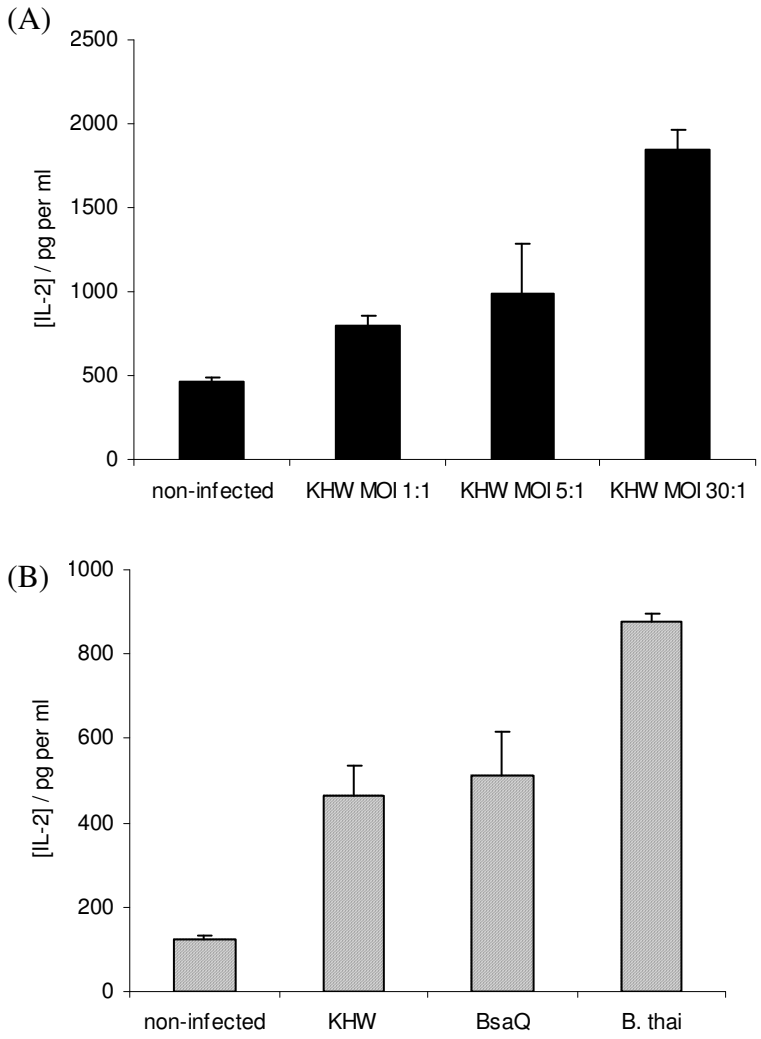


Figure 3.1 IL-2 production by Jurkat T cells infected with *B. pseudomallei* strain *KHW*, *bsaQ* mutant and *B. thailandensis* after exposure to TCR stimuli

Jurkat T cells were infected with *B. pseudomallei* strain *KHW* at an MOI of approximately 1:1, 5:1 or 30:1 (A) or with strain *KHW*, *bsaQ* mutant and *B. thailandensis* at MOI 30:1 (B) for 2 hours followed by 22 hour exposure to 2.5 μg/ml of surface-bound anti-CD3 mAb (TCR stimulation). IL-2 concentrations in the cell supernatants were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without bacterial infection (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

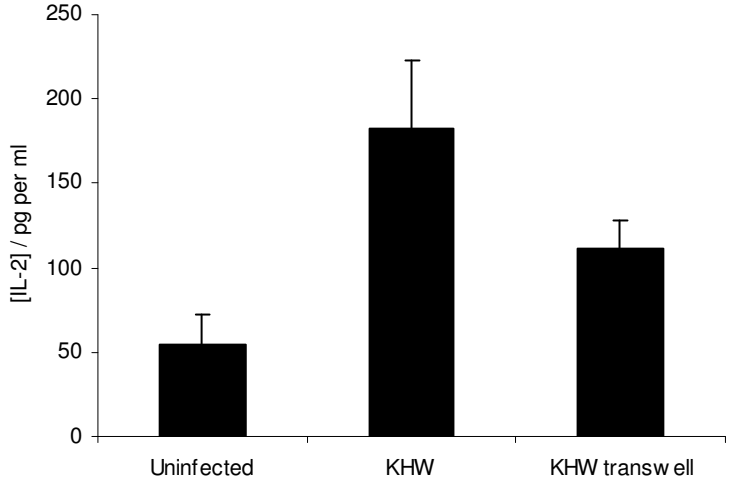


Figure 3.2 IL-2 production by Jurkat T cells incubated with *B. pseudomallei* strain *KHW* in a trans-well set up to exclude direct cell-bacteria contact

Jurkat T cells were incubated with *B. pseudomallei* strain *KHW* at an MOI of approximately 30:1 in the presence (KHW) or absence of bacteria-cell contact (KHW transwell) by adding the bacteria into a 0.4-micron membrane transwell placed within a 24-well containing the cells. Two hours later, the cells were incubated with 2.5 µg/ml of surface-bound anti-CD3 mAb (TCR stimulation) for 22 hours before IL-2 levels in the cell supernatants were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without bacterial infection (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

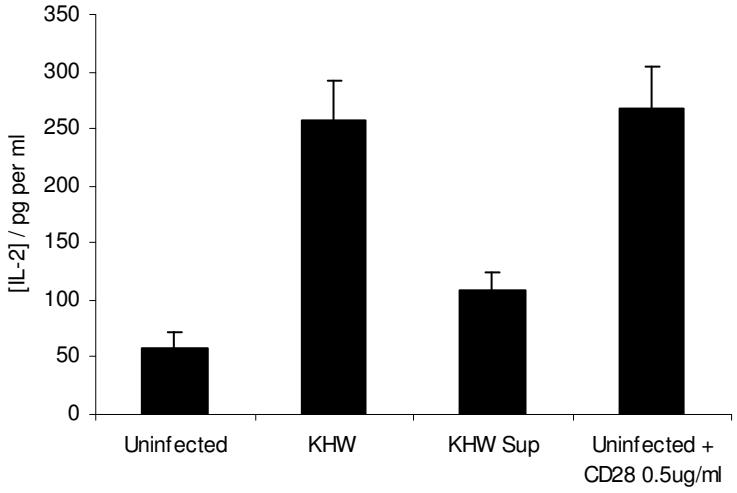


Figure 3.3 IL-2 production by Jurkat T cells treated with *B. pseudomallei* strain *KHW* bacterial culture supernatant

Jurkat T cells were incubated for 2 hours with live *B. pseudomallei KHW* at an MOI of approximately 30:1 (KHW) or log phase bacterial culture supernatant filtered through a 0.2 micron membrane (KHW Sup). Cells were then stimulated with 2.5 µg/ml of surface-coated anti-CD3 mAb for 22 hours. For the positive control, uninfected cells were incubated with anti-CD3 mAb together with 0.5 µg/ml of soluble anti-CD28 mAb. IL-2 levels in the cell supernatants were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without bacterial infection (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

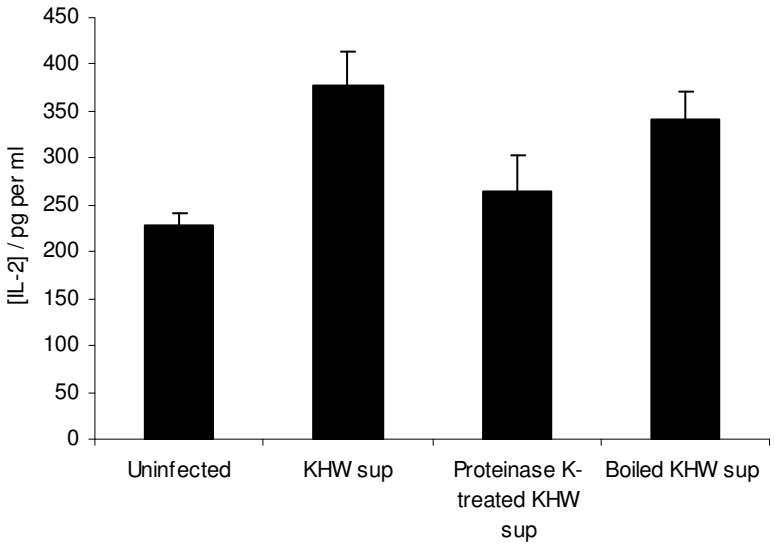


Figure 3.4 IL-2 production by Jurkat T cells treated with *KHW* bacterial culture supernatant

Jurkat T cells were incubated for 2 hours with log phase *KHW* Supernatant (KHW Sup), *KHW* supernantant pre-treated with 1 μg/ml proteinase K (Proteinase K-treated KHW Sup) or heat-treated *KHW* supernantant (Boiled KHW Sup). All bacterial supernatant was filtered through a 0.2 micron membrane. Cells were then stimulated with 2.5 μg/ml of surface-coated anti-CD3 mAb for 22 hours. IL-2 levels in the cell supernatants were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without treatment with the different bacterial supernatants (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

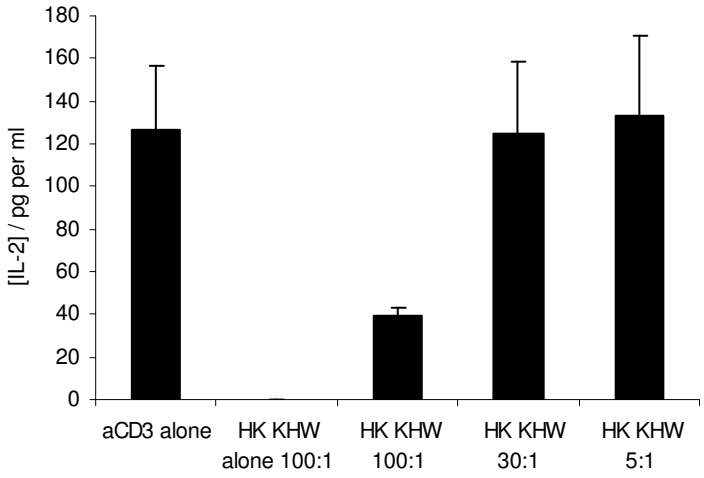


Figure 3.5 IL-2 production by Jurkat T cells treated with heat-killed *B. pseudomallei* strain *KHW*

Jurkat T cells were incubated with heat-treated *B. pseudomallei* strain *KHW* at bacteria-cell ratios of 5:1, 30:1 or 100:1 for 2 hours, followed by stimulation with 2.5 μg/ml of surface-bound anti-CD3 mAbs for 22 hours. IL-2 levels in the cell supernatants were determined by ELISA. IL-2 was not detected when cells were incubated with heat-killed bacteria alone (HK *KHW* 100:1 alone) and basal IL-2 levels were detected when untreated cells were stimulated with anti-CD3 mAbs (aCD3 alone). In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without treatment heat-killed bacteria supernatant (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

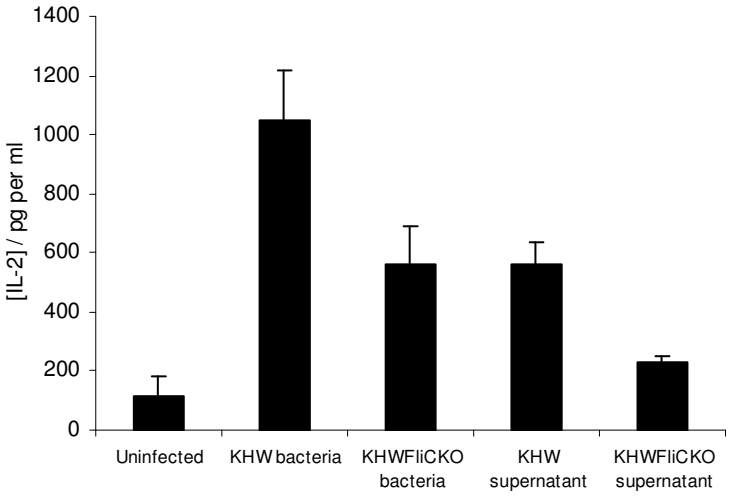


Figure 3.6 IL-2 production by Jurkat T cells infected with WT *B. pseudomallei* strain *KHW* or *KHWFliCKO* live bacteria and bacterial culture supernatant

Jurkat T cells were infected with live *B. pseudomallei* strain *KHW* or *KHWFliCKO* bacteria at an MOI of approximately 30:1. Supernatant from the same log phase WT (*KHW* supernatant) or FliC mutant (*KHWFliCKO* supernatant) bacterial culture that was used for infection was collected and filtered through a 0.22-micron membrane. Cells were incubated with live bacteria or supernatants for 2 hours before stimulation with 2.5 μg/ml of surface-coated anti-CD3 mAb for 22 hours. IL-2 levels in the cell supernatants were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without bacterial infection or treatment with bacteria supernatant (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

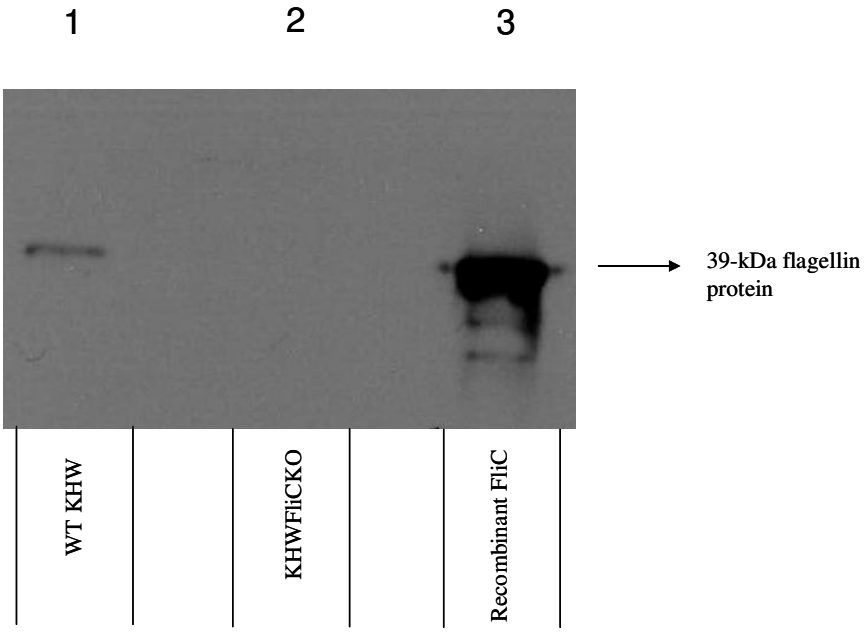


Figure 3.7 Detection of FliC protein in *B. pseudomallei* strain *KHW* culture supernatant by Western blot

Western blot analysis of flagellin protein, FliC, in protein precipitates prepared from bacteria-free WT *KHW* (Lane 1) or *KHWFliCKO* (Lane 2) bacterial supernatant. Approximately 5 µg portions of protein were separated on a SDS-10% polyacylamide gel and immunoblotted with polyclonal mouse anti-flagellin antibodies. Lane 3 contained 1 µg of recombinant *B. pseudomallei* FliC protein. The bands on the gel correspond to the 39-kDa flagellin protein.

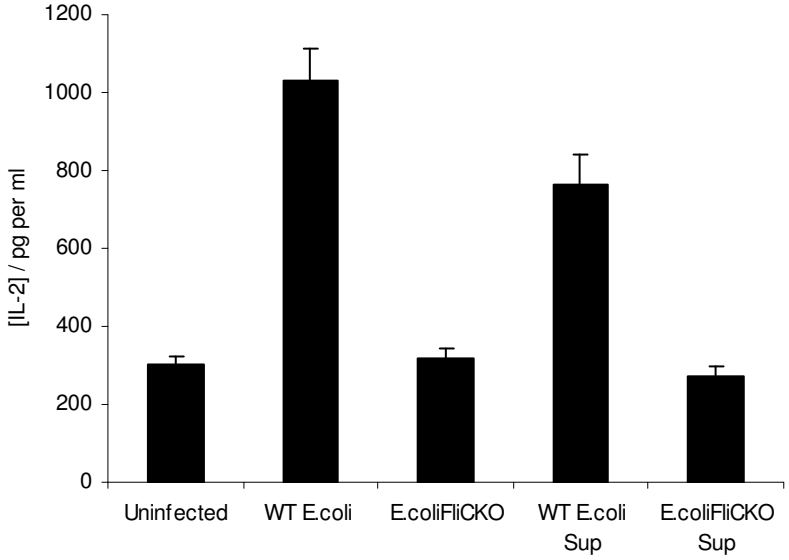


Figure 3.8 IL-2 production by Jurkat T cells infected with WT *E. coli* or *E. coliFliCKO* live bacteria and bacterial culture supernatant

Jurkat T cells were infected with live WT *E. coli* or *E.coliFliCKO* bacteria at an MOI of approximately 30:1. Supernatant from the same log phase WT (WT *E.coli* Sup) or *fliC* mutant (*E.coliFliCKO* Sup) bacterial culture that was used for infection was collected and filtered through a 0.22-micron membrane. Cells were incubated with live bacteria or supernatants for 2 hours before stimulation with 2.5 μg/ml of surface-coated anti-CD3 mAb for 22 hours. IL-2 levels in the cell supernatant were determined by ELISA. In the absence of anti-CD3 stimulation, there was no detectable IL-2 secretion by T cells with or without bacterial infection or treatment with bacteria supernatant (data not plotted). The experiment was repeated at least two times and error bars represent the standard deviation of three values.

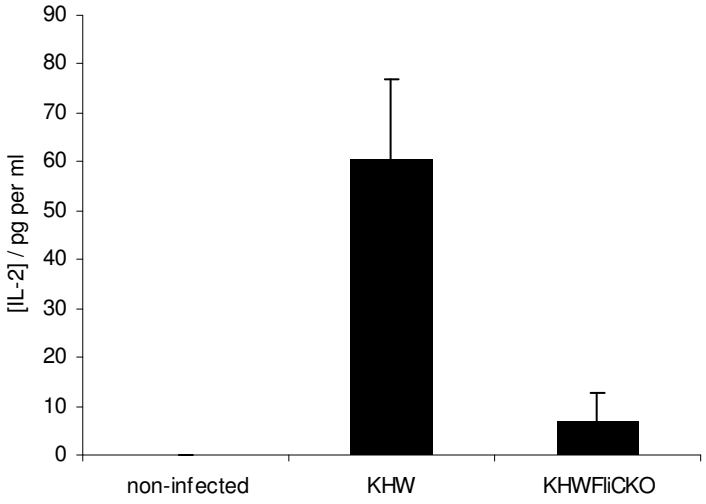


Figure 3.9 IL-2 production by purified human CD4$^+$ T cells infected with live *B. pseudomallei KHW*

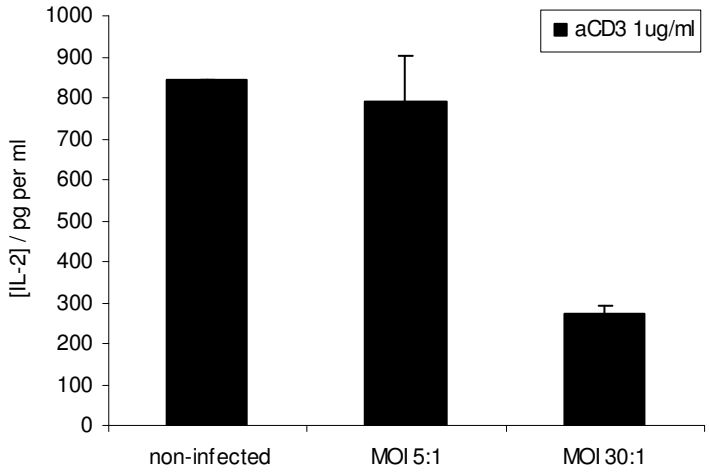
Primary human CD4$^+$ T cells were purified from human blood and infected with live *KHW* (KHW) or *fliC* deletion *KHW* mutant (*KHWFliCKO*) at an MOI of approximately 30:1 for 2 hours followed by 22 hour exposure to 2.5 μg/ml of surface-bound anti-CD3. IL-2 levels in the cell supernatants were determined by ELISA. There was no detectable IL-2 secretion by uninfected cells upon exposure to TCR stimulation. In the absence of anti-CD3 stimulation, there was also no detectable IL-2 secretion by T cells with or without bacterial infection (data not plotted). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

Reduced IL-2 production by B3Z T cells infected with *B. pseudomallei* strain *KHW*

Previously, we showed that *B. pseudomallei* could invade and replicate within mouse B3Z $CD8^+$ T cell lines. We decided to test if the costimulatory effects of *B. pseudomallei* strain *KHW* observed in human Jurkat T cells was also seen in mouse T cells. When B3Z T cells were infected with live *B. pseudomallei* strain *KHW* at MOI 30:1, followed by exposure to TCR stimuli supplied in the form of surface-coated anti-CD3 mAbs, they produced significantly less amounts of IL-2 as compared to uninfected cells (Fig 3.10A). B3Z T-cell line is a T cell hybridoma that recognizes SIINFEKL OVA peptide in association with $H\text{-}2K^b$. Hence, to determine if IL-2 response to antigenic stimulation was similarly affected, T cells infected at MOI 30:1 were exposed to OVA peptide presented by DC2.4 cells ($H\text{-}2^b$) and were similarly found to produce lower amounts of IL-2 as compared to uninfected cells (Fig 3.10B). Infection at MOI 5:1 however, did not result in an appreciable reduction in IL-2 production by infected cells in response to both TCR and peptide stimuli, indicating that larger numbers of bacteria are necessary to affect T cell function.

Cells were infected for 2 hours followed by 18 hour incubation with anti-CD3 mAb or DC2.4 cells with bound peptides, resulting in a total time of 20 hours of infection, at which time cell supernatant was assayed for IL-2 levels. Cell viability of infected B3Z T cells started to decrease at this time point onwards. Hence, the decrease in IL-2 amounts produced by infected cells could be attributed to bacteria-induced cell death. DC2.4 cells were fixed with 1% formaldehyde before pulsing with OVA peptides to exclude bacterial infection that would cause lysis of DCs.

(A)



(B)

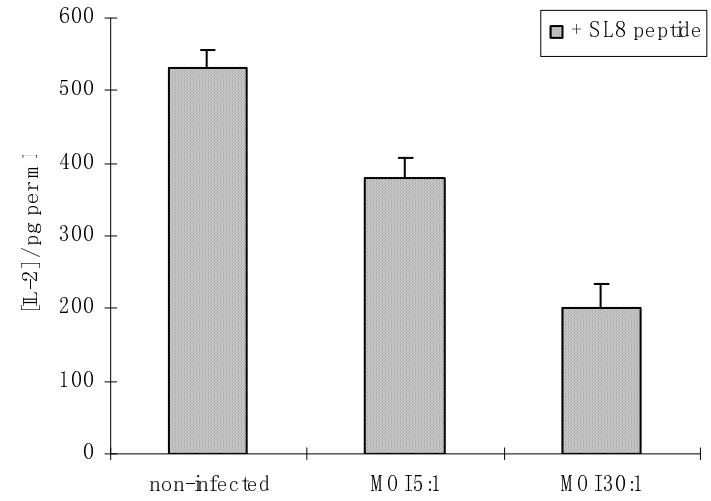


Figure 3.10 IL-2 production by *B. pseudomallei* strain *KHW*-infected B3Z T cells after exposure to TCR or antigenic stimuli

B3Z T cells were infected with *B. pseudomallei* strain *KHW* at an MOI of approximately 5:1 and 30:1 for 2 hours followed by an 18 hour exposure to surface bound anti-CD3 mAbs (TCR stimulation) (A), or SL8 OVA peptide pulsed on DC2.4 cell (antigenic stimulation) (B). IL-2 levels in the cell supernatant were determined by ELISA. In the absence of TCR or peptide stimulus, there was no detectable IL-2 secretion by T cells in the presence or absence of bacterial infection. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

DISCUSSION

B. pseudomallei was previously shown to be able to invade and replicate intracellularly within human and mouse T cell lines. However, rapid cell death was not observed in infected T cells as in dendritic cells. We postulated that during the earlier hours of infection when the infected cell is still viable, there is a time period of coexistence between the bacterium and the host cell, during which *B. pseudomallei* could alter host T cell function.

To test whether human Jurkat $CD4^+$ T cells infected with virulent *B. pseudomallei* strain *KHW* show any altered response to TCR stimulation, we measured IL-2 production by the infected cells upon engaging their TCR with CD3-specific antibodies, which are known to stimulate IL-2 production in Jurkat cells. The αβ-TCR heterodimer is associated on the cytoplasmic side with CD3 polypeptide chains that mediate signal transduction across the plasma membrane via motifs involved in linking surface TCR to intracellular signaling effectors (Weiss *et al*., 1984, Weiss and Littman, 1994, Abraham and Weiss, 2004). IL-2 is a crucial "T cell growth factor" that stimulates proliferation of antigen-primed T cells in an autocrine fashion (Kouttab *et al*., 1984).

Yersinia tyrosine phosphatase, YopH, targets and inhibits signaling intermediates crucial for TCR-mediated signaling such as Lck, a TCR-associated protein tyrosine kinase (Alonso *et al*., 2004, Gerke *et al*., 2005). Interestingly, we found *B. pseudomallei* did not negatively modulate T cell activation signaling pathways, at least during the first 22 hours of bacteria-host cell interaction. In fact, we observed an enhanced IL-2 production by *B. pseudomallei* infected Jurkat T cells as compared to uninfected T cells upon TCR engagement. This suggests that unlike infectious pathogens such as *Yersinia*,

B. pseudomallei did not compromise host T cell-mediated immune responses by affecting T cell activation.

In contrast, costimulatory effect of *B. pseudomallei* was not observed for mouse $CD8^+$ B3Z T cells. *B. pseudomallei* infected B3Z T cells produced less IL-2 than infected cells in response to both TCR and peptide stimuli. This can be correlated to earlier data that showed death of infected B3Z T cells at 22 hours after infection, the time which cellular supernatant was harvested and assayed for IL-2. Hence, future studies on bacterial costimulation of B3Z T cells should involve shorter assay time points. For instance, IL-2 mRNA expression can be measured instead of IL-2 secretion.

Enhanced IL-2 production upon TCR engagement was also observed when Jurkat cells were incubated with *B. pseudomallei* strain *KHW* in a transwell set up to prevent bacteria-cell contact or with the bacterial culture supernatant. This indicates that *B. pseudomallei*'s costimulation of T cells was not solely dependent on bacterial contact or entry, and that the bacteria could be releasing a factor that can also costimulate T cells resulting in their increased IL-2 production. In all cases, infection with live bacteria resulted in a significantly greater enhancement of IL-2 production as compared to incubation with bacteria in a transwell set up or with bacterial culture supernatant. This suggests that *B. pseudomallei's* costimulation of host T cells could be more potent in the event when bacteria are allowed to contact the cells while at the same time, costimulatory bacterial factors are also released by the bacteria onto the cells.

T cell stimulation by antigen follows a "two signal hypothesis" where the first signal is provided by ligation of TCR by peptide-MHC complexes that primes the T cell for proliferation and differentiation, and the second signal consists of a set of co-stimuli

conferred by counter-receptors expressed by APCs or by soluble factors including cytokines and chemokines. Co-stimuli are crucial for promoting T cell-cycle progression leading to naïve T cell clonal expansion and differentiation into effector T cells (Lafferty *et al*., 1980, Jenkins *et al*., 1990, Johnson and Jenkins, 1993). The most prominent T cell costimulatory receptor is CD28, which binds to CD80/ CD86 ligands expressed at high levels by activated professional APCs such as macrophages and dendritic cells (Jenkins and Johnson, 1993). Ligation of CD28 amplifies TCR-mediated T cell activation through activation of signaling effectors shared by the TCR. The lack of CD28-mediated costimulation results in reduced T cell proliferation and reduced T helper cell differentiation and cytotoxic T cell responses (Lucas *et al*., 1995, Rulifson *et al*., 1997, Prilliman *et al*., 2002).

CD28-deficient mice have reduced responses to infectious pathogens such as *Listeria monocytogenes* and *Leishmania major* (Mittrucker *et al*., 2001, Compton and Farrell, 2002). Hence, costimulation of T cells is a crucial hallmark for development of efficient T cell-mediated immune responses against pathogens. Our study provides evidence that, in addition to CD28 costimulatory signals provided by APCs, the bacterial pathogen can directly provide a similar costimulatory signal, either as a component attached to the live organism or a secreted factor, which enhances T cell activation by amplifying TCR-mediated signals leading to increased IL-2 production.

Flagellin is a known ligand for innate immune receptor, TLR5, that is expressed on T cell surface (Hornung *et al*., 2002, Hayashi *et al*., 2001, Smith and Ozinsky, 2002). The use of an isogenic *fliC* deletion mutant have shown that costimulatory effects of live *B. pseudomallei* is only partially due to the presence of flagellin and that there are other

costimulatory components associated with the bacteria that can act in synergy with TCR-mediated signals. In contrary, bacterial culture supernatant from the *fliC* deletion mutant was completely unable to enhance IL-2 production in T cells upon TCR stimulation. Hence, flagellin is likely to be the predominant costimulatory component secreted by *B. pseudomallei.* This is also seen with *E. coli*, where bacterial supernatant from *E. coli fliC* deletion mutant was clearly shown to be completely unable to costimulate Jurkat T cells.

Flagellin monomer makes up flagella filament and is known to be secreted by bacteria through an export machinery made up of the basal body, the hook, and the filament in the flagella structure (Shapiro, 1995, Macnab, 1999). Monomeric flagellin is normally found in supernatants of motile bacteria e.g. *Salmonella*, consistent with the detection of FliC in supernatants of *B. pseudomallei* in our study. Monomeric flagellin is also more stimulatory for TLR5 than polymeric flagellin (Smith *et al*., 2003). This explains that in the case of live *B. pseudomallei*, flagellin as part of the bacterial flagella is unlikely to be the main contributor to costimulatory effects seen. Instead, the live bacteria seem to possess other costimulatory components. The reduced costimulatory effect seen with the live *fliC* deletion *B. pseudomallei* mutant could be because no flagellin monomer is secreted onto the cells during live bacterial infection.

Heat-killed *B. pseudomallei* are completely unable to costimulate T cells, while heat-treated bacterial supernatant was still able to costimulate Jurkat T cells. This indicates that the epitope of the secreted flagellin monomer involved in binding to TLR5 is apparently not destroyed upon heat treatment. The absence of T cell costimulation by heat-killed *B. pseudomallei* could be because flagellin monomers were not being secreted onto T cells or other costimulatory components had been destroyed by heat. Hence, *B.*

pseudomallei's costimulation of T cells could either be due to the presence of heat-labile bacterial components or is essentially dependent on the bacteria being alive and hence able to interact with host T cells.

Interestingly, results from *bsaQ*, *B. thailandensis* as well as *E. coli* studies demonstrate that costimulation of T cells by *B. pseudomallei* is independent of a functional type III secretion apparatus and is not unique to a particular bacteria species. Both WT *B. pseudomallei* and *E. coli* bacteria and supernatant were also able to costimulate T cells and flagellin was found to be the predominant costimulatory component in the bacterial supernatant of both species. Thus, it is possible that the costimulatory components associated with live *B. pseudomallei* could likely be general pathogen-associated molecules such as flagellin.

Other unidentified costimulatory components in addition to flagellin were found to be associated with the live *B. pseudomallei*. However, this may not be the case for *E. coli*, since *fliC* deletion *E. coli* mutant bacteria was found to be unable to enhance Jurkat T cell IL-2 production compared to the WT *E. coli*. During the *E. coli* experiments, tetracycline or streptomycin was added after 2 hours of infection to suppress bacterial growth during the subsequent incubation period with anti-CD3 mAbs. However, the growth of the *E.coliFliCKO* bacteria was not found to be completely suppressed compared to the WT *E. coli*. The failure to observe any costimulatory effects with live *E. coliFliCKO* might be a result of the cells being unhealthy due to bacterial growth in the medium. Hence, it cannot be concluded if flagellin is the main costimulatory component associated with live *E. coli*.

On one hand, flagellin is a common PAMP molecule, while on the other, flagella function is an important virulence factor of pathogens such as *B. pseudomallei* and *Salmonella* (Carsiotis *et al*., 1984, Chua *et al*., 2003). This creates a paradox whereby the same virulence factor of the pathogen can also costimulate T cells. At the same time as bacterial costimulation helps the host to develop protective immunity more efficiently, excessive inflammation may occur if the costimulatory signal is too strong and poorly regulated leading to overexpansion of effector T cells. It is important for the strength of costimulatory signals to be controlled. In our study, *B. pseudomallei*'s interaction with host T cells alone cannot stimulate them. Enhancement in IL-2 production was observed only when T cells treated with bacteria are supplied with TCR stimulus. This specificity possibly controls for excessive inflammation due to overexpansion of effector T cells.

Our findings that *B. pseudomallei* can costimulate T cells together with TCR activation can be correlated to clinical studies on *in vitro* lymphocyte responses to *B. pseudomallei* antigens. Lymphocytes taken from subjects who have recovered from melioidosis or from seropositive yet healthy asymptomatic individuals, showed enhanced proliferation and IFNγ production in response to *B. pseudomallei* antigens, in comparison to serologically negative control subjects without clinical history of infection (Ketheesan *et al*., 2002, Barnes *et al*., 2004). The presence of memory antigen-specific T cells in these subjects provides evidence that CMI was developed during infection to control disease progression. In their studies, lymphocytes taken from subjects were stimulated with a cocktail of antigens prepared by sonication of *B. pseudomallei*. Such a lysate preparation could contain immunogenic antigens as well as costimulatory bacterial components. Note that PBMCs taken from subjects were stimulated as a whole

population. In the context of the T lymphocytes, the enhanced lymphocyte proliferation and IFNγ production could be a result of costimulation by bacterial costimulatory ligands in synergy with antigenic stimulation of TCR on lymphocytes. In these clinical studies, memory antigen-specific lymphocytes were tested for response to antigens prepared from *B. pseudomallei* lysate, while in our study, responses of Jurkat T cell line to live *B. pseudomallei* and their secreted factors are studied.

B. pseudomallei costimulation was also seen for primary $CD4^+$ T cells isolated from human PBMCs, supporting the physiological relevance of the *in vitro* observations with Jurkat T cells. It is evident from our work that the pathogen can directly costimulate primary human T cells. Future work should focus on determining if this bacterial costimulation can affect parameters of T cell activation, other than IL-2 production. Furthermore, effects of bacterial costimulation on different subsets of T cells including regulatory and memory T cells should be explored. Importantly, *B. pseudomallei* clearly possesses costimulatory components other than flagellin, which remains to be discovered. Discovery of potent T cell costimulatory components associated with the pathogen and how the host T cells respond to them will expand the list of vaccine candidates for protection against *B. pseudomallei* infection. This is especially so since effector T cells are known to be important for host defenses against this pathogen. At the same time, potent T cell costimulation by *B. pseudomallei* can help to explain the pathophysiology associated with septicemic melioidosis possibly as a result of overexpansion of T cells and their increased proinflammatory cytokine production.

CHAPTER 4

IN VITRO STUDIES ON INTERACTION OF *BURKHOLDERIA PSEUDOMALLEI* FLAGELLIN PROTEIN WITH HOST T CELLS

INTRODUCTION

Engagement of the TCR with antigenic peptides in association with MHC molecules leads to a series of intracellular signaling events resulting in transcription of genes involved in T cell proliferation and differentiation. However, TCR ligation alone is insufficient for T cell activation and a second costimulatory signal is required (Jenkins *et al*., 1990, Johnson and Jenkins, 1993). The latter can be triggered by ligation of costimulatory receptors on T cells. For example, CD28 and CD40L are two prominent T cell costimulatory receptors that recognize CD80/ CD86 and CD40 expressed at high levels on activated professional APCs respectively (Jenkins and Johnson, 1993, Mondino and Jenkins, 1994). In particular, CD28-mediated costimulation in T cells has been well studied. Many T cell responses are significantly compromised in CD28-deficient mice, which have reduced cell-mediated immune responses to infectious pathogens (Shahinian *et al*., 1993, Compton and Farrell, 2002).

Pattern recognition receptors such as TLRs that recognize bacterial PAMPs have generally been associated with innate cells such as macrophages and neutrophils which become activated to kill infectious pathogens upon ligation of their TLRs. Binding of PAMPs to TLRs on DCs are needed for their maturation into cells capable of activating T cells to initiate CMI responses (Aderem and Ulevitch, 2000, Iwasaki and Medzhitov, 2004). Interestingly, it was shown that T cells also express TLRs mRNA (Hornung *et al*., 2002, Zarember and Godowski, 2002). In addition to indirect effects on CMI via their action on DCs, TLRs have been shown to function as costimulatory receptors that can directly influence T cell function in the absence of APCs. For example, ligation of TLR2,

TLR7/8 and TLR5 synergize with TCR signals to enhance proliferation and cytokine production by human $CD4^+$ T cells (Caron *et al*., 2005, Xu *et al*., 2005, Liu *et al*., 2006).

Flagellin monomer is the known ligand for TLR5 and polymerizes to form bacterial flagella, a virulence factor for many pathogens such as *B. pseudomallei* (Hayashi *et al*., 2001, Chua *et al*., 2003). Flagellin has been shown to induce cytokine production by NK cells and monocytes (Hayashi *et al*., 2001, Chalifour *et al*., 2004). Now, there is growing evidence showing that flagellin, together with TCR signals, can costimulate $CD4^+$ T cells resulting in enhanced T cell responses as well as having direct effects on modulating regulatory T cell function (Crellin *et al*., 2005, Caron *et al*., 2005). Such a direct contact between TLRs and pathogenic costimulatory signals has been shown in *B. pseudomallei* interaction with T cells. I have shown in the previous chapter that live *B. pseudomallei*, as well as other bacterial species like *E. coli* and *B. thailandensis,* could potently costimulate T cells, leading to augmented IL-2 production. Flagellin was found to be one of the costimulatory components associated with live *B. pseudomallei* and flagellin secreted by the bacteria contributed mainly to the costimulatory effects of bacterial supernatant.

Here, we investigated the direct effects of recombinant purified *B. pseudomallei* flagellin on human Jurkat $CD4^+$ T cells, as well as primary human $CD4^+$ and $CD8^+$ T cells purified from PBMCs. To date, costimulatory effects of flagellin have not been tested on primary human $CD8^+$ T cells. Furthermore, enhanced cytokine production has so far been shown only at the protein level and not at the transcript level. CD28 costimulation is known to potently enhance IL-2 production (Jenkins *et al*., 1991) and the regulation of which can be attributed to enhancement of mRNA stability together with

increased transcript expression (Sanchez-Lockhart *et al*., 2004, Sanchez-Lockhart and Miller, 2006). Hence, through reverse transcription and real time PCR techniques, IL-2 transcript expression levels as well as mRNA stability upon flagellin costimulation of T cells were determined. Such information is useful for future signaling studies to elucidate how TLR5 signaling pathway synergizes with TCR-mediated signals at the molecular level to enhance T cell responses.

We also investigated the costimulatory effects of both LPS (TLR4 ligand) and PAM_3CysSK_4 (TLR2 ligand) on Jurkat T cells and primary human T cells. These results are interpreted together with that of flagellin costimulation, to ensure that any observed costimulatory effect of flagellin on augmenting IL-2 production in T cells was not due to contamination by bacterial LPS or lipoprotein in the recombinant flagellin preparation.

MATERIALS AND METHODS

Culture and maintenance of cell lines

Jurkat T cells and HEK293T were cultured and maintained as described in Chapter 2 Materials and Methods section, except DMEM medium (Sigma, St. Louis, MO) was used for HEK293T, a kind gift from Dr. Lu Jinhua (Dept. of Microbiology, NUS).

Isolation of human $CD4^+$ and $CD8^+$ T cells from blood

Around 50 ml of human blood was separated using Histopaque-1077 (Sigma, St. Louis, MO) according to the manufacturer's protocol. Buffy coat containing the peripheral blood mononuclear cells (PBMCs) were isolated and washed 2 times with PBS. Cells were resuspended in de-gassed buffer (PBS, 2 mM EDTA and 1 % FBS; pH 7.2) and magnetically labeled with 20 µl of CD4 or CD8 Microbeads (Miltenyi, Bergisch Gladbach, Germany) per 1 x 10^7 cells for 15 minutes on ice. After washing once with de-gassed buffer, cell suspension (up to 100 x 10^6 cells) was loaded onto a Magnetic cell sorting (MACS) column that is placed in the magnetic field of a MACS separator (Miltenyi). Magnetically labeled $CD4^+$ or $CD8^+$ cells were retained on the column while unlabelled cells were allowed to flow through the column. The bound column was washed 3 times with 0.5 ml de-gassed buffer each time. The column was removed from the magnet and retained labeled cells were eluted by applying the plunger filled with 2 ml of de-gassed buffer. $CD4^+$ or $CD8^+$ T cells were used for costimulation experiments. The purity of the separated $CD4^+$ and $CD8^+$ T cell fractions were analyzed by flow cytometry.

Bacterial proteins and antibodies

Ultra pure lipopolysaccharide (LPS) and Pam_3Cys-Ser-Lys_4 (PAM_3CysSK_4), which are TLR4 and TL2 ligands respectively were purchased from Invivogen, San Diego, CA. Anti-CD3 mAb that reacts with the human CD3 ε-chain, a 20-kDa subunit of CD3/ TCR complex was purchased from BD Pharmingen. Anti-CD28 mAb was purchased from BioLegend, San Diego, CA. Recombinant *B. pseudomallei* FliC protein was prepared by Ng H. L. in our laboratory.

Costimulation of cells

Jurkat T cells or primary human T cells were seeded at a cell density of 0.2 x 10^6 cells/ml into 96-well plates previously coated with 2.5 µg/ml of anti-CD3 mAb. Cells were costimulated with various concentrations of flagellin, anti-CD28 mAbs, LPS or PAM_3CysSK_4 (PAM) or 24 hours before supernatant was harvested and assayed for IL-2 levels.

Reverse-transcription PCR

Jurkat T cells were seeded at a cell density of 2.0 x 10^6 cells/ml in medium into 12-well plates previously coated with 2.5 µg/ml of anti-CD3 mAb. Recombinant *B. pseudomallei* flagellin was added at a concentration of 1 ng/ml as a costimulus. Cells were stimulated with anti-CD3 with or without flagellin for 5 hours at 37°C with 5 % CO_2. Total RNA was isolated from the cells using Trizol® (Invitrogen Life Technologies) according to the manufacturer's protocol. Briefly, 2.0 x 10^6 cells were pelleted and lysed in 1 ml of Trizol® reagent, followed by addition of 200 µl of chloroform. After centrifugation, the colorless upper aqueous phase containing RNA was transferred to a fresh tube. 0.5 ml of isopropanol was added to precipitate the RNA and the pellet was washed in 75 % ethanol, air-dried and redissolved in 10 µl of DEPC-treated RNAse-free

water. 5 μg of RNA was subjected to reverse transcription to cDNA using M-MLV reverse transcriptase enzyme and oligo-dT primers (Promega, Madison, WI) according to the manufacturer's protocol. PCR was performed using Taq polymerase (Promega) in 50 mM $MgCl_2$ buffer in a PTC-100 thermocycler (MJ Research Inc., Boston, MA). The cycling parameters for amplification with IL-2-specific primers IL-2F (5'AACTCACCAGGATGCTCACA3'); IL-2R (5'GCACTTCCTCCAGAGGTTTG3'), or TLR5-specific primers TLR5F (5'CATTGTATGCACTGTCACTC3'); TLR5R (5'CCACCACCATGATGAGAGCA3'), or GAPDH-specific primers GAPDHF (5'GAAGGTGAAGGTCGGAGTC3');

GAPDHR (5'GAAGATGGTGATGGGATTTC3') were as follows: 1 cycle of denaturation at 94°C for 5 min and 30 cycles at 94°C for 30s, 55°C (59°C for IL-2) for 30s, and 72°C for 1 min, followed by 1 cycle at 72°C for 10 min.

Real time PCR

Jurkat T cells were seeded at a cell density of 2.0 x 10^6 /ml in medium into 12-well plates previously coated with 2.5 μg/ml of anti-CD3 mAb. 1 ng/ml of recombinant *B. pseudomallei* flagellin or 1 μg /ml of soluble anti-CD28 mAb were added. Cells were stimulated with anti-CD3 with or without flagellin or anti-CD28 mAbs for 5 hours, after which 0.5 μg/ml of cyclosporin A (CsA) (Calbiochem, San Diego, CA) was added to inhibit transcription. At 0, 1 and 2 hours after addtition of CsA, total RNA was isolated from the cells using Trizol® (Invitrogen Life Technologies) according to he manufacturer's protocol. For each sample, 1 μg of RNA was subjected to reverse transcription to cDNA using TaqMan® reverse transcriptase reagents kit (Applied Biosystems, UK) according to the manufacturer's protocol. Real-Time PCR was

performed with Applied Biosystems 7500 Real-Time PCR system using 100 ng cDNA template for each sample that was mixed with target IL-2 or endogenous gene GAPDH - specific primers and Power SYBR® green master mix containing AmpliTaq Gold® polymerase (Applied Biosystems). The cycling parameters for amplification with the gene specific primers were as follows: 1 cycle of polymerase activation at 95°C for 10 min and 40 cycles at 95°C for 15s and 60°C for 1 min. PCR cycling for each cDNA sample was performed in triplicates. IL-2-specific primers used were IL-2F (5'CTGGAGCATTTACTGCTGGATTT3')

and IL-2R (5'TGGTGAGTTTGGGATTCTTGTAATT3'). GAPDH-specific primers used were GAPDHF (5'ATGGAAATCCCATCACCATCTT3') and GAPDHR (5'CGCCCCACTTGATTTTGG3').

Transfection and Detection of NF-κB activation

HEK293T cells were seeded at a cell density of 0.2 x 10^6 cells/ml in medium into 24-well plates 24 hours before transfection. The cells were transfected with 0.2 µg of two luciferase reporter plasmids; p5xNF-κB-luc (Stratagene, La Jolla, CA) and pRL-CMV (Promega, Madison, WI). Cells were also co-transfected with 0.1 µg TLR2 plasmid or pcDNA control plasmid, kind gifts from Dr. Lu Jinhua (Dept. of Microbiology, NUS). GenePORTER 2 reagent was used for the transfections (Gene Therapy System, La Jolla, CA). 24 hours after transfection, cells were stimulated with 1 ng/ml of flagellin or 1 µg/ml of PAM for 7 hours. After this, cells were lysed with passive lysis buffer (PBL) (Promega) in the wells. The plate was placed on a shaking incubator for 40 min to facilitate cell lysis. The lysate was diluted 50 x and 2 µl of the lysate was used for determining luciferase expression using the Dual Luciferase Assay Kit to detect NF-κB

activation (Promega). Luminescence emitted was expressed as a ratio: Firefly luciferase activity / Renilla luciferase activity. Background luminescence ratio of untreated pcDNA-transfected cells were subtracted from that of pcDNA or TLR2-transfected cells treated with flagellin or PAM.

Cytokine measurement

At time points indicated, cell supernatant was harvested and production of IL-2 cytokine by infected Jurkat and B3Z T cells upon TCR engagement was measured by ELISA according to manufacturer's protocol (BD Pharmingen).

Flow cytometric analysis

0.2 x 10^5 cells were incubated at 4°C for 1 hour with PE-conjugated anti-CD4 or CD8 monoclonal antibodies (BD Pharmingen). Fluorescence was measured using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA).

RESULTS

Costimulatory effects of *B. pseudomallei* flagellin and anti-CD28 mAb on IL-2 secretion by Jurkat T cells

When Jurkat T cells were incubated with surface-coated anti-CD3 mAb and flagellin for 24 hours, very high amounts of IL-2 cytokine were detected in the supernatant. In the presence of 0.5 ng/ml of flagellin and 2.5 µg/ml of anti-CD3 mAb, Jurkat cells secreted 8.5 fold higher amounts of IL-2 as compared to cells stimulated with anti-CD3 mAb alone. Even in the presence of low concentrations of 62.5 pg/ml flagellin, a 2.4 fold increase in IL-2 levels was detected compared to stimulation with anti-CD3 alone (Fig 4.1). Low levels of IL-2 can be detected when cells were stimulated with anti-CD3 alone, indicating that triggering the TCR-mediated signaling pathway is sufficient to result in basal levels of IL-2 production by T cells. However, IL-2 was not detected in supernatants of cells stimulated with flagellin alone in the absence of anti-CD3 stimulation (Fig 4.1). This shows that triggering the TLR5 signaling pathway in T cells via binding of flagellin is insufficient to result in IL-2 production. Instead, flagellin needs to be supplied as a costimulus together with a TCR ligand to exert effects on T cell function.

When Jurkat T cells were costimulated with 2 µg/ml or 50 ng/ml of anti-CD28 mAbs, they produced 6.9 fold and 3.4 fold higher IL-2 amounts than cells stimulated with anti-CD3 mAb alone, respectively (Fig 4.2). Flagellin costimulation seems to be more potent than CD28 costimulation where 0.5 ng/ml of flagellin can result in 8.5 fold increase in IL-2 production. In fact, when 1 µg/ml of anti-CD28 mAbs was added together with 100 pg/ml of flagellin, an enhancement of 10 times in IL-2 levels was

detected in the cellular supernatant (Fig 4.1 and 4.2). Therefore, both flagellin and CD28 costimulation have an additive effect in augmenting TCR-mediated signals.

Costimulatory effects of bacterial flagellin on expression of IL-2 mRNA transcript by Jurkat T cells

Semi-quantitative reverse transcription PCR was performed to determine if the increased amounts of IL-2 secreted by Jurkat cells upon flagellin costimulation with TCR stimulus is a result of increased levels of IL-2 mRNA transcripts. In the absence of any stimulus, IL-2 mRNA transcript was completely not detected. When anti-CD3 stimulus was supplied, low levels of IL-2 transcripts could be detected. Cells that were costimulated with anti-CD3 mAb alone exhibited a 1.3 fold increase in IL-2 transcript levels compared to non-stimulated cells (Fig 4.3A and B). Costimulation of T cells with both 1 ng/ml of flagellin and anti-CD3 mAb resulted in a 3.7 fold increase in IL-2 mRNA transcript levels compared to non-stimulated cells and a 2.9 fold increase in IL-2 transcript levels compared to cells stimulated with anti-CD3 mAb alone (Fig 4.3A and B). This indicates that flagellin costimulation results in increased transcription of IL-2 gene, which could account for the increase in IL-2 secretion by costimulated Jurkat T cells.

Using reverse-transcription PCR, Jurkat T cells were also found to express TLR5 mRNA transcript (Fig 4.4A). Unlike the observations for IL-2 mRNA transcript, basal levels of TLR5 transcript could be detected in non-stimulated cells (Fig 4.4A). Interestingly, stimulation of T cells with anti-CD3 mAb alone resulted in 1.7 fold increase in TLR5 transcript levels compared to non-stimulated cells (Fig 4.4A and B). Stimulation of T cells with anti-CD3 mAb and 1 ng/ml flagellin resulted in 1.1 fold higher amounts of TLR5 transcripts compared to non-stimulated cells (Fig 4.4A and B).

This shows that TLR5 gene expression could be up-regulated when TCR-mediated signaling pathways are triggered in Jurkat T cells.

Real time PCR was then carried out to provide a more accurate quantification of IL-2 transcripts and to determine if the increased IL-2 transcript levels were due to higher expression or enhanced stability of the mRNA. Relative quantitation gene expression was used to quantify differences in the expression level of the IL-2 target gene in Jurkat cells with different treatments. Cells stimulated anti-CD3 mAb alone, anti-CD3 and 1 ng/ml of flagellin, or anti-CD3 and anti-CD28 for 5 hours, exhibited a 3.2, 129.7 and 27.7 fold higher expression level of IL-2 gene than the untreated cells, respectively (Fig 4.5A). This indicates that flagellin costimulation significantly increases IL-2 mRNA transcript production to a greater extent compared to CD28 costimulation. During the first hour of CsA addition, IL-2 mRNA from flagellin-costimulated cells underwent rapid degradation (down to 14 %) at a faster rate compared to mRNA from CD28-costimulated cells (down to 51 %) (Fig 4.5B). However, even at 1 and 2 hours after addition of CsA, flagellin-costimulated cells were found to express a IL-2 mRNA fold change of 23.5 and 47 respectively, which is greater than that seen for CD28-costimulated cells (Fig 4.5A). CD28 costimulation appears to slightly stabilize IL-2 mRNA after 1 hour of CsA addition indicated by a lower rate of mRNA degradation compared to within the first hour of CsA addition (Fig. 4.5B). For flagellin stimulation, mRNA degradation was halted after 1 hour of CsA addition, showing a stabilization of IL-2 mRNA. Hence, flagellin costimulation primarily enhances IL-2 secretion in Jurkat cells through significantly augmenting mRNA transcript generation but at later time points also mRNA stability.

IL-2 response of Jurkat T cells to LPS and PAM_3CysSK_4 as costimulatory molecules

To rule out costimulation by any LPS or bacterial lipoprotein contaminants in purified flagellin preparations we used, we separately tested the costimulatory effects of LPS and PAM, which are agonists for TLR4 and TLR2 respectively. When 1 µg/ml of PAM or 2 µg/ml LPS were supplied together with 2.5 µg/ml of anti-CD3 mAb, amounts of secreted IL-2 detected was similar to that of cells stimulated with anti-CD3 mAb alone (Fig 4.6). High levels of IL-2 were detected in the positive control in which the cells were stimulated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 0.5 µg/ml of ionomycin (a calcium ionophore), indicating that the cells tested were capable of producing IL-2 higher than that of basal levels (Fig 4.6). The failure of PAM or LPS to enhance IL-2 production in response to anti-CD3 stimulation could mean that Jurkat T cells do not express TLR2 or TLR4. Indeed, we found that Jurkat cells do not express TLR 2 and 4 transcripts (data not shown). Altogether, the results indicate that in Jurkat cells, the costimulatory effect of flagellin with TCR stimulation on IL-2 production could not be due to LPS or lipoprotein contamination of flagellin purified from bacterial source.

Costimulatory effects of bacterial flagellin on IL-2 production by primary human $CD4^+$ and $CD8^+$ T cells

We isolated $CD4^+$ and $CD8^+$ T cell subsets from PBMCs and tested if they responded similarly to costimulation by bacterial flagellin as seen for the Jurkat E6-1 clone, a $CD4^+$ T cell line. $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs with anti-CD4 or -CD8 antibody-coated magnetic beads through MACS. For each experimental attempt, flow cytometric analysis revealed more than 98% purity for each $CD4^+$ or $CD8^+$ cell fraction (Fig 4.7).

When $CD4^+$ T cells and $CD8^+$ T cells were stimulated with 2.5 µg/ml of anti-CD3 mAbs together with 2 or 1 ng/ml of flagellin, they secreted an average of 1.85 fold and

3.7 fold higher amounts of IL-2, respectively, as compared to cells stimulated with anti-CD3 mAb alone (Fig 4.8A and B). Primary human T cells do not produce as much IL-2 in response to flagellin costimulation as compared to Jurkat E6-1 clone, a T cell leukemia line known to secrete very high amounts of IL-2 after stimulation. A longer period of costimulation of 30 hours was also required to detect IL-2 levels in supernatants of the human T cells. For both subsets of T cells, although the basal amount of IL-2 levels secreted in response to anti-CD3 stimulation alone varied across experiments, the fold increase in IL-2 levels after flagellin costimulation was consistent (Fig 4.8). The results from the human T cell costimulation study indicates that flagellin costimulation is able to enhance TCR-mediated signaling pathway leading to augmented cytokine production in human CD4 and CD8 expressing T cells.

IL-2 response of primary human T cells to LPS and PAM_3CysSK_4 as costimulatory molecules

In contrast to Jurkat T cells, primary human $CD4^+$ and $CD8^+$ T cells were previously found to express TLR2, TLR4 and TLR5 mRNA transcripts. Hence, we tested the costimulatory effect of LPS and PAM on IL-2 production in these cells. For both T cell subsets, when 1 µg/ml LPS was added together with 2.5 µg/ml of anti-CD3 mAb, IL-2 levels detected in the cellular supernatant were similar to that of cells stimulated with anti-CD3 mAb alone (Fig 4.9A and B). Hence, the costimulatory effect of flagellin with TCR stimulation on IL-2 production could not be due to LPS contamination of flagellin purified from bacterial source. In contrary to Jurkat T cells studies, CD4 and CD8 expressing T cells secreted 8.7 and 14.5 fold higher amounts of IL-2 in response to 1 µg/ml of PAM costimulation with anti-CD3 mAbs as compared to TCR stimulation alone (Fig 4.9A and B). This indicates that a more potent T cell costimulatory signal is

mediated through TLR2, since TLR2 costimulation could enhance IL-2 production in primary human T cells to a greater extent compared to flagellin costimulation.

To rule out bacterial lipoprotein (TLR2 ligand) contamination in the flagellin preparation used for costimulation, we tested if the same batch of flagellin could activate NF-κB in HEK293T cells transfected with a TLR2 plasmid. We found that unlike PAM, 1 ng/ml of flagellin was found not to activate NF-κB above basal levels in HEK293T cells transfected with TLR2 plasmid (Fig. 4.10). HEK293T cells are known to contain low levels of TLR5 expression, hence basal levels of NF-κB activation could be detected in pcDNA-transfected HEK293T cells stimulated with flagellin (Fig. 4.10). Hence, the flagellin preparation used for costimulation is unlikely to be contaminated with any bacterial lipoprotein and the costimulation effects on IL-2 production seen with primary human T cells are solely due to flagellin.

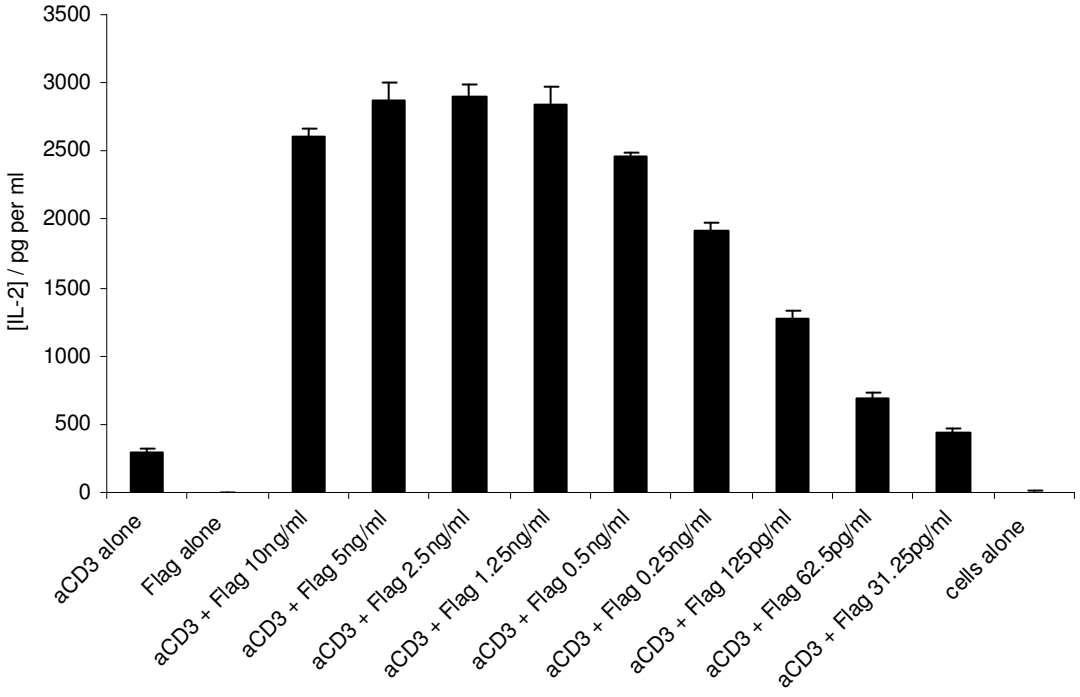


Figure 4.1 Costimulatory effects of *B. pseudomallei* flagellin protein on IL-2 secretion by Jurkat T cells

0.2×10^6 Jurkat T cells were stimulated with 2.5 µg/ml of anti-CD3 mAb coated onto a 96-well plate in the presence of various concentrations of flagellin protein (Flag) as a costimulus. 24 hours after costimulation, IL-2 levels in the cell supernatant were determined by ELISA. In the presence of anti-CD3 mAb alone, low levels of IL-2 were detected. In the absence of any stimulus (cells alone) or in the presence of 1 ng/ml flagellin alone (flag alone), there was no detectable IL-2 secretion by T cells. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

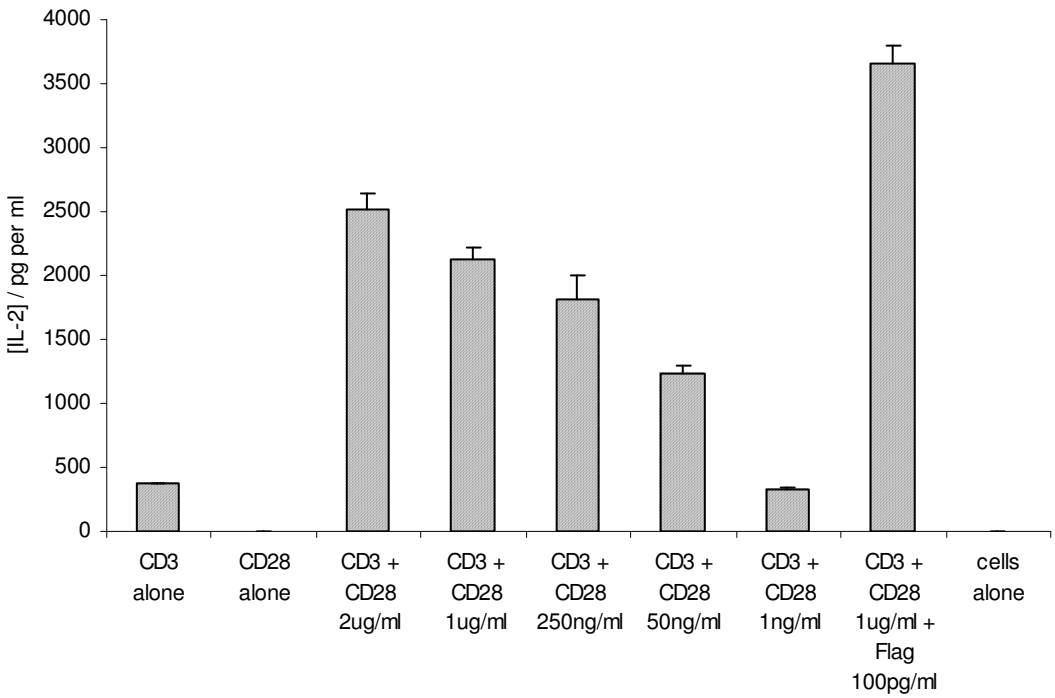


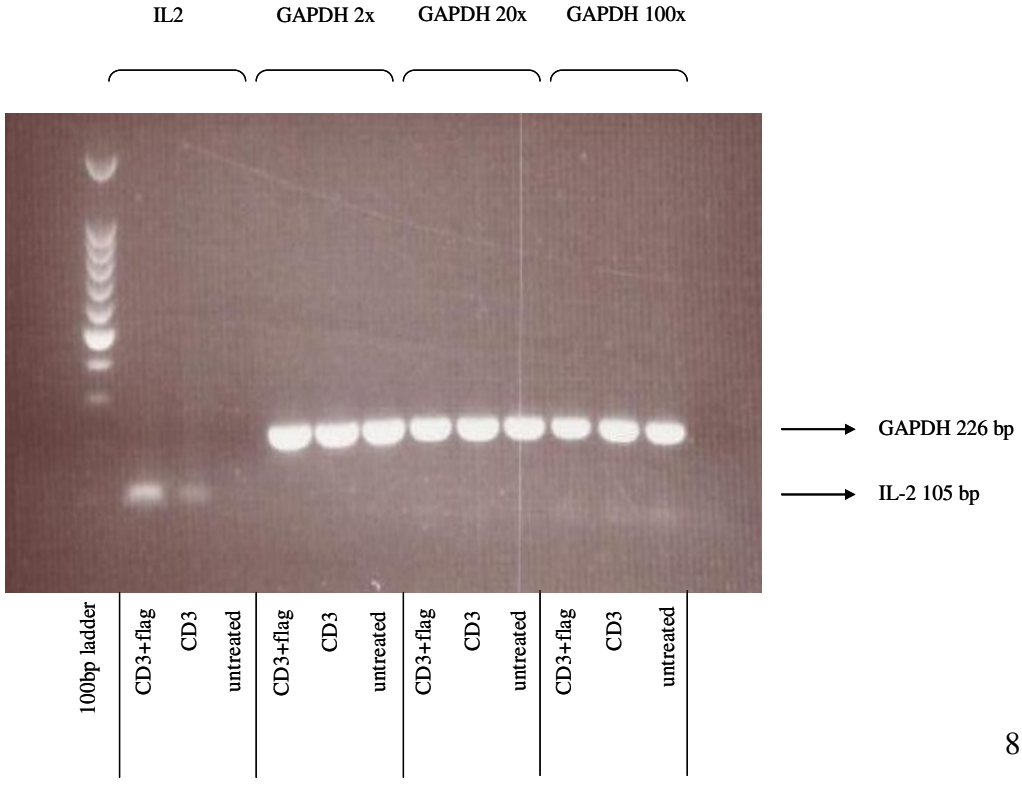
Figure 4.2 Costimulatory effects of CD28 agonist on IL-2 production by Jurkat T cells

0.2 x 10^6 Jurkat T cells were stimulated with 2.5 µg/ml of anti-CD3 mAb coated onto a 96-well plate in the presence of various concentrations of soluble anti-CD28 mAb as a costimulus. 24 hours after costimulation, IL-2 levels in the cell supernatant were determined by ELISA. In the presence of anti-CD3 mAb alone, low levels of IL-2 were detected. In the absence of any stimulus (cells alone) or in the presence of anti-CD28 mAb alone (CD28 alone), there was no detectable IL-2 secretion by T cells. The experiment was repeated at least three times and error bars represent the standard deviation of three values.

Figure 4.3 Costimulatory effects of *B. pseudomallei* flagellin protein on IL-2 mRNA transcript expression in Jurkat T cells – reverse transcription PCR

2.0×10^6 Jurkat T cells were incubated for 5 hours in the absence of stimulus (untreated), stimulated with 2.5 µg/ml of anti-CD3 mAb alone (CD3) or in the presence of 1 ng/ml of flagellin protein as a costimulus (CD3+ Flag). Total RNA was isolated from the cells followed by reverse-transcription PCR to determine IL-2 (A) expression. GAPDH served as the housekeeping gene and serial dilutions of its PCR products to determine a non-saturating concentration was subjected to gel electrophoresis. GAPDH transcript expression was unvaried across samples. A 1 Kb DNA ladder was used as a marker to identify IL-2 and GAPDH PCR product of 105 bp and 226 bp respectively. Intensity of the PCR products bands was quantified by densitometry for target gene IL-2 (B). Treated samples (CD3 or CD3+Flag) were compared to untreated samples and calculated by the following formula: signal of target gene = target gene signal of sample relative to untreated sample/ GAPDH (100x) signal of sample relative to untreated sample; where GAPDH and target gene signal in untreated samples were arbitrarily given a value of 1.

(A)



(B)

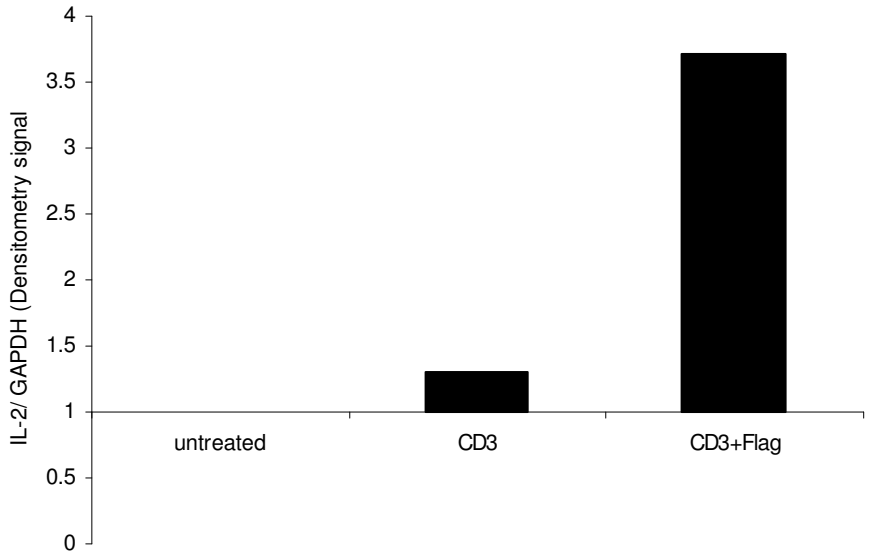
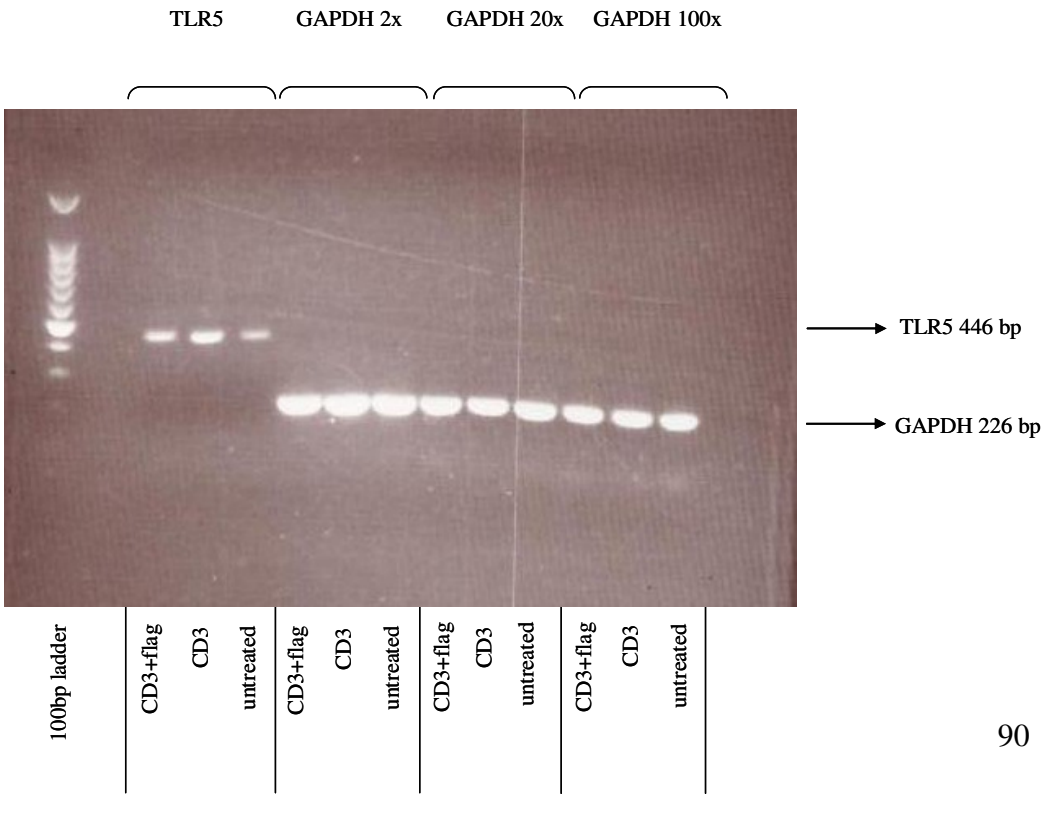

IL-2/ GAPDH (Densitometry signal
4
3.5
3
2.5
2
1.5
1
0.5
0
untreated
CD3
CD3+Flag

Figure 4.4 Costimulatory effects of *B. pseudomallei* flagellin protein on TLR5 mRNA transcript expression in Jurkat T cells – reverse transcription PCR

2.0×10^6 Jurkat T cells were incubated for 5 hours in the absence of stimulus (untreated), stimulated with 2.5 µg/ml of anti-CD3 mAb alone (CD3) or in the presence of 1 ng/ml of flagellin protein as a costimulus (CD3+ Flag). Total RNA was isolated from the cells followed by reverse-transcription PCR to determine TLR5 (A) expression. GAPDH served as the housekeeping gene and serial dilutions of its PCR products to determine a non-saturating concentration was subjected to gel electrophoresis. GAPDH transcript expression was unvaried across samples. A 1 Kb DNA ladder was used as a marker to identify TLR5 and GAPDH PCR product of 446 bp and 226 bp respectively. Intensity of the PCR products bands was quantified by densitometry for target gene TLR5 (B). Treated samples (CD3 or CD3+Flag) were compared to untreated samples and calculated by the following formula: signal of target gene = target gene signal of sample relative to untreated sample/ GAPDH (100x) signal of sample relative to untreated sample; where GAPDH and target gene signal in untreated samples were arbitrarily given a value of 1.

(A)



(B)

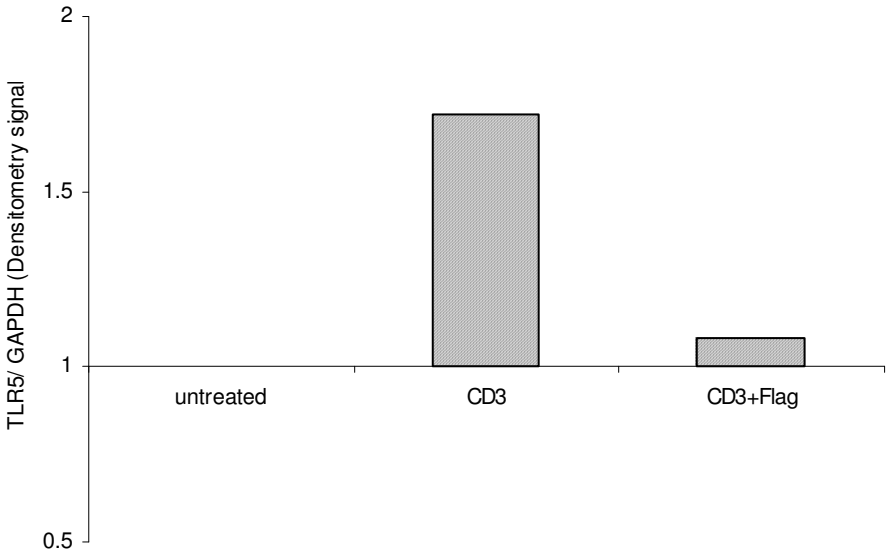
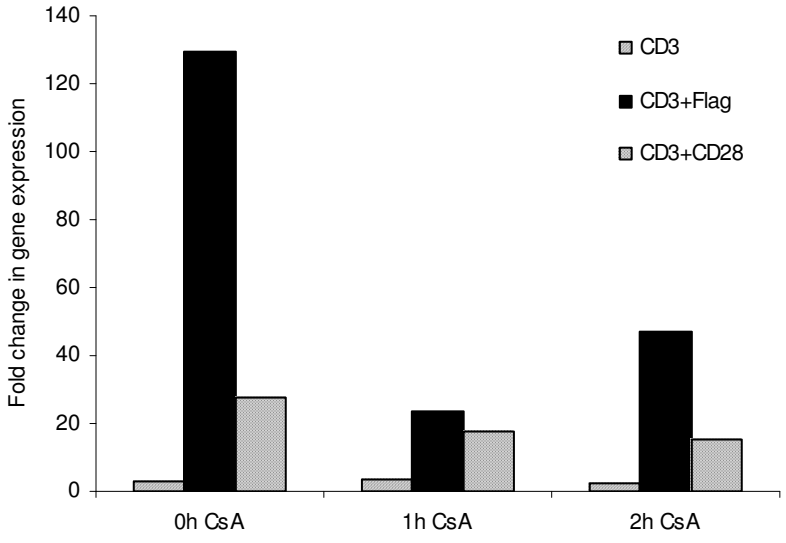

2
1.5
1
0.5
TLR5/ GAPDH (Densitometry signal
untreated
CD3
CD3+Flag

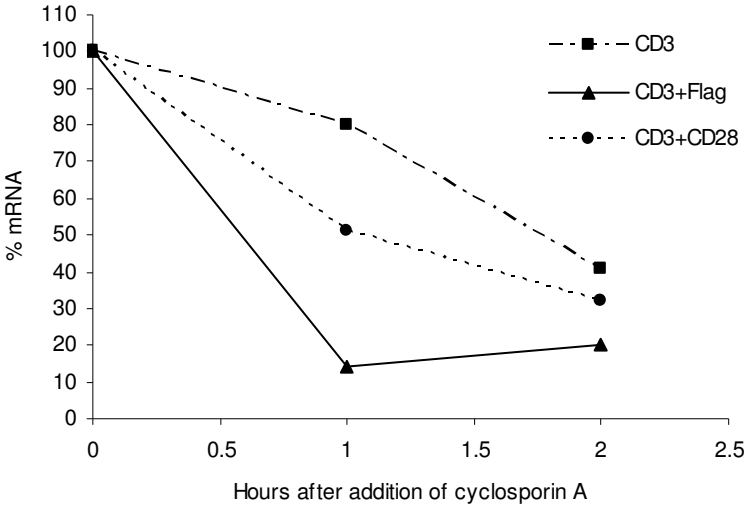
Figure 4.5 Costimulatory effects of *B. pseudomallei* flagellin protein on IL-2 mRNA transcript expression and stability in Jurkat T cells – real time PCR relative quantitative expression

2.0 x 10^6 Jurkat T cells were incubated for 5 hours in the absence of stimulus (untreated), in the presence of 2.5 µg/ml of anti-CD3 mAb alone (CD3), in the presence of anti-CD3 with 1ng/ml of flagellin protein (CD3+Flag) or with 1 µg/ml of anti-CD28 mAb (CD3+ CD28) as a costimulus. 5 hours after stimulation, 0.5 ug/ml cyclosporin A (CsA) was added to inhibit further transcription. Total RNA was isolated from the cells at 5 hours after costimulation and without CsA addition (0h CsA), after 1 hour (1h CsA) and 2 hours (2h CsA) of CsA addition. RNA was reverse transcribed to cDNA and the levels of both the target gene (IL-2) and endogenous control gene GAPDH were assessed by real time PCR. IL-2 expression levels in treated samples (CD3/ CD3+Flag/ CD3+CD28) are expressed as a fold change of expression levels relative to that in the calibrator sample (untreated cells at 0h CsA/ 1 h CsA/ 2h CsA) (A). Within each treatment (CD3/ CD3+Flag/ CD3+ CD28), % IL-2 mRNA in samples at 1h CsA and 2h CsA are expressed relative to the calibrator sample (CD3/ CD3+Flag/ CD3+CD28 at 0h CsA) (B). In both the treated and calibrator samples, the endogenous control gene was used to normalize the input amounts of the target gene cDNA templates used for real time PCR reaction. The experiment was repeated twice to give similar results.

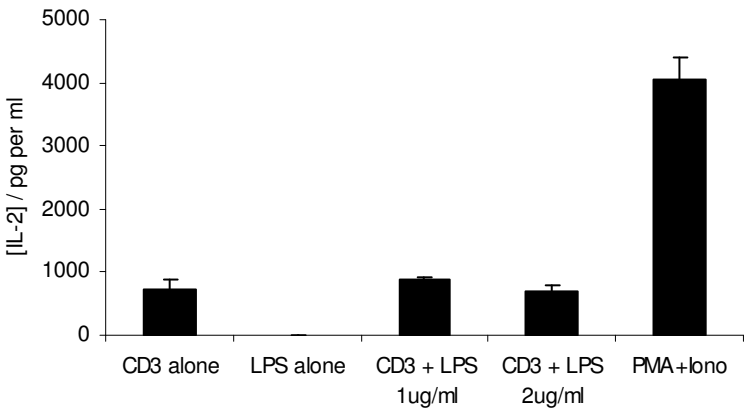
(A)



(B)


110
100
90
80
70
60
50
40
30
20
10
0
% mRNA
0
0.5
1
1.5
2
2.5
Hours after addition of cyclosporin A
CD3
CD3+Flag
CD3+CD28

(A)



(B)

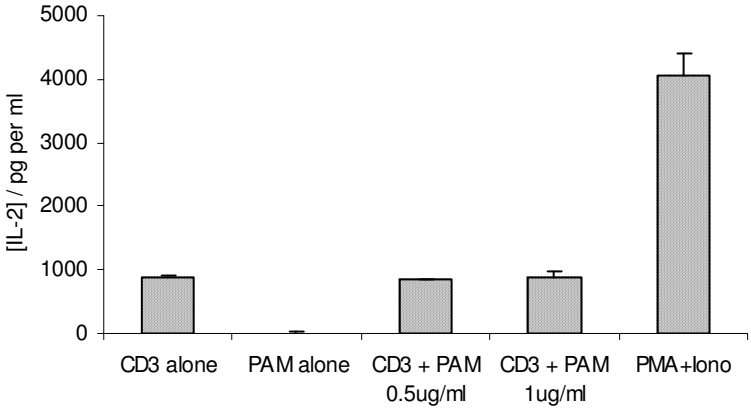
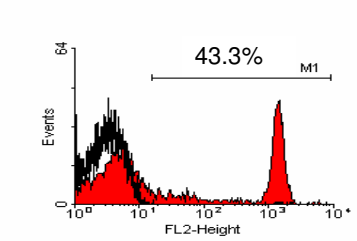


Figure 4.6 Costimulatory effects of LPS and PAM_3CysSK_4 on IL-2 secretion by Jurkat T cells

0.2 x 10^6 Jurkat T cells were stimulated with 2.5 µg/ml of anti-CD3 mAb coated onto a 96-well plate in the presence of different concentrations of LPS (A) or PAM (B) as costimuli. 24 hours after costimulation, IL-2 levels in the cell supernatant were determined by ELISA. In the absence of any stimulus or in the presence of LPS or PAM alone, there was no detectable IL-2 secretion by the cells. Error bars represent the standard deviation of three values.

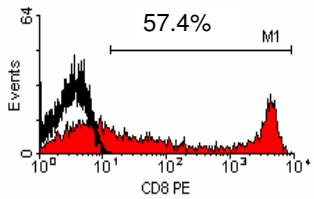
(A)

PBMCs (before separation) stained with CD4-PE



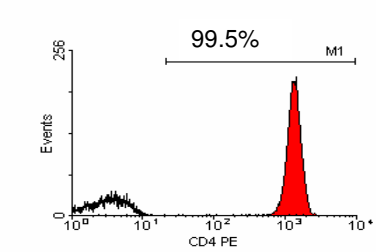
(B)

PBMCs (before separation) stained with CD8-PE



(C)

CD4+ fraction stained with CD4-PE



(D)

CD8+ fraction stained with CD8-PE

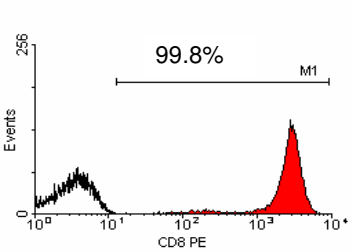
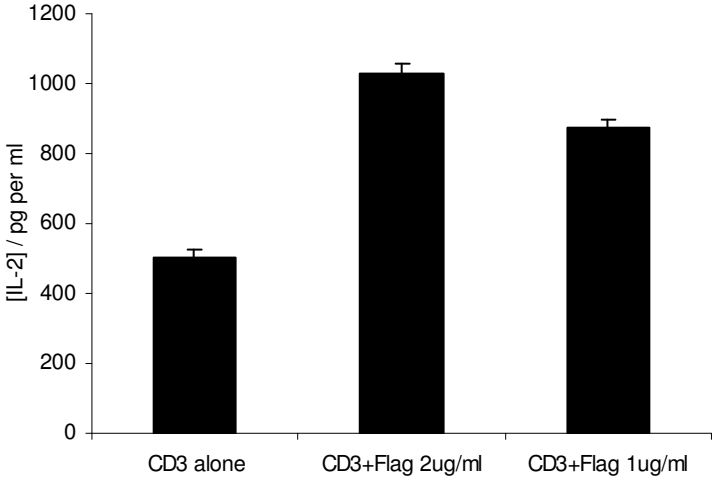


Figure 4.7 Flow cytometric analysis of $CD4^+$ and $CD8^+$ cell fractions purified from PBMCs

0.2 x 10^6 of the cells PBMCs before separation were incubated with PE-conjugated anti-CD4 (A) or anti-CD8 mAbs (B). 0.2 x 10^6 cells of the separated $CD4^+$ or $CD8^+$ cell fractions were stained with PE-conjugated anti-CD4 (C) or anti-CD8 mAbs (D). Following 1 hour of staining on ice, fluorescence was measured using the flow cytometer. This is one representative of at least three separate experiments.

(A)



(B)

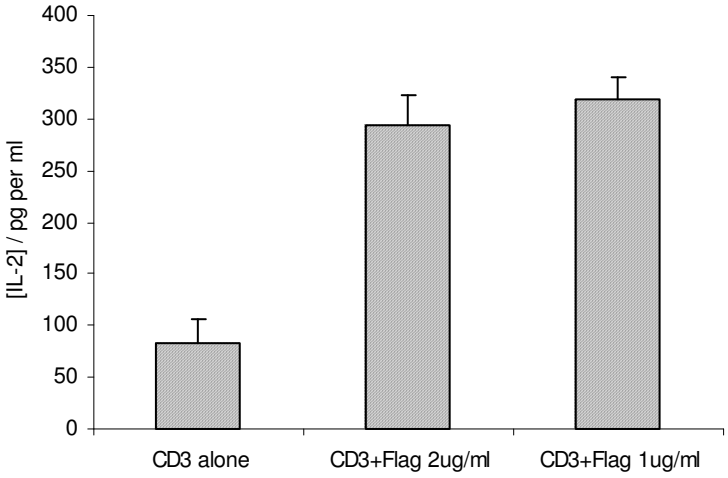
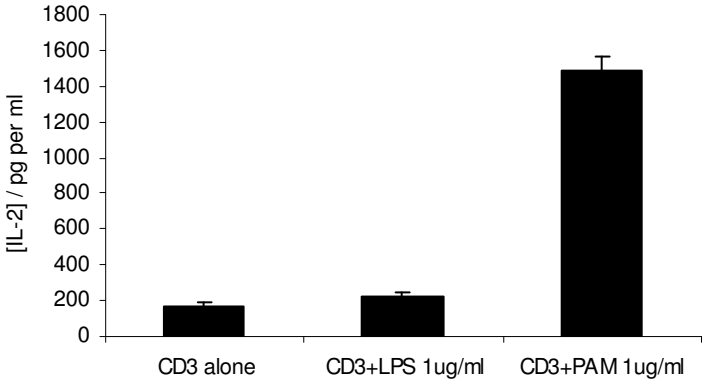


Figure 4.8 Costimulatory effects of *B. pseudomallei* flagellin protein on IL-2 secretion by primary human $CD4^+$ and $CD8^+$ T cells

0.25 x 10^6 $CD4^+$ (A) or $CD8^+$ (B) T cells were stimulated with 2.5 µg/ml of anti-CD3 mAb coated onto a 96-well plate in the presence of 1 ng/ml of flagellin protein as a costimulus. 30 hours after costimulation, IL-2 levels in the cell supernatant were determined by ELISA. In the absence of any stimulus or in the presence of flagellin alone, there was no detectable IL-2 secretion by T cells (not shown). The experiment was repeated at least three times and error bars represent the standard deviation of three values.

(A)



(B)

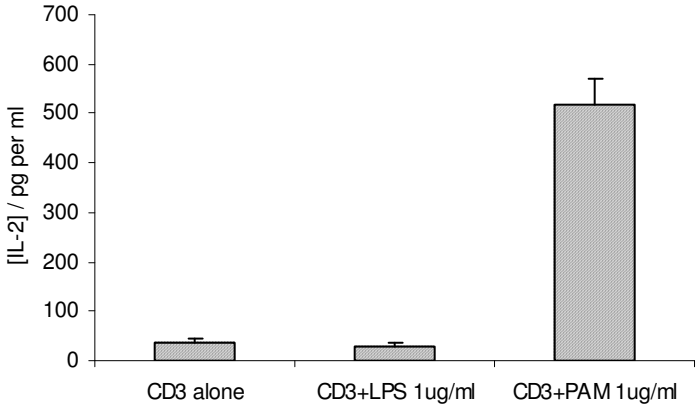


Figure 4.9 Costimulatory effects of LPS and PAM_3CysSK_4 on IL-2 secretion by primary human $CD4^+$ and $CD8^+$ T cells

0.2 x 10^6 $CD4^+$ (A) or $CD8^+$ (B) T cells were stimulated with 2.5 µg/ml of anti-CD3 mAb coated onto a 96-well plate in the presence of 1 µg/ml of concentrations of LPS or PAM as costimuli. 30 hours after costimulation, IL-2 levels in the cell supernatant were determined by ELISA. In the absence of any stimulus or in the presence of LPS or PAM alone, there was no detectable IL- 2 secretion by the cells. Error bars represent the standard deviation of three values.

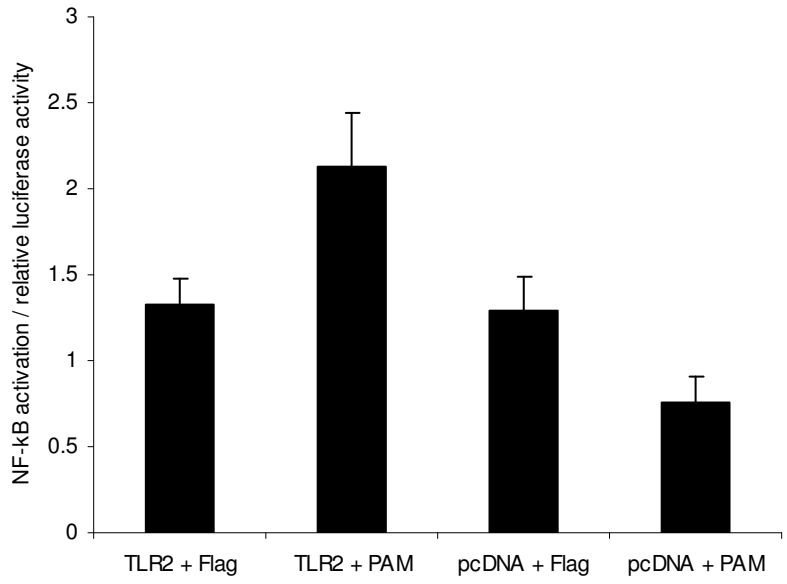


Figure 4.10 Effects of flagellin and PAM_3CysSK_4 on NF-κB activation in HEK293T cells transfected with TLR2 plasmid

HEK293T cells were co-transfected with two luciferase reporter plasmids and TLR2 or pcDNA control plasmid, after which cells were stimulated with 1 ng/ml of flagellin (TLR2 + Flag/ pcDNA + Flag) or 1 μg/ml of PAM (TLR2 + PAM/ pcDNA + PAM). 7 hours after stimulation, NF-κB activation was detected by measuring luciferase expression using Dual Luciferase Assay Kit as described in Materials and Methods section. Luminescence emitted was expressed as a ratio: Firefly luciferase activity / Renilla luciferase activity. Background luminescence ratio of untreated pcDNA-transfected cells were subtracted from that of pcDNA or TLR2-transfected cells treated with flagellin or PAM. Error bars represent the standard deviation of three values.

DISCUSSION

Our previous data show that potent costimulatory effects of live *B. pseudomallei* on enhancing IL-2 secretion by Jurkat T cells upon their TCR engagement can be attributed to bacterial flagellin. To further confirm this finding, we tested the direct costimulatory effects of recombinant *B. pseudomallei* flagellin protein on Jurkat T cells as well as primary $CD4^+$ and $CD8^+$ T cells. We found that *B. pseudomallei* flagellin is indeed a strong T cell costimulatory molecule, where minimal concentrations of recombinant flagellin supplied together with anti-CD3 stimulation (TCR stimulus) are sufficient to result in increased IL-2 production by Jurkat T cells, equivalent and even superior to that achieved by CD28 costimulation. It is evident that both TLR5 and CD28 costimulation can synergize with TCR-mediated signals to upregulate IL-2 production in T cells. Furthermore, when anti-CD28 and flagellin are supplied at the same time with TCR stimulus, there was an additive effect on the enhancement of IL-2 secretion by Jurkat T cells. This suggests that TLR5 and CD28 signaling could activate similar signaling effectors shared by the TCR, leading to enhancement of transcription factors that control IL-2 gene transcription. Flagellin costimulation also resulted in increased IL-2 secretion by primary human $CD4^+$ and $CD8^+$ T cells purified from PBMCs. This supports the previous observations with transformed Jurkat T cell line.

Our results pointing to TLR5-flagellin signaling as a potent costimulatory signal for T cell activation are consistent with recent studies reporting the direct effects of TLR ligands on T cells. It was shown that bacterial lipoprotein BLP, a TLR2 agonist, induced proliferation of $CD4^+$ effector as well as regulatory T cells (Sutmuller *et al*., 2006, Liu *et al*., 2006). Flagellin, the TLR5 ligand, similarly enhanced proliferation and cytokine

production by human $CD4^+$ T cells in synergy with TCR activation (Caron *et al*., 2005, Crellin *et al*., 2005). In particular, flagellin up-regulated T cell proliferation and IFN-γ production more efficiently in memory CD4+ T cells than naïve T cells (Caron *et al*., 2006). In our study, bulk $CD4^+$ and $CD8^+$ T cells isolated from PBMCs could contain both naïve and memory T cell population. Hence, we cannot differentiate which population of T cells flagellin is costimulating. Future work has to include separating the memory and naïve T cell subsets to be tested with flagellin costimulation.

It is evident that functions of TLRs are no longer only associated with the activation of cells of innate immunity such as dendritic cells. T cells also express TLRs, which can directly recognize costimulatory components associated with the pathogen, such as *B. pseudomallei*, to activate a costimulatory signaling pathway to augment T cell activation in synergy with TCR-mediated signals. Expression of TLRs by host cells to directly recognize bacterial costimulatory signals represents a mechanism for them to become activated leading to more efficient development of CMI responses against the pathogen. For instance, naive $CD8^+$ and $CD4^+$ T cells upon activation by antigenic and costimulatory signals, will proliferate and differentiate into effector T cells such as $CD8^+$ cytotoxic T cells and cytokine secreting $CD4^+$ helper T cells. We found that flagellin could costimulate both $CD4^+$ and $CD8^+$ T cells. Both subsets of T cells, by becoming activated through recognition of antigen-MHC complexes together with bacterial flagellin costimulus could lead to development of an efficient T cell-mediated immune response against the pathogenic bacteria.

$CD4^+$ T cells have been shown to be important for late host resistance to *B. pseudomallei* infection (Haque *et al*., 2006). *B. pseudomallei* flagellin can costimulate

$CD4^+$ T cells, as shown for Jurkat $CD4^+$ T cell line and primary human $CD4^+$ T cells. During an infection, the bacterium itself can provide costimulatory signals leading to clonal expansion of antigen-specific $CD4^+$ effector T cells, which are essential for mounting a cell-mediated immune response.

Our study is also the first to demonstrate the costimulatory effects of flagellin on human $CD8^+$ expressing T cells. These primary $CD8^+$ T cells which we tested could consist of naïve or memory T cells, which were previously found in our laboratory to express TLR5 mRNA. CD44-high expressing memory $CD8^+$ T cells isolated from spleen of uninfected mice were found to take part in bystander activation, where they could produce IFN-γ upon stimulation with gamma-irradiated *B. pseudomallei* (Lertmemongkolchai *et al*., 2001). These $CD8^+$ memory T cells should express TLR5 since memory $CD4^+$ T cells expressed high levels of TLR5 (Crellin *et al*., 2005). The bystander activation could be a result of costimulation with *B. pseudomallei* flagelin. However, it is intriguing as to what is the source of the primary stimulus since the $CD8^+$ T cells were taken from uninfected mice. Liu and co-workers (2006) have recently showed that IL-2 produced by effector T cells, can replace TCR signals (anti-CD3 stimulation) in the model of BLP (TLR2 ligand) costimulation of regulatory T cells. In the above study by Lertmemongkolchai and group (2001) irradiated *B. pseudomallei* was used to stimulate bulk spleen cells, which would contain antigen-presenting cells (APCs). The APCs could be stimulated by the *B. pseudomallei* antigens to produce cytokines. At the same time, a small population of naïve T cells that express TCRs specific to *B. pseudomallei* antigens could be activated to produce IL-2. Cytokines produced by the APCs and antigen-specific T cells could serve as the primary stimulus, together with *B.*

pseudomallei costimulatory molecules such as flagellin, resulting in activation of memory T cells in a bystander fashion. Such as bystander activation of T cells could explain severe inflammation seen in acutely infected patients associated with high proinflammatory cytokine profiles. For instance, serum concentrations of soluble IL-2 receptors and IFN-γ were observed to be highly elevated in septicemic melioidosis patients (Lauw *et al*., 1999, Brown *et al*., 1991). This could be a result of potent costimulatory signals on inducing T cell activation during infections with high bacterial loads. Future studies could examine whether flagellin could costimulate T cells in synergy with cytokine-mediated signals in addition to TCR signals.

Ironically, flagellin that can potently costimulate T cells is also an essential component that makes up bacterial flagella, a virulence factor of *B. pseudomallei* (Chua *et al*., 2003). Hence, at the same time that bacterial costimulation can promote development of efficient host T cell responses, excessive inflammation may also occur if the costimulatory signal is inappropriately regulated leading to overexpansion of effector T cells. For instance, TLR2 agonist BLP was found to abrogate the suppressive function of regulatory T cell (Tregs) during the first 15 hours of stimulation, after which Tregs regained their suppressive capacity. This indicates that BLP-mediat ed abrogation of Tregs-mediated suppression of effector T cells is only a transient effect (Liu *et al*., 2006). Such an occurrence helps to prevent overexpansion of effector T cells that might lead to unwanted over-inflammation. In contrast to TLR2 studies, Crellin and co-workers showed that flagellin stimulation enhanced suppressive capacity of Tregs and FOXP3 expression, a transcription factor involved in suppressive functions of T regs. However, this is likely debatable since FOXP3 expression in Tregs was assessed 48 hours

after flagellin and anti-CD3 costimulation (Crellin *et al*., 2005). Hence, any transient abrogation of Treg function during earlier hours of stimulation with flagellin could be overlooked in this study. Further work is needed to study the effects of flagellin-TLR5 costimulation in regulatory T cells.

It has been shown that TLR4 ligand, LPS, did not possess costimulatory effects on upregulating effector T cell proliferation or cytokine production (Xu *et al*., 2005, Caron *et al*., 2005, Crellin *et al*., 2005). However, conflicting data is seen for regulatory T cells (Crellin *et al*., 2005, Caramalho *et al*., 2003). Recently, there are also several reports on TLR2 ligand BLP as a potent T cell costimulatory signal in enhancing proliferation of human $CD4^+$ T cells (Xu *et al*., 2005, Liu *et al*., 2006). Hence, we investigated the costimulatory effects of both LPS and PAM on Jurkat T cells and found that both TLR agonists were unable enhance IL-2 production in response to anti-CD3 stimulation. Jurkat T cells were shown not to express TLR2 or TLR4 mRNA in our laboratory, which could explain the observations. Importantly, the results indicate that in Jurkat cells, the costimulatory effect of flagellin with TCR stimulation on IL-2 production could not be due to LPS or lipoprotein contamination of recombinant *B. pseuomallei* flagellin purified from *E. coli*. We also showed that LPS similarly had no costimulatory effect on primary human $CD4^+$ and $CD8^+$ T cells. In contrast, PAM could potently enhance IL-2 production by these cells upon TCR stimulation, consistent with current reports demonstrating potent costimulatory effects of BLP on human $CD4^+$ T cells. Furthermore, HEK293T cells studies showed that the recombinant flagellin preparation used to costimulate the primary T cells was not contaminated by BLP. Therefore, it can be concluded that the enhanced IL-2 production in flagellin

costimulated Jurkat $CD4^+$ T cells as well as human $CD4^+$ and $CD8^+$ T cells was due to the effects of flagellin-TLR5 costimulation.

$CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ Treg cells, which have not been activated with TCR signals, were shown to express TLR5 at levels within the range of monocytes. In particular, T regs expressed significantly higher amounts of TLR5 than the effector counterparts (Crellin *et al*., 2005). It was also shown that TLR5 mRNA levels were reduced dramatically in $CD4^+$ effector as well as regulatory T cells after 24 hours of activation with anti-CD3/ anti-CD28 mAbs (Crellin *et al*., 2005). In contrast, we detected an upregulation of TLR5 mRNA transcript level at 5 hours after anti-CD3 stimulation. This suggests that TLR5 mRNA could be transiently upregulated shortly after TCR stimulation. Since TCR ligation alone is insufficient for T cell activation and a second costimulatory signal is required, naïve T cells primed by antigenic signals could transiently upregulate TLR5 expression to recognize bacterial costimulatory signals to enable full T cell activation.

Enhanced cytokine production by flagellin costimulation has so far been detected only at the protein level (Caron *et al*., 2005, Crellin *et al*., 2005). Using reverse transcription and real time PCR techniques, we demonstrated that the increased IL-2 secretion by Jurkat T cells upon CD3 and flagellin costimulation could be correlated to increased IL-2 mRNA transcript expression levels. This suggests that TLR5 signaling synergizes with that of TCR to enhance IL-2 production largely through the activation of transcription to promote increased IL-2 gene transcription.

Although flagellin costimulation resulted in very high levels of IL-2 mRNA transcript levels, we found that it did not result in enhanced IL-2 mRNA stability during

the first hour of cyclosporin A (CsA) addition. However, enhancement of mRNA stability could be observed between 1 to 2 hours after CsA addition. Later time points after CsA addition should be looked at in future to better conclude on the effects of flagellin on mRNA stabilization. It has been reported that CD28-mediated activation of PI3 kinase (PI3K) led to an upregulation of IL-2 transcription in DO11.10 TCR-transgenic mice T cells, which accounted for the increased IL-2 secretion by the T cells upon CD28 costimulation. At the same time, CD28 costimulation also led to enhanced IL-2 secretion through posttranscriptional regulation involving the stabilization of IL-2 mRNA mediated through a PI3-kinase independent pathway (Sanchez-Lockhart *et al*., 2004 and Sanchez-Lockhart and Miller, 2006).

In our study, we found that flagellin costimulation enhanced IL-2 transcript expression to a significantly greater extent compared to CD28 costimulation, although both costimuli resulted in a similar degree of enhancement in IL-2 secretion in T cells. It is possible that CD28-mediated enhancement of IL-2 mRNA stability could account for high levels of IL-2 secretion even though transcript expression was enhanced to a smaller extent. In fact, CD28 costimulation appeared to stabilize IL-2 mRNA after the first hour of inhibition of T cell transcription by CsA compared to untreated cells, while IL-2 mRNA in flagellin costimulated T cells dropped to very low levels quickly during the first hour of CsA addition. Hence, flagellin costimulation could primarily enhance IL-2 secretion in Jurkat cells through significantly augmenting mRNA transcript generation and possibly by stabilizing mRNA at later time points.

The stability of IL-2 mRNA is mainly controlled by adenosine- or uridine-rich elements (ARE) in the 3'-untranslated region (Kracht and Saklatvala, 2002, Raghavan *et*

al., 2002 and 2004). IL-2 mRNA stability is controlled by c-Jun amino-terminal kinase (JNK) (Chen *et al*., 1998). p38 MAP kinase has also been implicated in mediating mRNA stability of IL-2 (Mestas *et al*., 2005). However, proximal signals induced by CD28 costimulatory receptor to induce IL-2 mRNA stability have not yet been elucidated (Sanchez-Lockhart and Miller, 2006). Nonetheless, our study suggests some differences in the mechanism by which TLR5 and CD28 costimulation synergizes with TCR signaling to enhance IL-2 production in T cells.

TLR5 and TCR signaling pathways have been well studied. Ligation of TLR5 is followed by recruitment of receptor-proximal adaptor MyD88 that binds to IRAK-1, which phosphorylates TRAF6 leading to activation of IKK molecules, degradation of IκB and subsequent activation of NF-κB transcription factor (Hayashi *et al*., 2001, Gohda *et al*., 2004). In innate immune cells such as DCs, flagellin-TLR5 signaling stimulates their maturation and cytokine production with NF-κB activation being detected in these cells (Means *et al*., 2003, Didierlaurent *et al*., 2004). TCR ligation also leads to NF-κB and AP1 activation through PKCθ involving Carma1, a scaffolding protein required for the coupling of TCR ligation to activation of IKK signalsome (Wang *et al*., 2004, Thome and Tschopp, 2003). Hence, synergistic effects of TLR5 signaling with TCR signals could be achieved through enhancing the activation of transcription factors such as NF-κB to promote increased IL-2 gene transcription.

CD28 is a crucial T cell costimulatory receptor that potently enhances IL-2 production by antigen-specific human T cells (Jenkins *et al*., 1991). T cells stimulated with antigen in the absence of the CD28 signal also become anergic (Jenkins *et al*., 1987). The CD28 response element (CD28RE) has also been identified as the DNA

element mediating IL-2 gene activation by CD28 costimulation (Fraser *et al*., 1991). Functional regulation of the CD28RE within the IL-2 promoter is mediated by NF-κB transcription factors (Verweij *et al*., 1991, Ghosh *et al*., 1993, Lai *et al*., 1995). CD28 as well as TLR5 costimulation, can trigger NF-κB activation to enhance TCR-mediated signals as seen by our observations of additive effects on augmenting IL-2 production in T cells upon TCR engagement when TLR5 ligand flagellin was supplied together with anti-CD28. This could possibly be a result of additive effects on enhancing NF-κB activation. Other transcription factors involved in activation of IL-2 gene transcription by TCR and CD28 includes nuclear factor of activated T cells (NFAT) (Maggiwar *et al*., 1997) as well as AP1 (c-fos and c-jun) (McGuire and Iacobelli, 1997). TRAF6 adaptor molecule downstream of TLR signaling mediates AP1 activation through MAP kinases that activate AP1 components (Bradley and Pober, 2001, Dong *et al*., 2002, Tanoue and Nishida, 2003). Hence, TLR5 signaling could also synergize with TCR signaling pathways at junctures other than NF-κB activation.

Unlike other TLRs found to be localized within intracellular compartments, TLR5 signaling occurs at the cell membrane and does not require internalization (West *et al*., 2005). Thus, we should study in future whether TLR5 is colocalized with TCR within the central supramolecular activation cluster (c-SMAC), which has been found to be enriched for TCR, CD4 and CD28 coreceptors along with crucial signaling effectors such as PKCθ and Lck (Huppa and Davis, 2003). Colocalization of TLR5 within the c-SMAC could indicate that both signaling pathways merge at this site as in the case for CD28. CD28 costimulation has been reported to activate PI3K to induce recruitment of PKCθ into the c-SMAC region as well as nuclear localization of NF-κB (Sanchez-Lockhart *et al*., 2004).

VAV1 signaling intermediate also potently upregulates CD28-induced activation of transcription factors that regulate IL-2 gene transcription such as NF-κB and NFAT (Michel *et al*., 2000, Marinari *et al*., 2002). It has also been shown that NF-κB activation is mediated by recruitment of signaling effectors other than PKCθ, such as Bcl-10 and IκB kinase beta to the immunological synapse involving Carma 1 (Wang *et al*., 2004). Hence, future work could include determining if crucial TCR signaling intermediates such as PKCθ are similarly recruited to the c-SMAC region or if CD28 costimulatory effectors like VAV1 are also involved in NF-κB activation upon TLR5 costimulation.

CHAPTER 5

CONCLUSION

We showed that *B. pseudomallei* could invade and replicate intracellularly in T cells. T cells are central players of cell-mediated immunity, which is important for the eradication of intracellular pathogens such as *B. pseudomallei*. Intracellular persistence of *B. pseudomallei* could account for latent infections and disease relapse from reactivation of an endogenous bacterial source.

Upon infection, it is necessary for the host to efficiently evoke an appropriate immune response to eradicate the pathogen before overwhelming bacterial loads from uncontrolled bacterial replication tears down the body's system. Recent clinical studies have demonstrated the development of cell-mediated immunity during *B. pseudomallei* infection (Ketheesan *et al*., 2002 and 2004) $CD4^+$ T cells have also been shown to be important for late host resistance to *B. pseudomallei* infection in a mouse model (Haque *et al*., 2006). Altering cellular functions or inducing cell death of host immune cells such as T cells represents means by which *B. pseudomallei* could cripple host adaptive immunity. We found that live virulent *B. pseudomallei* did not induce rapid cell death in T cells. In addition, during the period of bacterial and host cell coexistence, the pathogen did not negatively alter T cell activation signaling. Instead, enhanced T cell activation, indicated by increased IL-2 production by T cells, in response to TCR antigenic signals was observed. IL-2 is an essential autocrine T cell growth factor cytokine that leads to clonal expansion of antigen-primed effector T cells.

Such a costimulation effect by bacterial flagellin on host T cells could represent a host mechanism to more efficiently develop CMI responses against the pathogen. During infection, *B. pseudomallei* can provide the primary antigenic signal as well as costimulatory signal such as flagellin, to result in enhanced T cell activation and

subsequent clonal expansion of antigen-specific effector $CD4^+$ and $CD8^+$ T cells to better fight against the pathogen. Our study is also the first to show that bacterial flagellin can costimulate $CD8^+$ expressing T cells. The discovery of potent T cell costimulatory molecules associated with the pathogen and how the host immune cells respond to them will also expand the search for vaccine candidates that are capable of evoking efficient CMI protection in the host during *B. pseudomallei* infection.

However, such potent T cell costimulatory effects by *B. pseudomallei* could also account for overt pathophysiology associated with acute septicemic melioidosis. In such a case, overwhelming bacterial load could lead to an overexpansion of effector T cells and their increased pro-inflammatory cytokine as a result of strong T cell costimulation by bacterial components. For instance, it has been shown that IL-2 produced by effector T cells could replace primary antigenic signals in the model of costimulation of regulatory T cells by TLR2 ligand (Liu *et al*., 2006). Cytokines produced by APCs and by antigen-specific T cells could serve as the primary stimulus, which together with *B. pseudomallei* costimulatory molecules, can activate memory T cells. Such a bystander activation could account for severe inflammation seen in acutely infected patients associated with a high pro-inflammatory cytokine production profile.

Furthermore, although flagellin has been shown to enhance the suppressive capacity of regulatory T cells, it is not sure if flagellin could transiently abrogate suppressive function of regulatory T cells as in the case of TLR2 ligand, which could lead to over expansion of antigen-specific effector T cells (Liu *et al*., 2006). Hence, unregulated potent costimulation of T cells by bacterial costimulatory components may result in unwanted excessive inflammation leading to disease pathophysiology.

CHAPTER 6

FUTURE STUDIES

Despite the clinical importance of melioidosis especially in endemic regions, the pathogenesis of *Burkholderia pseudomallei* is not fully understood. Understanding the pathogenesis of the bacterium is important for effective therapeutic developments.

By employing an *in vitro* cell culture method, we showed that *B. pseudomallei* has the ability to invade and replicate intracellularly within T cell lines. The mechanisms by which the bacteria can survive within T cells has not been elucidated. Future work could include mutagenesis studies to identify virulence components responsible for invasion and intracellular replication within these cells. *Ex vivo* studies should also be carried out to examine the capacity of *B. pseudomallei* to invade and replicate in primary human DCs and T cells isolated from PBMCs.

Others in the laboratory had previously shown that *B. pseudomallei* induced rapid cell death in human DCs (Sun *et al*., 2005). The bacterium has previously been shown to induce rapid cell death in macrophages via a caspase-1-dependent pathway. Interestingly, we did not observe rapid cell death in *B. pseudomallei* infected T cell lines. Future studies could focus on how T cells can overcome bacterial induced cell death during early hours of infection.

Both experimental and clinical studies have demonstrated the importance of CMI responses, especially that mediated by T cells, in the control of *B. pseudomallei* infection. However, it is uncertain whether *B. pseudomallei* could directly alter functions of T cells, the essential players in adaptive immune responses. Our study is the first to reveal that infection with live *B. pseudomallei* did not negatively affect T cell activation in terms of their ability to produce IL-2. In fact, we found that live *B. pseudomallei* could costimulate T cells to enhance TCR-mediated signals resulting in increased IL-2

production compared to uninfected T cells. Memory lymphocytes taken from individuals with prior exposure to *B. pseudomallei* were found proliferate in response to *B. pseudomallei* lysate (Ketheesan *et al*., 2002). *B. pseudomallei* costimulatory signals could enhance such lymphocytic responses. Therefore, it is also important to examine the effects of bacterial costimulation on different subset of T cells including regulatory and memory T cells.

Flagellin protein, found in *B. pseudomallei* supernatant, was found to be the main costimulatory component secreted by the bacteria. Our study essentially provides new insights as to how the pathogen can directly costimulate naïve human T cells. Future studies could include determining other parameters of T cell activation such as proliferation, upregulation of surface activation markers, cytotoxic function and production of other cytokines as well as maintenance of memory T cells. Importantly, future work should focus on discovery of costimulatory components other than flagellin that appear to be associated with live *B. pseudomallei*. The discovery of potent T cell costimulatory components associated with the pathogen and how the host T cells respond to them will extend vaccine candidate options for protection against *B. pseudomallei* infection. This is especially so since effector T cells are known to be important for host defenses against this pathogen.

Recently, a group has reported that BLP (TLR2 ligand), together with IL-2 produced by effector T cells, could result in enhanced proliferation of regulatory T cells (Liu *et al*., 2006). This indicates that IL-2 can replace TCR signals (anti-CD3 stimulation) in the model of BLP costimulation of T regs. Hence, future studies could

include examining whether *B. pseudomallei* costimulatory molecules can synergistically enhance T cell responses with cytokine-mediated signals as the primary stimuli.

B. pseudomallei flagellin is able to costimulate T cells, and is at the same time a virulence factor (Chua *et al*., 2003). This has an implication in overt pathology seen in septicemic patients or acute infection mice models, which could be accounted for by an overexpansion of effector T cells and increased production of proinflammatory cytokine triggered by potent costimulatory signals associated with the pathogen.

Costimulatory signals are necessary to prevent T cell anergy after antigen recognition (Jenkins *et al*., 1987). Costimulation by *B. pseudomallei* could prevent bacterial-induced activation-induced cell death in antigen specific T cells. For instance, CD28 costimulation was associated with an increased expression of anti-apoptotic protein Bcl-xL that could prevent activation induced cell death (Khoshnan *et al*., 2000). It will be interesting to find out if *B. pseudomallei* costimulation of T cells similarly resulted in enhanced expression of anti-apoptotic proteins, which could explain why infected T cells might not be susceptible to bacterial-induced death during early hours of infection.

Recent studies have been focused on elucidating the roles of TLRs as T cell costimulatory receptors. Our study showing that flagellin associated with live bacteria as well as recombinant *B. pseudomallei* flagellin could potently augment IL-2 production in synergy with TCR signals is consistent with current literature reports. However, it will be important to discover next how TLR5 signaling synergizes with the TCR signaling pathway at the molecular level. CD28 costimulation has been well characterized over the years. Hence, initial signaling investigations can be based on paralleling TLR5 costimuation pathways to that mediated by CD28 to identify any similarity in molecular

mechanisms of synergy with TCR pathways. For instance, we can determine whether as in the case of CD28, TLR5 colocalizes with TCR within the central supramolecular activation cluster (c-SMAC) that is known to be enriched for TCR, its associated coreceptors as well as crucial signaling effectors. This can help us to identify the site at which the TLR and TCR signaling pathways merge. We can also examine if TLR5 costimulation results in recruitment of important TCR signaling molecules such as PKCθ and Lck to the c-SMAC region. Our data suggest an additive effect with flagellin and CD28 costimulation. Hence, we could determine if TLR5 costimulation involves activation of transcription factors shared by CD28 such as NF-κB, NFAT and AP1 that regulate IL-2 gene transcription, and whether upstream effectors such as those shared by CD28 are also involved in triggering the activation of the transcription factors.

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APPENDIX

Reagents:

Complete RPMI 1640: RPMI 1640, 10 % FCS, 200 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin

Blocking buffer for ELISA: PBS, 1.0 % BSA

Blocking buffer for Western Blotting: TBS-T, 5 % BSA

LB Medium: 10 g / liter bacto-tryptone, 5 g / liter bacto yeast extract, 5 g / liter NaCl. pH was adjusted to 7.0 with 2N NaOH

Lysis Buffer: PBS, 1 % Triton X-100

MACS de-gassed buffer: PBS, 2 mM EDTA, 1 % FBS, pH 7.2

PBS: 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4

Stop solution for ELISA: deionized water, 0.5 M H_2SO4

TBS-T: 20 mM Tris-HCL, 500 mM NaCl, 0.05% Tween-20, pH 7.5.

TSA: Pancreatic digest of casein 15.0 g / liter, Papaic digest of soybean meal 5.0 g / liter, NaCl 5.0 g / liter, Agar 5.0 g / liter, pH 7.3

Wash buffer for ELISA: PBS, 0.05 % Tween-20