Macrophage Activation in the Presence of *Burkholderia pseudomallei*

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DECLARATION OF ETHICS

The research presented in this thesis was conducted within the guidelines of the James Cook University Statement and Guidelines on Research Practices which is based on the NHMRC Australian Code for the Responsible Conduct of Research (2007). The proposed research methodology received approval from the James Cook University Animal Ethics Committee (A1069).

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January 2012

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ABSTRACT

Melioidosis is a potentially fatal disease caused by the soil dwelling bacterium *Burkholderia pseudomallei*. The disease is endemic in tropical and subtropical regions of the world but is primarily located in southeast Asia and in northern Australia. Acute melioidosis is characterised by a fulminating septicaemia that can result in death within days of exposure. The generation of sepsis results from a prolonged and or exaggerated stimulation of host immune cells by pathogens which culminates in the hyperproduction of inflammatory mediators. Stimulation of host cells by pathogens is facilitated by pattern recognition receptors which bind to microbial structures and initiate downstream signalling cascades. The most well studied pattern recognition receptors are those in the Toll-like receptor family (TLRs). Over the past decade a tremendous amount of work has been conducted to identify TLR specific ligands, TLR structures, associated signalling proteins and the cytokines produced by TLR activation. Understanding the nature of TLR mediated signalling in host cells is fundamental to elucidating the pathogenesis of sepsis and the improvement of clinical management. Up until 2007, no extensive publications had surfaced regarding TLR recognition of *B. pseudomallei* and the role of TLRs still remains an area of intense study. Therefore, the major focus of the research outlined within this thesis was the characterisation of TLR activation by *B. pseudomallei* during acute infection. This was achieved using infection studies in murine and human cell lines as well as in primary cells isolated from a previously characterised murine model of acute melioidosis. Primary cells were also isolated from partially resistant murine hosts and used in infection studies.

To ascertain the degree of TLR activation by 26 *B. pseudomallei* isolates with varying levels of virulence, standard antibiotic protection assays were performed on RAW 264.7 macrophages and peritoneal exudate cells (PEC) challenged with *B. pseudomallei*. Reverse transcriptase-polymerase chain reaction (RT PCR) was performed to determine TLR2, TLR4, TLR5, and TLR9 expression. Internalisation and killing of bacteria were determined at the early stages of infection. ELISAs were performed to determine total protein levels of tumor necrosis factor alpha (TNF-α) from cultured supernatants. Griess assays were used to assess nitrite production by

macrophages as a measure of cytotoxic activity. Up to 2 h post infection *B. pseudomallei* failed to significantly increase TLR4, TLR5 and TLR9 expression in both cell types. However, TLR2 expression was increased, irrespective of isolate virulence in RAW 264.7 macrophages. Levels of TNF-α and nitrite were significantly attenuated in RAW 264.7 macrophages and no correlation was found between the level of virulence of the infecting strain and TLR expression, bacterial uptake or killing. The ability of *B. pseudomallei* to evade detection by macrophages may be in part, due to possible signal dampening of TLR receptors at the early stages of infection.

Susceptibility to *B. pseudomallei* infection is determined by host immunocompetence as well as bacterial virulence. During acute melioidosis, excessive levels of pro-inflammatory cytokines are found systemically and lead to fatal septicaemias. Using RT PCR analysis, we found that *B. pseudomallei* can induce TLR4, TLR5, and TLR9 expression in peritoneal exudate cells (PECs) derived from susceptible and partially resistant mice. Induction of TLR4, TLR5, and TLR9 expression, in addition to TNF-α and interleukin 12, p40 subunit (IL-12p40), was greater in susceptible hosts. These results indicate the importance of genetic factors in regards to TLR recognition and response to *B. pseudomallei* and indicate more pronounced TLR activation in susceptible hosts.

To determine if macrophage activation is ubiquitous in the presence of *B. pseudomallei* isolates of different origin, we examined the role of TLR2 and TLR4 in the recognition of clinical isolates of high and low virulence. Using quantitative real time polymerase chain reaction (qRT PCR) analysis, transfection assays and ELISA, we determined transcription profiles of TLRs and cytokine secretion in macrophages co-cultured with *B. pseudomallei*. Our findings demonstrate that there are differences in TLR transcription profiles between isolates and that contrary to previous reports the degree of TLR2 and TLR4 mediated NF-κB activation may be dependant on the individual *B. pseudomallei* isolate.

In summary, the results in the present study have provided a basic understanding of TLR involvement in *B. pseudomallei* recognition. They provide supporting evidence regarding the role of TLRs during melioidosis and the establishment of different TLR

transcription in hosts with different susceptibilities to infection. These results also suggest that *B. pseudomallei* activation of TLRs may be different between isolates.

PUBLICATIONS

Publications resulting from this Thesis:

1. **Feterl ML,** Engler C, Govan B, Norton E, Ketheesan N. 2006. Activity of tigecycline in the treatment of acute *Burkholderia pseudomallei* infection in a murine model of melioidosis. *International Journal of Anitmicrobial Agents* 28(5): 460-4.

2. **Feterl ML,** Govan B, Ketheesan N 2008. The effect of different *Burkholderia pseudomallei* isolates of varying levels of virulence on Toll-like Receptor Expression. *Transactions for the Royal Society of Tropical Medicine and Hygiene* 102 Suppl S1: S82-8.

3. **Feterl ML,** West TE, Govan B, Ketheesan K. 2010. Differential TLR mediated activation of NF-κB by *Burkholderia pseudomallei*. Infection and Immunity (in preparation)

4. **Feterl ML,** West TE, Govan, B, Ketheesan K. 2010. Toll-like receptor activation is influenced by *Burkholderia pseudomallei* virulence. World Melioidosis Congress 2010, Townsville, Australia. (oral presentation)

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LIST OF COMMONLY USED ABBREVIATIONS

ATCC – American type culture collection
ANOVA – analysis of variance
bp – base pairs
CD14 – cluster of differentiation 14
cfu – colony forming units
CpG DNA - cytosine guanine deoxyribonucleic acid
cps – counts per second
DEPC - diethylprocarbonate
DMEM – Delbecco's modified eagle medium
cDNA – complementary DNA
DNA – deoxyribonucleic acid
dNTP – deoxynucleoside triphosphate
ELAM – endothelial leukocyte adhesion molecule
ELISA – enzyme linked immunosorbent assay
ER – endoplasmic reticulum
FBS – foetal bovine serum
HEK293 – human embryonic kidney cell 293
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
IFN-γ – interferon gamma
IL-1β – interleukin-1 beta
IL-12 – interleukin-12
iNOS – inducible nitric oxide synthase
IRAK – interleukin receptor associated kinase
IPTG - Isopropyl β-D-1-thiogalactopyranoside
iv – intravenous
LB – laurien broth
LD_{50} – fifty percent lethal dose
LPS – lipopolysaccharide
Mal – MyD88 adaptor like
MD-2 – myeloid differentiation protein
MHC – major histocompatibility complex
mRNA – messenger ribonucleic acid
MOI – multiplicity of infection
MyD88 – myeloid differentiation factor 88
NCTC – national collection of type cultures
NF-κB – nuclear factor kappa B
NK – natural killer
NO – nitric oxide
OD – optical density
O-PS – O-antigenic polysaccharide
PAMP – pathogen associated molecular patterns
PEC – peritoneal exudate cells
PBS – phosphate buffered saline
PCR – polymerase chain reaction
qRT PCR – quantitative real time PCR
RAW 264.7 – mouse leukeamic macrophage cell line
RNA – ribonucleic acid
RNI – reactive nitrogen intermediates

ROI – reactive oxygen intermediates
RPMI – roswell park memorial institute
RT PCR – reverse transcription PCR
SBA – sheep blood agar
SD – standard deviation
SEM – standard error of the mean
SOC – super optimal catabolite repression
TAE – Tris-acetate EDTA buffer
T_H1 – T Helper (type) 1
T_H2 – T Helper (type) 2
TIR – Toll/Interleukin-1 receptor-like domain
TIRAP - TIR domain-containing adaptor protein
TLR1 - Toll-like receptor 1
TLR2 - Toll-like receptor 2
TLR6 – Toll-like receptor 6
TLR5 – Toll-like receptor 5
TLR4 – Toll-like receptor 4
TLR9 – Toll-like receptor 9
TNF-α – tumor necrosis factor α
TRAF – TNF receptor associated factor
TRAM – TRIF related adaptor molecule
TRIF - TIR domain-containing inducing interferon
TTSS – type 3 secretion system

CHAPTER 1

GENERAL INTRODUCTION

Melioidosis is a potentially fatal tropical disease caused by the soil dwelling saprophyte *Burkholderia pseudomallei*. This Gram-negative bacterium is most commonly found in Southeast-Asia and Northern Australia, although isolated reports of *B. pseudomallei* in soils have been reported around the globe. Infection can occur though cuts and abrasions in the skin, or through inhalation and ingestion of contaminated soil and surface water. Many cases go largely unrecognised due to lack of awareness and diagnostic facilities where the organism is present. However, following the *Bacillus anthracis* scare in the United States in 2001, the National Institute of Health began collecting "priority pathogens" for investigations into the pathogenesis of potentially dangerous biological organisms (www.cdc.gov/nczved). As such, *B. pseudomallei* was classified as a category B bio-warfare agent and studies into virulence determinants, pathogenic mechanisms, host immune responses and diagnostic methodologies regarding the bacteria are expanding world wide.

The clinical manifestations of melioidosis are often classified into four categories: acute, subacute, chronic and subclinical. Acute melioidosis is characterised by a potentially fulminating septicaemia with high mortality ranging from 19% in Australia to 50% in northeast Thailand (Peacock, 2006). Subacute melioidosis is more common and generally less severe than acute disease and infection can be localised or disseminated. Chronic melioidosis is the most common form of infection and is characterised by a latent localised foci of infection within almost any organ, in addition to the presence of superficial abscesses (Dance, 1991). Patients may remain asymptomatic for years until immunocompromised, when asymptomatic infection can recrudesce and rapidly progress into septicaemia (Kingston, 1971). Individuals suffering from diseases associated with immunosuppression such as renal failure, alcoholism, and diabetes mellitus are at higher risk of infection (Puthucheary *et al.*, 2001). Reactivation of latent infection is more often associated with these co-morbidities. Symptomatic infections are correlated with a high incidence of mortality (Chaowagul *et al.*, 1989), slow to minimal response to antibiotic therapy and a high

rate of relapse despite prolonged and apparently successful treatment of primary infection (Sookpranee *et al.*, 1992).

Determining the pathogenesis of *B. pseudomallei* infection relies on the use of animal models. In our laboratory, we have characterised a model of acute and chronic melioidosis in BALB/c and C57BL/6 mice respectively (Leakey *et al.*, 1998). BALB/c mice are highly susceptible to infection and disease progression is rapid. Within 48 hours substantial bacterial loads are present in the spleen and liver and mortality due to sepsis occurs within 96 hours of infection. The bacteraemia present in these animals resembles the clinical progression of the disease in patients presenting with acute melioidosis. In contrast, C57BL/6 mice are relatively resistant to infection, often remaining asymptomatic up to six weeks following infection. Therefore, C57BL/6 mice provide an adequate model for the study of chronic melioidosis. Within the current study, cells derived from BALB/c and C57BL/6 mice are used to study the early stages of *B. pseudomallei* infection.

In the early stages of infection the role of the innate immune system is to contain the progression of pathogen growth in order to allow time for the host to mount an adaptive immune response. Macrophages are one of the first host immune cells to migrate to the site of infection and are capable of clearing the pathogen by phagocytosis. In addition, macrophages release inflammatory cytokines and chemokines to facilitate a protective host response. However, while infiltration of immune cells and the release of inflammatory cytokines is critical for effective clearance, it often contributes to the pathogenesis of septic disease (DeVries *et al.*, 1999; de Jong *et al.*, 2010). Depletion of macrophages during *B. pseudomallei* infection causes an increase in mortality in murine models of melioidosis and demonstrates an important role for this cell type in early host defense (Breitbach *et al.*, 2006). In this study the function and activation of macrophages will be assessed in the presence of several *B. pseudomallei* isolates.

The innate immune system functions as a sentinel for the host and is activated via the recognition of microbial structures and nucleic acids. These structures are crucial for pathogen survival and are termed pathogen associated molecular patterns (PAMPs). These PAMPs are derived from fungi, viruses, pathogenic bacteria and parasitic-

protozoa. Recognition of PAMPs occurs through the Toll-like receptors (TLRs), an evolutionary conserved family of receptors that are expressed in various immune and non-immune cells of the mammalian host (Kumar *et al.*, 2009). To date, twelve members of the mammalian TLR family have been identified and are located on the cell surface and intracellularly, depending on their corresponding ligand (Akira and Takeda, 2004). The sentinel function of TLRs against invading pathogens is facilitated by the induction of inflammatory cytokines and type I interferons in response to appropriate TLR/PAMP binding. Once binding occurs, TLRs elicit a series of downstream signalling events that tailor both humoral and adaptive immune responses necessary to clear the pathogen. In this study, TLRs pertinent to bacterial recognition will be investigated in relation to *B. pseudomallei* interactions with host macrophages.

The mechanism of host recognition and signalling in response to *B. pseudomallei* infection is still under investigation in several innate immune cell types. Determination of TLR interactions with *B. pseudomallei* in otherwise healthy and immunocompromised hosts and how those interactions correlate with inflammatory responses, will help contribute to the understanding of disease progression during the early stages of infection. Therefore the broad aims for the work outlined in subsequent chapters of this thesis are:

1. To investigate the effect of *B. pseudomallei* isolates with differing virulence on the activation of murine macrophages (Chapter 4)

2. To demonstrate altered TLR expression in human macrophage cell lines in the presence of high and low virulence *B. pseudomallei* isolates (Chapter 5)

3. To demonstrate differences in TLR expression on host macrophages derived from susceptible and partially resistant hosts in the presence of *B. pseudomallei* (Chapter 6)

4. To determine activation of TLR2 and TLR4 by high and low virulence *B. pseudomallei* isolates in TLR transfected human cells (Chapter 7)

CHAPTER 2
A REVIEW OF BACKGROUND LITERATURE

2.0 HISTORICAL BACKGROUND AND TAXONOMY

Melioidosis is caused by the Gram-negative, motile, non-spore forming, and facultative anaerobic bacillus *Burkholderia pseudomallei.* The organism was first described by Whitmore and Krishnaswami in 1921 while treating 38 fatal cases of pneumonia in Rangoon (Whitmore and Krishnaswami, 1912). The bacterium was described as *Pseudomonas pseudomallei,* until 1992, when Yubuuchi *et al.* reclassified the organism into the new genus *Burkholderia* based on RNA and DNA sequencing data (Yabuuchi *et al.*, 1992). The term melioidosis is derived from the greek root "melis" meaning "a distemper of asses" and "eidos", meaning "resemblance" (Puthucheary and Vadivelu, 2002). This connotation arose from clinical and pathophysiological similarities to glanders, a debilitating condition afflicting equines caused by *Burkholderia mallei* infection. Outcome of disease varies, ranging from benign and localised foci of infection, to acute fulminating septicaemia and mortality (Chaowagul *et al.*, 1989).

2.1 GEOGRAPHICAL DISTRIBUTION OF *BURKHOLDERIA PSEUDOMALLEI*

Burkholderia pseudomallei is prevalent in southeast Asia and northern Australia (Figure 2.1). The bacterium is a soil microbe and endemic areas are primarily located between 20° N and 20° S of the equator (Brown *et al.*, 1991). Isolates of *B. pseudomallei* have also been found on the Indian subcontinent, Papua New Guinea, Africa, Asia, Europe, and the Americas (Chen *et al.*, ; Brown *et al.*, 1991; Currie *et al.*, 2008). Following the 2004 Asian tsunami several sporadic cases have been diagnosed, revealing the distribution of *B. pseudomallei* in Indonesia and other areas in the Pacific affected by the disaster (Currie *et al.*, 2008). However, distribution of *B. pseudomallei* still remains markedly under recognised due to lack of awareness and inadequate facilitates to isolate and culture the organism.

Figure 2.1 Geographic distribution of *B. pseudomallei*.

2.2 CLINICAL PRESENTATION

The clinical manifestations of melioidosis cover a wide spectrum of symptoms making the diagnosis of the disease cumbersome, particularly in isolated regions where proper diagnostic facilities are not accessible. Clinical signs can range from benign skin and soft tissue infections, abcess formation, fever and pneumonia, to a rapidly progressive and often fatal septicaemia with multiple organ involvement (Chrispal *et al.*, ; Dhodapkar *et al.*, 2008; Phuong *et al.*, 2008)(Chaowagul *et al.* 1989). A latent form of infection also exists that can remain quiescent for several years before patients become symptomatic (Chaowagul *et al.*, 1993; White, 2003). Recrudescence is generally triggered by sepsis syndrome, disseminated vascular coagulation, long bone fractures and reduced immune competence (Puthucheary *et al.*, 2001)

Melioidosis typically presents as an acute pulmonary illness marked by prostration and toxicity, which are frequently inconsistent with initial physical diagnosis and chest radiographs (Puthucheary and Vadivelu, 2002). Melioidosis has been characterised with non apparent infections, transient bacteraemia, asymptomatic pulmonary infection, acute pulmonary infection, localised septicaemic infection, disseminated septicaemic infection, and chronic suppurative infection (Chaowagul *et al.*, 1993; Puthucheary *et al.*, 2001; Puthucheary and Vadivelu, 2002 ; Currie, 2003).

Due to the ambiguity of clinical manifestations and the prevalence of multi-organ involvement of the disease, uniform classification is problematic (Puthucheary and Vadivelu, 2002). The most functional classification from a clinical standpoint is a division between septicaemic and non-septicaemic melioidosis (Puthucheary and Vadivelu, 2002).

Clinical presentation of septicaemic melioidosis is variable, from a bacteraemia with no apparent foci of infection, to a fatal disseminated bacteraemia with fulminant shock and multiple organ failure (Chaowagul *et al.*, 1989; White, 2003). Sepsis caused by *B. pseudomallei* differs from other Gram negative bacilli induced sepsis in several ways (Puthucheary and Vadivelu, 2002). Typically, patients presenting with community acquired infections have a history of fever and no primary foci of infection (Chaowagul *et al.*, 1989). In contrast, *B. pseudomallei* produces a rapidly progressing septicaemia, with dissemination from a primary site of infection. Initial radiographic diagnosis illustrates multiple nodular lesions and subcutaneous abscesses in conjunction with joint swelling (Cheng *et al.*, 2003). Often the course of fulminant shock is too rapid to be reversed even with intensive care management (Sookpranee *et al.*, 1992).

2.3 MOUSE MODEL OF *BURKHOLDERIA PSEUDOMALLEI* INFECTION

Due to the often rapidly fatal outcome of patients with acute *B. pseudomallei* infection, the development of a suitable animal model to investigate the pathogenesis of infection was essential. In 1998, a murine model of melioidosis was characterised using BALB/c and C57BL/6 mouse strains (Leakey *et al.*, 1998). Following initial intravenous challenge with a minimum infective dose (MID) of *B. pseudomallei*, BALB/c mice developed a rapidly progressing bacteraemia 72 h to 96 h post infection and succumbed to septic shock. In contrast, C57BL/6 mice demonstrated no viable bacteria in the blood 96 h post infection. Mortality rates were 100% for BALB/c mice 72 h post infection, while mortalities of C57BL/6 occurred from 2 to 6 weeks. Post-mortem results confirmed multiple abscesses in the spleen and liver of the BALB/c strain 72 h post infection, with viable blood counts of more than 11,000 cfu/ml (Leakey *et al.*, 1998). All C57BL/6 mice demonstrated gross splenomegaly with scattered foci of necrosis and infection. However, organ bacterial loads were typically

100-1000-fold less than BALB/c in the same period (Leakey *et al.*, 1998). Using a similar animal model, these results were confirmed by Hoppe *et al*. (1999).

In humans, disseminated intravascular coagulation and multiple organ failure due to septic shock are characteristic of acute infection (Chaowagul *et al.* 1989, Vateharapreechasakul *et al*.,1992). In this respect, the symptomatic manifestation of the disease and susceptibility of the BALB/c strain were similar to human cases of acute *B. pseudomallei* infection. In contrast, C57BL/6 mice were capable of resisting systemic infection and a fatal outcome during the initial stages. However, bacterial persistence within the spleen and liver of the C57BL/6 strain indicated incomplete resistance. Based on these findings the C57BL/6 strain provided a model of the chronic form of *B. pseudomallei* infection. Numerous studies have used this model to investigate the pathogenesis of melioidosis *in vivo* .

2.4 HOST FACTORS AND MELIOIDOSIS

It appears that a primary determinant of fatal septicaemic melioidosis is the presence of predisposing host risk factors. The most common risk factors particularly documented in Australia, include alcoholism, renal disease, and type 2 diabetes (Currie, 2003). In a 10 year prospective study of melioidosis patients conducted in Darwin, 20% of those studied had no verified risk factors, and only one fatality occurred in this group (Currie *et al.*, 2000). In contrast, a 19% mortality rate was observed in the remaining patients with verifiable risk factors. Similar observations have been made in studies of Indigenous Australian populations (Cheng *et al.*, 2003). These results suggest a correlation between risk factors and severity of illness and disease outcome.

The effects of immunosuppression and susceptibility to *B. pseudomallei* have been investigated in a diabetic rat model (Woods *et al.*, 1993). Preliminary work using both *in vitro* and *in vivo* analysis determined that insulin significantly inhibited the growth of *B. pseudomallei* in isolated lung tissues. However, a later investigation by the same group reported that the reduced growth of *B. pseudomallei* may have been caused, or influenced by, the presence of a contaminant in the insulin preparations (Simpson *et al.*, 2000b). A clinical review of diabetic patients with melioidosis in Thailand found that less than 10% of the patient population studied had type I diabetes. These

findings suggest that insulin deficiency is not a contributing factor to disease outcome in diabetic patients with *B. pseudomallei* infection (Simpson *et al.*, 2003). Moreover, the likely determinant of infection in these patients is contingent upon the dysfunctional immune response due to diabetes mellitus (Geerlings and Hoepelman, 1999). Evidence supporting this was found in a study examining macrophage uptake of *Pseudomonas aeruginosa*, a pathogen resistant to phagocytosis (Barghouthi *et al.*, 1995). Barghouthi *et al*. 1995 found that *P. aeruginosa* uptake by murine macrophages was mediated in a glucose dependant manner, indicating the need for a glucose trigger to stimulate phagocytosis (Barghouthi *et al.*, 1995). It is possible that *B. pseudomallei* invasion of macrophages utilises the excessive amounts of glucose in the serum of diabetic patients for uptake. Further investigations into the effect of diabetes on immune cell function in melioidosis patients would provide useful insights into potential mechanisms responsible for the severity of disease.

2.5 CURRENT ANTIMICROBIAL THERAPY

Current antimicrobial therapy for the treatment of acute melioidosis indicates the use of the third generation cephalosporin ceftazidime, 120mg/kg/day (Chaowagul *et al.*, 1989). The seminal therapeutic study evaluating ceftazidime versus the former conventional therapy (chloramphenicol 100mg/kg/day, doxycycline 4mg/kg/day, trimethoprim 10mg/kg/day, and sulphamethoxazole 50mg/kg/day) confirmed a 50% reduction in overall mortality in patients presenting with acute infection (Chaowagul *et al.*, 1989). Combination regimens of ceftazidime and co-trimoxazole are also accepted and have been shown to lower mortality rates in comparison to mono-therapeutic regimes (Sookpranee *et al.*, 1992). Clinical trials using combination therapy demonstrated a faster eradication rate, lower mortality (30.7% vs 82.3%), and lower incidence of relapse in 27 patients treated for septicaemic melioidosis in comparison to conventional therapy (Sookpranee *et al.*, 1992). Despite successful management of severe melioidosis using ceftazidime, mortality rates remain unacceptably high (~40%) (Sookpranee *et al.*, 1992). Therefore, the pursuit of novel therapies is paramount to future success in disease management.

Recently, carbapenem antibiotics have received increasing attention for the treatment of severe melioidosis (Simpson *et al.*, 2000b). Potential benefits of the carbapenems include greater activity *in vitro* (Smith *et al.*, 1996; Simpson *et al.*, 2000a; Jenney *et*

al., 2001), decreased endotoxin release (Simpson *et al.*, 2000b), and a post antibiotic effect (PAE) (Smith *et al.*, 1995). Clinical trials comparing the use of meropenem to that of ceftazidime for the treatment of severe melioidosis in 63 patients were recently reported (Cheng *et al.*, 2003). Mortality rates were almost identical for each treatment (19% meropenem, (n = 63), 18% ceftazidime, (n = 154). However, selection criteria for meropenem therapy included a higher proportion of patients presenting with sepsis and bacteraemia. Another comparative trial between ceftazidime and the carbapenem, imipenem, was conducted in Thailand (Simpson *et al.*, 1999). Data from this investigation showed that ceftazidime demonstrated a greater incidence of failure of clearance within the first 48h, however there were no differences in mortality at the conclusion of this period (Simpson *et al.*, 1999).

Similar results have been observed in murine models of acute infection. Ulett *et al.*, (2003), using BALB/c mice, found no viable cfu bacterial counts in the spleen following 10 days of i.p therapy (12.5µg/ml, 12 h intervals) using ceftazidime in combination with co-trimoxazole (Ulett *et al.*, 2003). Combination therapy was more efficacious than ceftazidime or co-trimoxazole alone, and more effective than cefriprome (Ulett *et al.*, 1999).

2.6 NOVEL TREATMENT STRATEGIES FOR MELIOIDOSIS

Symptomatic *B. pseudomallei* infections are correlated with a high incidence of mortality (Chaowagul *et al.*, 1989), slow to minimal response to antibiotic therapy, and a high rate of relapse despite prolonged and successful treatment of primary infection (Sookpranee *et al.*, 1992). Because of the limited number of human cases encountered, clinical trials for potentially useful agents are restricted.

Studies have indicated that some strains of *B. pseudomallei* may develop resistance to not only ceftazidime but a spectrum of other antimicrobials (Tribuddharat *et al.*, 2003). Resistance of *B. pseudomallei* to a range of ß-lactams, aminoglycosides, macrolides, cephalosporins, and tetracyclines have been observed (Ashdown, 1988; Moore *et al.*, 1999; Ho *et al.*, 2002). Due to the rapid rise of multi-resistant *B. pseudomallei* strains to these antimicrobials, treatment options are limited. Therefore, the search for novel treatment modalities is imperative in light of the emerging threat of resistant organisms.

Tigecycline, a 9-t-butylglycylamino derivative of minocycline, possesses a broad spectrum of activity against a variety of clinically relevant Gram positive and Gram negative organisms (Sum and Petersen, 1999; Fritsche *et al.*, 2004; Pankey, 2005). This tetracycline derivative has demonstrated excellent activity in several studies against clinically relevant strains including methicillin resistant *S. aureus,* vancomycin resistant *E. faecalis*, and extended spectrum β-lactamase producing *E. coli* (Biedenbach *et al.*, 2001; Petersen *et al.*, 2002; Fritsche *et al.*, 2004; Kitzis *et al.*, 2004). Furthermore, tigecycline is well tolerated and is observed to exhibit pharmacokinetic properties similar to those of the tetracycline class in preliminary human trials. A retrospective *in vitro* susceptibility test using Kirby-Bauer disk diffusion and Etest assays demonstrated a 91% to 100% activity of tigecycline against 184 non duplicate *B. pseudomallei* isolates from Malaysia (Sam *et al.*, 2010). Similar results were reported elsewhere (Thamlikitkul and Trakulsomboon, 2006). In addition, the *in vivo* efficacy of tigecycline was assessed in a murine model of melioidosis, where combination therapy of ceftazidime and tigecycline was the most effective treatment and conferred a greater survival advantage over seven days (Feterl *et al.*, 2006). To date, no clinical trials determining tigecycline efficacy in human melioidosis are underway.

2.7 ANTIBIOTIC RESISTANCE OF *BURKHOLDERIA PSEUDOMALLEI*

The classic mechanisms of antibiotic resistance include enzymatic modification of the drug, substrate specificity and efflux systems. The aforementioned mechanisms are also supplemented by newly recognised multidrug efflux systems, which are capable of recognising a broad range of compounds (Moore *et al.*, 1999). *B. pseudomallei* is intrinsically resistant to various antimicrobials including the β-lactams, macrolides, aminoglycosides, and polymyxins (Ashdown, 1988). AmrAB-OprA, a multidrug efflux system of the resistance nodulation division (RND) family, is responsible for the efflux of aminoglycosides and macrolides by *B. pseudomallei* (Moore *et al.*, 1999). Using transposon mutagenesis, *B. pseudomallei* 1026b was mutagenised with Tn5-OT182 to construct aminoglycoside susceptible mutants. Results indicated that two mutants, RM101 and RM102, were susceptible to a variety of aminoglycosides demonstrating 16 to 128 fold reductions in MIC values for streptomycin, kanamycin, tobramycin, gentamicin, and the macrolide erythromicin. However, MICs for the ampicillin and cephalosporins did not differ from the parent strain.

DNA flanking transposon insertions from these mutants were isolated by self cloning and analysed for sequencing homology (Moore *et al.*, 1999). These analyses indicated sequence homology to RND type multi drug resistance proteins. The *B. pseudomallei* multi efflux system AmrAB-OprA, encoding amrA and amrB (aminoglycoside and macrolide resistance) showed strong sequence homology to the membrane fusion protein MexC (50%) and MexB (54%) in *P. aeruginosa*, as well as the AcrD (57%) in *E. coli*. Additionally, partial homology between an outer membrane protein associated with the efflux operon *mexC-mexD-oprJ* in *P.aeruginosa* was also reported. Despite the similarity between the *B. pseudomallei amr* genes and the *mex* genes in *P. aeruginosa*, no evident similarity existed for substrate (drug) specificity. *P. aeruginosa mex* genes conferred enhanced resistance to tetracycline, chloramphenicol, β-lactams, and flouroquinolones, while the *amr* genes in *B. pseudomallei* demonstrated no increased resistance to these compounds in this investigation.

β-lactamase expression is the most prevalent mechanism of bacterial resistance to the β-lactam family of antibiotics, which includes the penicillins and cephalosporins (Lee *et al.*, 2002). β-lactamase enzymes function by breaking open the β-lactam ring in the penicillin and cephalosporin molecules (Lee *et al.*, 2002). Perturbation of the molecular structure of the molecule prevents binding of the target enzyme responsible for peptidoglycan synthesis, an essential component of the bacterial cell wall (Lee *et al.*, 2002). Tribuddharat *et al.* (2003) recently examined resistance motfis of clinical *B. pseudomallei* isolates encoding the *penA* gene, which is responsible for expression of a class A β-lactamase. Enzyme kinetic analysis was performed on ceftazidime resistant *B. pseudomallei* mutants (316c) generated by single amino acid changes at position 167 within the catalytic site. The rates of ceftazidime hydrolysis of β-lactamase were not directly measured, but the mutant 316c enzyme recognised ceftazidime as a competitive substrate according to calculated K_i, k_{cat}/K_m values. The authors contend that increased affinity for ceftazidime by the 316c enzyme may account for increased resistance to this agent in laboratory derived mutant strains (MIC 64μg/ml). Ho *et al.* (2002) found similar results in that single amino acid substitutions at residue 167 (Pro→Ser) converted wild type BPS-1 *B. pseudomallei* strains into ceftazidime hydrolysing β-lactamase (BPS-1m) strains. In addition, point mutations in the 392f motif resulted in decreased susceptibility to clavulanic acid

inhibition (β-lactamase inhibitor) in both mutant *B. pseudomallei* and *E. coli* strains (Tribuddharat *et al.*, 2003).

2.8 PATHOGENICITY AND VIRULENCE DETERMINANTS

Holden *et al.* 2004 sequenced the genome of a clinical *B. pseudomallei* isolate. The genome is comprised of two chromosomes of 4.07 and 3.17 megabase pairs (chromosomes 1 and 2) respectively, and a functional partitioning of genes exists between the two. The chromosome 1 is conserved across related species and is responsible for gene products essential for cell growth. Chromosome 2 harbours accessory genes housed on genomic islands that enable survival in various environments. Soil and invasive isolates contain various groupings of these genomic islands that are absent in the most closely related organism *B. mallei*. The authors contend that the genetic evolution of the organism and its pathogenic characteristics are acquired via horizontal gene transfer on the small chromosome.

Pathogenicity could be defined as the ability of a microorganism to cause disease and is determined by that pathogen's ability to gain entry into the host organism, evade host defence mechanisms, colonise, and cause damage to infected tissues (Dance, 2002; Liu *et al.*, 2002). While not a natural human pathogen, *B. pseudomallei* has the capability of transcending its natural soil habitat and eliciting disease in humans via opportunistic infection (Puthucheary and Vadivelu, 2002).

Primarily, infection occurs through direct entry into abraded skin or by ingestion or inhalation of contaminated soil and surface water (Dance, 2002). Whether or not an individual develops symptomatic disease following exposure is thought to be dependent on several known factors, including: inoculum size, pathogen virulence, iron bioavailability, host immunocompetence, and potentially, host genetic variation (Barnes *et al.*, 2001a). Investigation into the microbial diversity and evolution of virulence determinants in *B. pseudomallei* is paramount to understanding its pathogenicity.

2.8.1 Siderophore Production, Adaptive Mechanisms for Iron Metabolism

Iron is essential for bacterial growth and the ability of pathogens to sequester iron from the host organism is essential for the establishment and maintenance of infection (Sunderplassman *et al*. 1999). The majority of iron in the mammalian host is predominantly bound to globular proteins such as lactoferrin and transferrin and is inaccessible to microorganisms for use (Yang *et al*. 1991). In order to circumvent this phenomenon, many microorganisms synthesise and excrete low molecular weight, iron specific chelators or siderophores, to sequester iron from the host (Neilands, 1981). In 1991, Yang *et al*. validated siderophore production by *B. pseudomallei* U7 via the chrome azurol S (CAS) assay. Chemical analysis identified a water soluble molecule with a molecular weight of 1 KDa, classified as malleobactin (Yang *et al.*, 1991). Malleobactin production is upregulated in iron deficient conditions *in vitro* and is capable of stimulating colony growth even in the presence of transferrin. The characterisation of malleobactin in *B. pseudomallei* assisted in understanding one potential mechanism of pathogen survival and replication in host tissue and blood.

2.8.2 Role of Type III Secretion Systems in Intracellular Survival

Burkholderia pseudomallei is a facultative intracellular pathogen that is capable of invading a broad spectrum of epithelial and macrophage cell types (Stevens *et al*., 2003; Jones *et al.,* 1996). The ability of this pathogen to invade variant cell lines and subvert cellular processes may be responsible for the amalgam of clinical presentations observed. Studies indicate that a cluster of *B. pseudomallei* genes demonstrate sequence homology to loci in *Salmonella typhimurium* (*Inv/Spa/Prg*) and *Shigella flexneri* (*Ipa/Mxi/Spa*) that encode type III secretion systems (TTSS) (Stevens *et al.* 2003). The Inv/Spa/Prg type III secretion system of *Salmonella* species (TTSS-1) and the *Shigella* species Ipa/Mxi/Spa apparatus are crucial components for epithelial cell infiltration by these pathogens (Holden *et al.*, 2004a). Type III Secretion Systems function as “molecular syringes”, injecting effector bacterial proteins into the plasma membrane and cytosol of their eukaryotic cell hosts (Plano *et al.*, 2001; Stevens *et al.*, 2002a) (Figure 2.2). These effector proteins often mimic eukaryotic enzymes in structure and function, altering host cellular activity and facilitating pathogen uptake (Holden *et al.*, 2004a).

One mechanism of Gram negative intracellular pathogen infiltration results from induced subcortical actin cytoskeletal rearrangements, or "membrane ruffling", which promotes macropinocytotic vesicle formation (Plano *et al.*, 2001; Stevens *et al.*, 2004). Bacterial pathogens are able to enter from the extracellular space into the cell via these vesicles (Plano *et al.*, 2001). *Burkholderia pseudomallei* has evolved a TTSS system to enter and escape eukaryotic cells by manipulating actin polymerisation (Stevens *et al.*, 2002a). Stevens *et al*. (2002) found that *B. pseudomallei* encodes an Inv/Mxi-Spa-like type III secretion gene cluster known as *Burkholderia* secretion apparatus (Bsa). Bsa type III secretion proteins enable bacterial entry into nonphagocytic (HeLa) cells as well as lysis and escape from endosomal membranes (Stevens *et al.*, 2002a). Mutations introduced in several *bsa* loci greatly reduced intracellular invasion in murine macrophages, indicating an important role for bsa secreted proteins in bacterial uptake (Stevens *et al.*, 2002a; Stevens *et al.*, 2003). Deletion of cluster 3 TTSS systems in *B. pseudomallei* abrogated full virulence *in vivo*, and virulence was not attributed to one effector molecule (Stevens *et al.*, 2004; Warawa and Woods, 2005). Putative autosecreted proteins of *B. pseudomallei* and selected mutants led to the discovery of *bimA*, which is required for intracellular motility and subversion of cellular actin dynamics (Stevens *et al.*, 2005). Mutations of *bimA* inhibited actin-based motility of internalised *B. pseudomallei* in a murine macrophage cell line. The *bimA* proteins responsible for polymerizing actin based movement are localised at one polar end of the bacterium and motility is directionally based. This process facilitates the localisation of the organism subcortically at the sites of host actin polymerisation, resulting in the formation of *B. pseudomallei* containing protrusions. These protrusions may assist in cell to cell spread and the evasion of other host immune mechanisms (Utaisincharoen, 2001; Stevens *et al.*, 2005). Mutant strains lacking the *bsaQ* gene, showed a marked decrease in secretion of BopE effector, and BipD translocator proteins and the reduced invasion efficiency into J774.1 macrophages and the ability to cause cell protrusions (Muangsombut *et al.*, 2008).

Six type VI secretion (T6SS) system clusters have been identified in *B. pseudomallei*, the largest number recorded for a pathogen with complete genomic sequencing and these include, *P. aeruginosa*, *Vibrio parahaemolyticus* and *Yersinnia pseudotuberculosis* (Shalom *et al.*, 2007). The T6SS comprise 2.3% of the

overall *B. pseudomallei* genome and 4.5% of the small chromosome (Shalom *et al.*, 2007). Holden et al. 2004 have demonstrated that the small chromosome is important for integrating accessory functions for survival in response to environmental stimuli. In line, the *B. pseudomallei* T6SS and are important for invasion into macrophages and demonstrate T6SS conferred virulence (Shalom *et al.*, 2007). Studies assessing *B. pseudomallei* mutant T3SS strains have also shown a regulatory role for the expression of other virulence factors by *bsa* TTSS cluster. Recently, the secreted virulence factor TssM was identified in *B. pseudomallei* which shared exact sequence homology to the TssM gene in *B. mallei* (Tan *et al.*, 2010). Active secretion of TssM resulted in the deubiquination of critical signalling molecules in transfected HEK293 cells as well as in murine RAW 264.7 macrophages. *In vivo* infection of BALB/c mice with TssM mutants showed a marked increase in IFN-β and IL-6 transcripts in addition to higher levels of IL-6 and TNF-α in the pulmonary compartment (Tan *et al.*, 2010). The role of secretion systems and their involvement in the pathogenesis of *B. pseudomallei* is complex and multi-faceted. Further investigation into the function of new and identified secretion system proteins will hopefully lead to potential targets for treatment of disease.

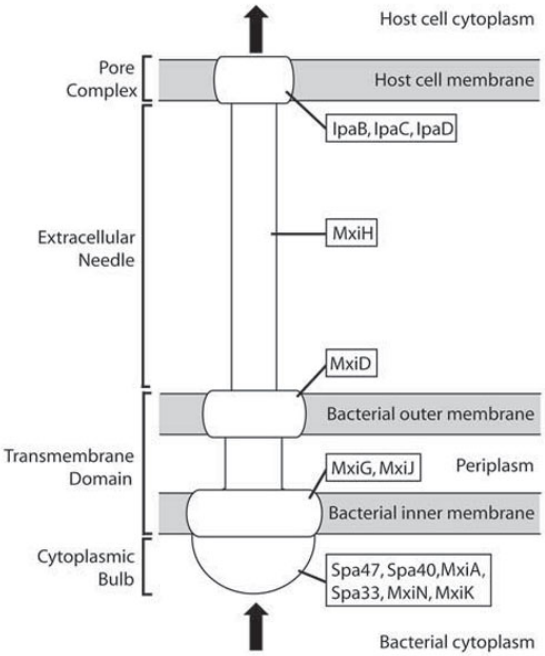

Figure 2.2 Basic structure of a type III secretion system in *Shigella* spp. *Burkholderia pseudomallei* shares sequence homology and function with these structural proteins (Deane *et al.* 2010).

2.8.3 *Burkholderia pseudomallei* Lipopolysaccharide

In order to establish infection in a susceptible host, a pathogen must avoid host immune responses and microbicidal activity. Many Gram-negative bacteria are susceptible to innate bactericidal mechanisms. However, some pathogens are capable of circumventing these host defences. In addition to complement and opsonic mediated bacteriolysis resistance (Egan and Gordon, 1996; DeShazer *et al.*, 1998), *B. pseudomallei* may avoid host elimination via invasion and survival within phagocytic and epithelial cells (Jones *et al.*, 1996; Stevens *et al.*, 2002a). Several studies have investigated the role of lipopolysaccharide (LPS) as a potential virulence determinant for *B. pseudomallei* persistence in the host.

Cell surface associated and secreted antigens including exopolysaccharride (EPS) and LPS have been identified in *B. pseudomallei* (Perry *et al.*, 1995; Reckseidler *et al.*, 2001). In 1995, Perry *et al.* found that variant *B. pseudomallei* strains isolated from different sites express heterogeneous LPS in the cell wall. In addition, LPS structure in *B. pseudomallei* was classified into two distinct O-polysaccharride moieties: type I and type II O-PS (Perry *et al.*, 1995).

DeShazer *et al*. (1997b) demonstrated that type II O-PS biosynthesis was essential for *B. pseudomallei* serum resistance and virulence. Microtitre plate assays of *B. pseudomallei* clinical isolates demonstrated proliferation in 10-30% normal human serum five logs greater than *E. coli* controls. Similarly, the clinical isolates incubated in normal human serum were three fold higher than induced serum sensitive mutants lacking the type II O-PS moiety (DeShazer *et al.*, 1997). Parallel experiments using serum from guinea-pig, infant diabetic rat, and hamsters also demonstrated three to four log increases in *B. pseudomallei* compared to *E. coli* controls. A polysaccharide capsule has also been identified in *B. pseudomallei* and was recognised in sera of 13 melioidosis patients demonstrating its antigenic properties (Masoud *et al.*, 1997). Furthermore, capsular presence minimised the effectiveness of host opsonisation and phagocytosis by reducing complement C3b deposition on the bacterial surface *in vitro* (Reckseidler-Zenteno *et al.*, 2005). These results indicate that the type II O-PS moiety and extensive polysaccharide complexes are essential components for serum resistance and virulence in *B. pseudomallei* infection.

Recently the antigenic structure of *B. pseudomallei* was compared with the closely related species *Burkholderia thailandensis* (Novem *et al.*, 2009). The major lipid A species of *B. pseudomallei* consists of a biphosphorylated disaccharide backbone, modified with 4-amino-4-deoxy-arabinose at both phosphates with penta-aclyated fatty acids. The acylation pattern of *B. pseudomallei* lipid A at C_{14} was not found in *B. thailandensis* and *B. pseudomallei* LPS was less stimulatory of RAW 264.7 and THP-1 macrophages (Novem *et al.*, 2009). The weakly immunogenic properties of *B. pseudomallei* LPS may help facilitate invasion into host cells and studies analysing host recognition of *B. pseudomallei* are currently an area of interest.

2.8.4 Flagella: Implication as a Virulence Factor

Flagella are commonly recognised as important virulence determinants in bacterial pathogens. In *Burkholderia* species, flagellum conferred motility may correspond with increased dissemination from local foci of infection (Brett *et al.*, 1997; Tomich *et al.*, 2002). In *B. pseudomallei,* several motility gene clusters with sequence homology to *E. coli* and *S. typhimurium* flagellar (*fliC)* proteins have been identified using transposon mutagenesis (Brett *et al.*, 1997). Differences in virulence between wild type and aflagellate induced mutants were further investigated in various *in vivo* models of infection (DeShazer *et al.*, 1997). No significant difference in virulence of non-motile mutants and wild type *B. pseudomallei* strains were found using intraperitoneally infected diabetic rat and Syrian hamster models (DeShazer *et al.*, 1997). These findings suggest that flagellar proteins are not essential virulence determinants in these models of *B. pseudomallei* infection. In contrast, Chua *et al*. (2003) determined flagella as critical virulence factors in acute *B. pseudomallei* infection. An isogenic deletion mutant was designed lacking the *fliC* gene that is essential for flagellum production. Results indicated that aflagellate, non motile *B. pseudomallei* mutants were non virulent in BALB/c mice infected via intraperitoneal and intranasal routes (Chua *et al.*, 2003). However, flagellate strains were highly virulent. The discrepancy in results between these two studies could have resulted from the different animal models used. Analysis of pattern recognition receptor expression on host cells that bind the flagellin moiety would have been insightful in this investigation to assist in understanding host immune responses as a recent study has shown *B. pseudomallei* is capable of activating downstream signalling via TLR5, a receptor responsible for flagella recognition (Hii *et al.*, 2008).

2.9 IMMUNE RESPOSE

The role of the innate immune defence system is the formation of an initial barrier to the establishment of infection. On a daily basis an individual will encounter numerous microbes and only rarely do these interactions result in infection. Most organisms are detected and destroyed in a short period by cellular and humoral defence systems that do not require the synthesis of antigen specific components. The innate immune system is capable of recognising a broad spectrum of pathogens and functions to isolate these infectious agents until an adaptive immune response can be initiated. In the following sections the humoral and cellular components of the immune response will be discussed. In particular the role of macrophages in innate immunity and the receptors responsible for pathogen recognition will be evaluated.

2.9.1 Complement: Humoral Mechanisms of Pathogen Destruction

A group of proteolytic enzymes collectively known as complement, are a heat-labile component of normal plasma that can recognise pathogens in the extracellular space and enhance the uptake of bacteria. Complement can be activated by three separate means, the classical, alternate, and mannose binding lectin (MASP) pathways (Figure 2.3). The classical pathway is activated by antibodies, which bind to invasive microbes and facilitate opsonisation. The alternate pathway is dependant upon the plasma glycoprotein properdin, which is equipped with a set of cofactors that directly identify microbes via cofactor B and the microbial surface. The MASP pathway is activated by mannose-binding protein engagement of mannosyl terminal residues on the surface of invasive microbes (Beutler, 2004).

The cumulative effect of complement activation via any of the aforementioned pathways triggers a cascade of events including: 1) the activation of C3b, which binds to pathogens and facilitates opsonic mediated phagocytosis; 2) the production of C5a, an inflammatory and chemotactic mediator; 3) activation of C5 through C9, the latter of which forms a ring-shaped assembly of protein subunits known as the membrane attack complex (MAC). The MAC is capable of lysing Gram negative bacteria in addition to inactivating virus.

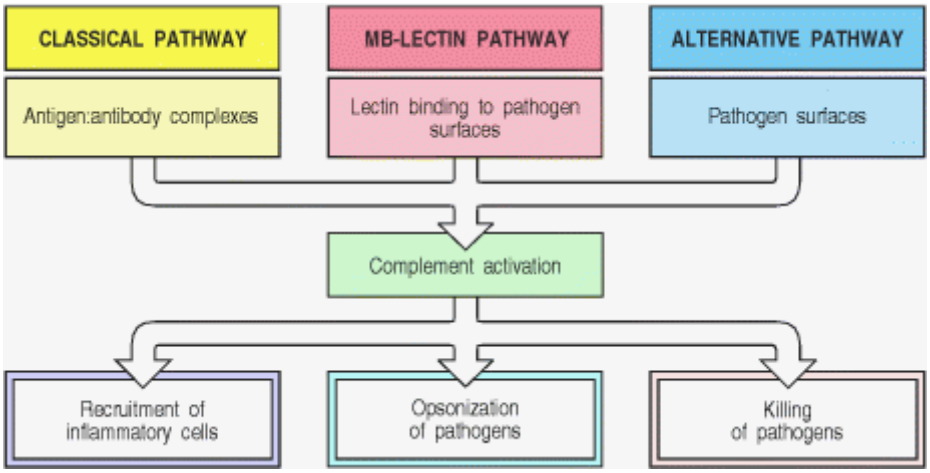

Figure 2.3 Schematic demonstrating the three pathways of complement activation in host plasma (Janeway *et al.* 2010).

2.9.2 Cellular Responses to Infection

In mammals, innate immunity is largely dependent on cells of myeloid origin, which are professional immunocytes that phagocytose and eliminate pathogens (Beutler, 2004). They can function independently or work in concert with the cells and proteins of the adaptive immune system. For example, lymphocyte derived antibodies that coat antigens on bacteria are targeted by myeloid cells for destruction.

Leukocytes are a diverse group of cells involved in the immune response. Mononuclear phagocytes or macrophages, a leukocyte subset, reside in host tissues at rest and in times of inflammation. Macrophages are derived from blood monocytes which circulate through the blood and lymph and are recruited to sites of infection by stimulation of chemokine receptors and adhesion molecules that facilitate migration into infected tissue (Swirski *et al.*, 2009). Macrophages and dendritic cells, another leukocyte subset, are involved in bacterial clearance and antigen presentation. Antigens presented on the surface of macrophages and dendritic cells are done so in conjunction with either one of two molecular class molecules known as the major histocompatibility complex (MHC). The site of antigen recognition in the cell determines the class of MHC molecule that delivers antigen to naïve lymphocytes to begin the process of developing long term immunological memory. Pathogens present in the cytosol of macrophages are presented in association with MHC class I molecules to $CD8^+$ T cells (Janeway and Medzhitov, 2002). Extracellular pathogens phagocytosed by macrophages and dendritic cells are recruited into host phagosomes

where proteolytic enzymes and reactive nitrogen and oxygen species break down the pathogen. Peptides released during bacterial eradication are in turn presented by MHC class II molecules at the plasma membrane. MHC class II presentation of antigen activates $CD4^+$ cells (Figure 2.4) that release pro-inflammatory cytokines and chemotactic molecules to initiate macrophage activation at the site of infection (Beutler, 2004).

Macrophages and dendritic cells are often the first to infiltrate foci of infection where they serve to clear bacteria and release potent immunomodulators (Silva, 2010). Clearance of pathogens by dendritic cells in infected peripheral tissues and the subsequent migration to draining lymph nodes is one of the first steps in developing adaptive immunity (Romagnani, 1997). The presentation of antigen to naïve T cells (Figure 2.4) initiates T cell activation and differentiation (Netea *et al.*, 2005). Following the activation of T cells, pro-inflammatory cytokines and chemokines released from macrophages and dendritic cells at sites of infection culminates in the differentiation of a T_H1-type or T_H2-type response. Which T_H phenotype develops is dependent on the pathogen and the subsequent pro-inflammatory cytokines released by macrophages and dendritic celss following pathogen recognition (Taylor *et al.*, 2005). As such, the type of cytokine profile present at the time of T cell stimulation determines T_H cell differentiation. T_H1-type responses are induced by IL-12 family members and alpha interferon, while T_H2-type responses are induced by IL-4, IL-5, IL-6 and IL-13 (Kelso, 1998). T_H1-type cells produce mediators that induce B cells to produce antibodies necessary for cellular mediated responses, including IL-2, IFN-γ and TNF-β (Kelso, 1998; Netea *et al.*, 2005). The principle function of T_H1-type responses is to promote the aggregation and activation of phagocytic cells along with antibody assisted cytotoxicity for the eradication of intracellular pathogens (Netea *et al.*, 2005). For example, during *B. pseudomallei* infection, intracellular growth in macrophages is suppressed when stimulated with IFN-γ along with increased expression of inducible nitric oxide synthase (iNOS), an important enzyme involved in the lysis of internalised bacteria (Ulett *et al.*, 1998; Utaisincharoen, 2001). Phagocytosis of intracellular bacteria induce the production of IL-12 and IFN-γ from antigen presenting cells in addition to IFN-γ from NK and $CD8^+$ T cells, which in turn further drives the development of a T_H1-type response (Romagnani, 1997). However,

while critical for pathogen clearance, dysregulation and or excessive activation of this response can have deleterious effects on the host (Kelso, 1998).

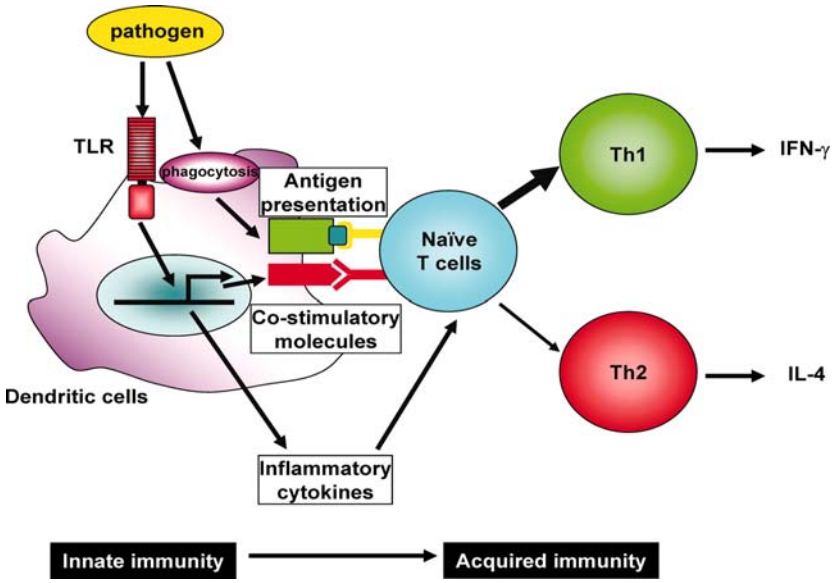

Figure 2.4 Schematic of a dendritic cell presenting antigen to naïve T cell in the lymph node.

The type of response Th1 or Th2 is determined by the location of the antigen in the cell. Th1 responses are indicative of cytosolic pathogens while Th2 responses correlate with pathogens in the phagosome (Takeda *et al.* 2001).

2.10 TOLL-LIKE RECEPTORS AND ADAPTIVE IMMUNITY: BRIDGING THE GAP

One of the unique features of the immune system is the reliance on phagocyte movement for patrolling, containment, and destruction of invasive pathogens. The phagocytosis of invading pathogens is paramount to the induction of an effective immune response. Degradation of pathogens by mononuclear phagocytes leads to the presentation of pathogen-derived proteins to naïve lymphocytes in addition to the release of pro-inflammatory cytokines that drive T_H1-type or T_H2-type cell differentiation. Over the past decade research has shown that the production of cytokine profiles by phagocytic cells is governed by specific innate immune receptors involved in pathogen recognition at the plasma membrane and in host phagocytic vesicles (Netea *et al.*, 2005). Abnormalities in pro-inflammatory cytokine production as well as in phagosome maturation and function by macrophages and dendritic cells have been observed during infection with several Gram negative species, including *B. pseudomallei* (Utaisincharoen, 2001; Utaisincharoen *et al.*, 2003a; Blander and Medzhitov, 2004; Puthucheary *et al.*, 2006). In the following sections, the structure

and signalling mechanisms of specific innate immune receptors involved in pathogen recognition and clearance will be discussed.

2.10.1 Toll-Like Receptors are Responsible for Pathogen Recognition and the Initiation of the Immune Response

As mentioned, the immune response to microbial pathogens is dependent on both innate and adaptive components. The immediate, innate response is primarily facilitated by white blood cells such as neutrophils, macrophages, and dendritic cells, which detect and kill invading pathogens (Aderem and Underhill, 1999). Furthermore, these antigen presenting cells mediate the induction of additional adaptive responses via the release of an array of pro-inflammatory cytokines. But how do these cells recognise the multitude of invasive organisms and communicate to the body that a pathogen is present?

A substantial challenge to innate immunity is the discrimination between a multitude of potentially infectious agents from "self" components using a limited number of receptors for pathogen recognition. This challenge is further exacerbated by pathogen mutation, whereby genetic alterations of the pathogen could affect host recognition. In order to address these obstacles the cells of the innate immune response have evolved a series of receptors that recognise a spectrum of conserved pathogen motifs that are not characteristic in higher eukaryotes (Aderem and Ulevitch, 2000). These motifs, or more specifically, these pathogen associated molecular patterns (PAMPs), are critical for pathogen survival and have changed minimally over the course of evolutionary history (Janeway and Medzhitov, 2002). Similarly, host organisms have evolved a series of germ-line encoded receptors that are constitutively expressed either on the cell surface or intracellularly within endosomal membranes (Roach *et al.*, 2005). Signalling and expression of these receptors is PAMP directed, leading to distinct immunological responses via activation of the transcription factor NF-κB or the production of type I interferons (Kawai and Akira, 2005). The basic mechanisms for innate immune recognition are highly conserved across species, ranging from fruit flies to mammals (Roach *et al.*, 2005). The role of the TLR family in the innate immune response has primarily been clarified by bacterial and viral challenge in mice lacking a specific TLR receptor and or adaptor molecule. It is evident that the most critical function of the TLR family during acute infection is the production of effector

molecules, inflammatory cytokines and chemokines at the sites of invasion (O'Neill, 2006). Several studies have addressed the affect of *B. pseudomallei* on pro-inflammatory mediators but never in relation to ligand binding and TLR function until 2007, and these will be addressed later in this review. In the following sections four of the eleven Toll-like receptors and their structure, specific ligands, and signalling mechanisms will be discussed. These receptors have been selected because they represent PAMPs that have been characterised in *B. pseudomallei* and are likely targets for TLR recognition of the bacteria.

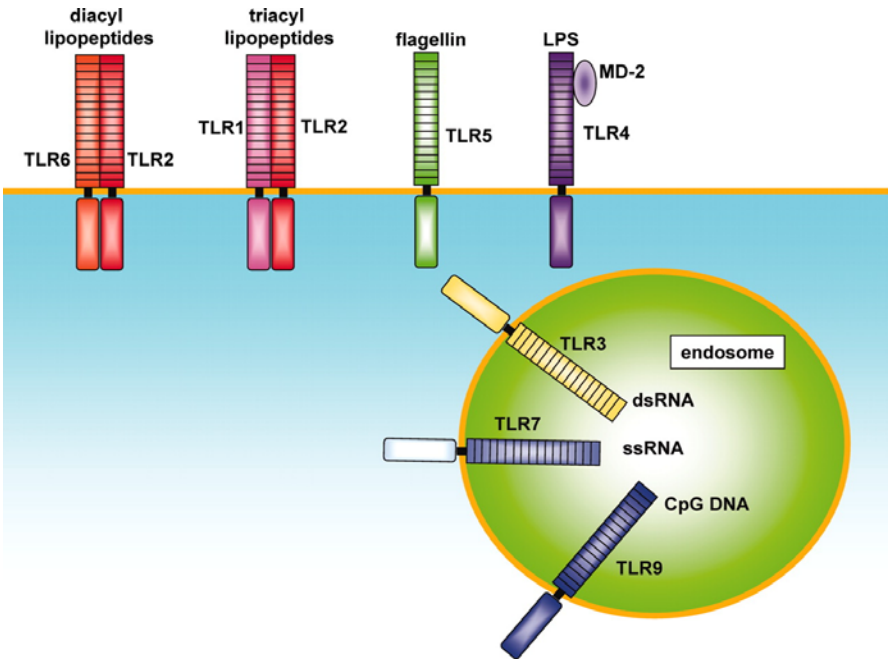

Figure 2.5 Location of some members of the Toll-like receptor family on the plasma membrane and intracellularly. (Takeda *et al.* 2005).

2.10.2 Toll like Receptor Structure

Investigations into the complex structural domains of the TLR family and the interaction of these receptors with their respective ligands have made substantial progress over the past decade. Ten functional human TLRs have been identified. TLRs are type I transmembrane glycoproteins composed of extra- and intra-cellular signalling domains (Gay and Gangloff, 2007). The extracellular domain consists of a large horseshoe shaped solenoid that possesses twenty-three leucine rich repeat units.

These repeats are stabilised by hydrogen bond networks made with asparagine residues and are involved with ligand binding (Choe *et al.*, 2005).

Toll-like receptors share a conserved cytoplasmic domain (TIR) with the interleukin-1 receptor family. The overall structure of the TIR domain consists of a five stranded central parallel β-sheet that is surrounded by five α-helices (Xu *et al.*, 2000). Despite the conservation of the TIR structure between IL-1R and the TLR subfamily, variation in the size and conformation of TIR domains exist. The differences in TIR domain structure confer specificity on the intracellular surface and ensure the correct recruitment and orientation of signalling molecules. Three types of TIR domain interactions exist: 1) the R face 2) the A face 3) the S face. The R face mediates the oligimerisation of TIR receptor domains which are induced by the binding of TLR ligands on the extracellular surface. These extracellular associations are mediated by variations in the amino acid and carbohydrate composition of the receptor complex (Choe *et al.*, 2005). The A face mediates the oligimerization of adaptor signalling molecules and the TIR domain on the subcortical surface. The S interface mediates the formation of the receptor and adaptor TIR domain complexes that are necessary for signal transduction (Underhill *et al.*, 1999). On the surface of the S interface a conserved protrusion known as the BB loop (Figure 2.6) is thought to be involved in intracellular signalling. Point mutations at the BB loop abrogated LPS signalling activity of TLR4 receptors in transfected kidney cells *in vitro* (Xu *et al.*, 2000). Mutant cells transfected with wild type BB loop encoding genes retained proper LPS induced signalling. It would be of value to use the same methodology to examine the changes in TLR receptor and TIR domain structure from mRNA isolated from *B. pseudomallei* challenged cells *in vivo*. A three dimensional representation of the TLR receptor using crystallisation methods would give direct visual confirmation of any alterations induced by the pathogen and its associated PAMPs at the TIR domain. To date, the atomic detail of three TLR complexes (TLR2/TLR1, TLR4/MD-2/Eritoran, TLR3) have been characterised and they provide tremendous detail on the interaction of ligand specific interaction with the receptor and induction of TLR dimerisation (Jin and Lee, 2008). Determining pathogen effects on TLR receptor complexes, signalling initiation and intracellular adaptor protein recruitment is paramount to understanding pathogenesis.

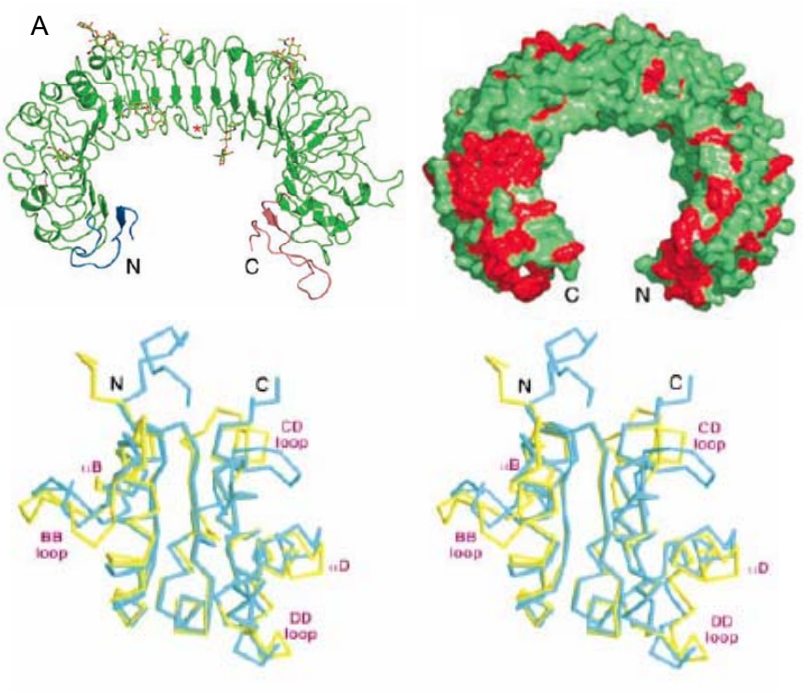

Figure 2.6 The superposition of the TIR domains of human TLR1 and TLR2 on the cytoplasmic surface.

Top left: overall architecture of TLR1 in a ribbon representation with the N terminal cap (blue), leucine repeats (green) and the C terminal cap (purple). TLR2 is depicted on the right, with red surfaces indicating negative charge, while green represents positive**.** Surfaces of variation are labelled on the bottom two diagrams (TLR1 on the left, TLR2 on the right). The BB loop is the interface between the TIR domain and host adaptor intracellular signalling molecules. (Adapted from Choe *et al*. 2005).

2.10.3 TLR2: Getting By With A Little Help From Its Friends

In regard to TLR specificity, TLR2 represents the broadest range of PAMP recognition and this phenomenon is thought to be attributed to TLR2 heterodimer formation with TLR1 and TLR6 (Weber *et al.*, 2004). Lipoproteins and lipopeptides are a major structural component of the cell walls of Gram positive bacteria and are the primary ligand for TLR2 receptor complexes (Jin *et al.*, 2007). Recognition of bacterial peptidoglycan, lipoprotein, and yeast zymosan by TLR2 was found in cells challenged with both Gram positive and Gram negative organisms (Brightbill *et al.*, 1999; Takeuchi *et al.*, 1999). Signalling via TLR2 is mediated via heterodimer formation with either TLR1 or TLR6, which have structural similarities (Takeuchi *et al.*, 2001; Alexopoulou *et al.*, 2002). Inhibition of TLR2 or TLR6 abolishes signalling in macrophages (Ozinsky *et al.*, 2000a). The structural makeup of the lipoprotein present determines which TLR2 complex is activated. Triacylated lipoproteins are

recognised by the TLR2/TLR1 complex while diacylated lipoproteins are recognised by the TLR2/TLR6 complex (Takeuchi *et al.*, 2002; Takashi *et al.*, 2007). The lipid chains of the ligand bridge the two TLRs where they are inserted into binding pockets to form a stable structure (Jin *et al.*, 2007). By working in concert with other TLR receptors, TLR2 ligand recognition is the most encompassing and diverse of the TLR family. As a result TLR2 has been shown to be stimulated by certain structural variants of LPS including LPS from *B. pseudomallei* (Takeuchi *et al.*, 1999; Wiersinga *et al.*, 2007c; West *et al.*, 2008a; Wiersinga *et al.*, 2008a; Yang and Joyee, 2008)

Several investigations have assessed bacterial activation of TLR2 in both *in vitro* and *in vivo* models of infection. For example, *Yersinia pestis*, *Yersinia pseudotuberculosis,* and *Yersinia enterocolitica*, have a conserved virulence plasmid (pYV) that confers resistance to innate immune mechanisms by regulating the type III secretion protein LcrV (Cornelis *et al.*, 1998; Hoiczyk and Blobel, 2001). Alveolar macrophages are unable to phagocytose *Yersinia* species in the presence of LcrV. Treatment of TLR2 deficient murine macrophages as well as human MonoMac6 cells with LcrV, down regulated TNF-α expression in these cells in comparison to wild type macrophages via influence from IL-10 secretion in a dose dependant manner, whilst the *Pseudomonas aeruginosa* homologue (PcrV) did not (Sawa *et al.*, 1999). Similar results were seen in *Y. enterocolitica* infected C57BL/6 peritoneal macrophages and MonoMac6 cells, where LcrV proteins were found to target the TLR2 receptor complex, that ultimately lead to immunosuppression (Sing *et al.*, 2002). The authors contend that this may be a mechanism for *Yersinia* species to evade phagocytosis. Due to the known prevalence of type III secretion systems in *B. pseudomallei*, similar LcrV proteins are likely to exist given the intracellular niche *B. pseudomallei* occupies. However, during acute infection, *B. pseudomallei* stimulates upregulation of TLR2 and pro-inflammatory cytokines in patients with septic melioidosis (Suputtamongkol *et al.*, 1992; Wiersinga *et al.*, 2007c).

In a murine C57BL/6 genital tract infection model with *Chlamydia trachomatis*, infected macrophages were shown to have a severe bacterial burden in TLR2 deficient mice (Darville *et al.*, 2003). Pro-inflammatory cytokine production of TNF-α, IL-6, and IFN-γ as well as the chemokine MIP-2 were decreased. The binding ligand of

C. trachomatis to TLR2 was not defined however the likelihood of *C. trachomatis* activating the TLR4 receptor simultaneously to TLR2 is probable considering LPS is a component of that pathogen. This phenomenon was later confirmed in other chlamydial infection models where TLR2 activation served as a critical component of the immune response in addition to those initiated by TLR4 (Yang and Joyee, 2008). Certain Gram negative bacterial species have been reported to signal through TLR2 and subsequently have a negative impact on host immune responses. In bone marrow derived macrophages and TLR deficient mice infected with *L. pneumophila*, TLR2 and not TLR4 was deleterious to the host response (Archer and Roy, 2006). Similar findings have been observed in *Porphyromonas gingivalis*, another Gram negative intracellular pathogen (Burns *et al.*, 2010).

2.10.4 TLR4: LPS Recognition and the Gateway to Sepsis

In 1996, Lemaitre and colleagues found that the hToll protein in *Drosophila melangaster* was an essential receptor for host defence against fungal infections. In addition, hToll was shown to possess structural and functional similarities to the IL-1R receptor (Lemaitre *et al.*, 1996). One year later, a mammalian homologue of the hToll like receptor was shown to induce expression of inflammatory genes in humans (Medzhitov *et al.*, 1997). Further analysis of TLRs over the ensuing years showed a broad constitutive expression of the receptor family in a variety of organisms and to date 10 functional human TLRs have been identified (Luke, 2008). Based on this information it became evident that the Toll-like receptor family, much like its PAMP ligand counterpart, had remained relatively conserved in eukaryotic organisms throughout evolutionary history.

The best characterised Toll-like receptor is TLR4, which is responsible for the recognition of Gram negative bacterial LPS (Medzhitov *et al.*, 1997). Lipopolysaccharide is one of the most potent stimulatory ligands of TLR4 in host mononuclear cells (Erridge *et al.*, 2002). The structure of LPS is composed of a hydrophopic lipid A backbone linked to a hydrophyllic polysaccharide component (Erridge *et al.*, 2002). The polysaccharide and acyl chain composition, along with variations in chemical linkage and sequence make LPS highly diverse (Jin and Lee, 2008). Analysis of the immunogenic capacity of several LPS variants from *E. coli* has shown that lipid A consisting of six lipid chains and two phosphate groups is

optimal for TLR4 activation and signalling (Erridge *et al.*, 2002). Lipid A with either five or seven acyl chains is 100 fold less stimulatory and variations in structure can effect TLR4 activation. The structural characteristics of *Burkholderia pseudomallei* lipid A by mass spectrometry have shown a penta acylated structure and Ara4N-modified phosphate groups (Novem *et al.*, 2009). Evasion and modulation of TLRs may be a particular advantage to organisms that cause chronic infection. However, the data regarding this matter is inconclusive. Certain Gram negative bacteria are capable of modifying the LPS structure of their outer cell walls making them less stimulatory to TLR4. The primary structure that varies across species is lipid A. As mentioned previously, lipid A extracts from *E. coli* and *S. typhimurium* are potent stimulators of TLR4 in macrophages (Poltorak *et al.*, 1998) however, modified LPS structures from *Heliobacter pylori* and *Chlamydia trachmatis* are less stimulatory to TLR4 in the gastric and urogenital epithelia (Ernst *et al.*, 1999). The interaction of *B. pseudomallei* and TLR4 will be outlined later in this review.

At the plasma membrane TLR4 forms a signalling complex with LPS binding protein (LBP) along with the co-receptors CD14 and MD-2 (Shimazu *et al.*, 1999; Lu *et al.*, 2008). Upon stimulation with LPS, the TLR4 complex utilises intracellular signalling molecules to induce the production of multiple pro-inflammatory cytokines and chemokines via the activation of transcription factors (Akira and Takeda, 2004).

Stimulation of TLR4 receptors by LPS can induce a massive proliferation of inflammatory cytokines which can result in sepsis. Septic shock is caused by the dissemination of the lipid A moiety into the bloodstream and is associated with severe Gram negative infections. Pneumonia derived sepsis induced by *B. pseudomallei* is the most common presentation of the disease in north east Thailand and northern Australia (Cheng and Currie, 2005). Modifications to TLR4 either endogenously or experimentally alter host responses to LPS. In murine RAW 264.7 macrophages stimulated with low concentrations of LPS a transient decrease in TLR4 expression was observed over a three hour period (Poltorak *et al.*, 1998). This finding may demonstrate endotoxin tolerance in healthy cells via the down-regulation of TLR4. Whether or not the mechanism responsible for LPS tolerance involves the down-regulation of TLR4 at the cell surface is complex. Pre-treatment of murine macrophages with LPS has been reported to inhibit TLR4 expression (Nomura *et al.*,

2000). Moderate inhibition has also been noted in human THP-1 monocytes (Hajishengallis *et al.*, 2002). In contrast, some studies have noted no variation in surface expression of TLR4 but rather its association with intracellular adaptor molecules (Medvedev *et al.*, 2002).

In a population study where individuals were exposed to aerosol LPS it was found that TLR4 gene polymorphisms can result in hyporesponsiveness. Interestingly, some of the subjects with mutations in TLR4 demonstrated a normal response while some wild type subjects were hyporesponsive (Arbour *et al.*, 2000). Furthermore, Polotrak and others have found that selective mutations in mice deficient in TLR4 were unresponsive to endotoxin and succumbed to septic shock (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). This was later found to be a result of a point mutation in the BB loop of the TLR4 receptor (Xu *et al.*, 2000). Northern blot and RT PCR analysis of murine mRNA indicated a lack of TLR4 amplicons from mutant TLR4 deficient splenocytes challenged with *S. typhimurium* LPS (Poltorak *et al.*, 1998). These results indicate host variation in LPS recognition and TLR4 mediated signalling.

Understanding of TLR4 signalling is contingent on a systematic approach to evaluating host receptors, signalling molecules, and cytokine expression. The purpose of this investigation is the continuation of understanding TLR4 signalling in macrophages infected with *B. pseudomallei* and the influence on disease outcome.

2.10.5 TLR5: The Counter Measure to Bacterial Flagella

In 2001, Hayashi *et al.* found that purified supernatants from both Gram positive and Gram negative bacteria were able to stimulate TLR5 in Chinese hamster ovary cells (CHO) and murine cells, subsequently inducing NF-κB mobilisation and the secretion of TNF-α (Hayashi *et al.*, 2001). Tandem mass spectrometry of *Listeria monocytogenes* conditioned medium identified the TLR5 ligand as flagellin, a principle component of bacterial flagella which is a known virulence factor of many bacterial species, including *B. pseudomallei* (Iyoda *et al.*, 2001; Chua *et al.*, 2003; Bouillaut *et al.*, 2005). Supernatants from cultures of *L. monocytogenes* and *S. typhimurium* lacking the flagellin genes abrogated TLR5 stimulation in both CHO and murine cells according to PCR analysis and also failed to elicit cytokine responses in controls (Hayashi *et al.*, 2001). TLR5 forms homodimers on the cell surface upon

stimulation with flagellin and activates TIR domain interactions on the cytoplasmic face to recruit and signal through the MyD88 pathway (Miao *et al.*, 2007).

Expression of TLR5 has also been observed in intestinal epithelial cells. Where, in contrast to commensal species, pathogenic *S. typhimurium* secreting flagellin across the intestinal epithelia contacting the basolateral membrane, induced pro-inflammatory gene expression (Gewirtz *et al.*, 2001). In addition, flagellin activates TLR5 in human lung epithelial cells. Hawn *et al*. 2003 demonstrated that a common stop codon polymorphism in the ligand binding domain of TLR5 abrogates its inflammatory signalling pathway and is associated with increased susceptibility to *Legionella pneumophila* (Hawn *et al.*, 2003). In contrast, a deleterious effect of TLR5 in mice orally infected with *S. typhimurium* has also been shown, suggesting that the benefits and or consequences of TLR5 activation are dependent on the pathogen (Uematsu *et al.*, 2006). Together, these findings suggest that TLR5 plays a critical role in microbial recognition on the mucosal surfaces of the body. Because of its known route of infection via inhalation, it would be of interest to ascertain if TLR5 expression in the pulmonary compartment is in some way affected by *B. pseudomallei*. In a recent study *B. pseudomallei* has been shown to activate NF-κB via TLR5 (Hii *et al.*, 2008).

2.10.6 TLR9: An Intracellular Toll-like Receptor

In general, individuals with a robust immune system show relatively low reactivity with self antigens and are thus able to distinguish autologous DNA from that of a pathogen. Various structural and sequence specific differences between the host and the pathogen have allowed cells of the immune system to evolve a recognition system that accounts for these discrepancies. This delineation has been proven experimentally, whereby both splenic B cells and dendritic cells exposed to bacterial and mammalian DNA, are only reactive to prokaryotic genomes and their products (Krieg, 1996; Lipford *et al.*, 1998). Specifically, the activation of immune cells is attributed to certain palindromic sequences containing CpG nucleotides (Kuramoto *et al.*, 1992). Structurally, bacterial and vertebrate DNA differ in their content of pyrimidine clusters, the prevalence of adenosine methylation, and the absence of CpG suppression or methylation (Krieg, 1996). Approximately 80% of the cytosine residues within mammalian CpG dinucleotides are methylated, while those in bacteria

are unmethylated (Bird, 1986). While these early investigations noted the differences in CpG DNA between bacterial and mammalian genomes, it was not until December 2000 that a suitable receptor candidate for CpG recognition emerged. Northern blot analysis of TLR9 deficient mice demonstrated that TLR9 is the principle receptor for the CpG DNA motif (Hemmi *et al.*, 2003). In the aforementioned study, TLR9 transcripts were more prevalent in splenic tissue and proliferation of TLR9 was abrogated in splenocytes and peritoneal macrophages isolated from TLR9 deficient mice in both *in vitro* and *in vivo* systems. Furthermore, pro-inflammatory cytokine (IL-6, IL-12, and TNF-α) secretion was also not detectable in macrophages and dendritic cells harvested from TLR9 deficient mice. Other investigations have reported similar results (Bauer *et al.*, 2001). In patients presenting with melioidosis elevated levels of pro-inflammatory cytokines have been observed (Suputtamongkol *et al.*, 1992). Due to its ability to persist in host phagosomes, one would expect a down regulation of TLR9 and the subsequent cytokines released via its activation in *B. pseudomallei* infected cells.

Synthetic oligodeoxynucleotides containing the unmethylated CpG motif have been found to mimic bacterial DNA in immune response stimulations (Krieg, 2002). Two structurally distinct CpG DNA clusters, termed types B/K and A/D, were identified (Verthelyi *et al.*, 2001). These synthetic CpG DNAs are phosphorothioate-modified and produce specific immunostimulatory effects (Verthelyi *et al.*, 2001). The B/K type CpG DNA is conventional, and is a potent inducer of cell proliferation, IgM production by B cells, as well as IL-6 and TNF-α secretion by monocytes and dendritic cells. The A/D type CpG DNA preferentially stimulates IFN-γ release by natural killer cells, some IFN-α production and minimal release of IL-12 (Anne Krug *et al.*, 2001; Verthelyi *et al.*, 2001). Toll-like receptor 9 has been shown to recognise both types of CpG DNA in dendritic cell subsets, with differential responses in cytokine production (IFN-α, IL-12) between splenic and bone marrow derived cells (Hemmi *et al.*, 2003). In addition to variations in cell stimulation, particular CpG DNA sequences stimulate an immune response in a species specific manner (Bauer *et al.*, 2001).

The distribution and recruitment of TLR9 prior to cellular activation via CpG DNA has been elucidated (Latz *et al.*, 2004). Some bacteria are internalised via a clathrin-

dependant endocytic pathway and localised in lysosomal compartments where the acidic and reducing conditions lead to the degradation of DNA and the exposure of CpG DNA motifs. Both TLR9 and the adaptor signalling molecule MyD88 are rapidly recruited from the endoplasmic reticulum to these sites of CpG DNA accumulation (Latz *et al.*, 2004). Bafilomycin and chloroquine, two compounds that block endosomal maturation and acidification, inhibit CpG DNA signalling via TLR9 (Hacker *et al.*, 1998).

Few studies have assessed the effects of live intracellular bacterial pathogens on macrophage TLR expression in response to invasion. Real-time PCR results from a sublethal infection study of C57BL/6 mice with *S. enterica* serovar *Typhimurium* indicated a transient increase in TLR1, TLR2, and TLR9 mRNA expression over 14 days (Totemeyer *et al.*, 2005). Up regulation of the aforementioned TLRs correlated with a plateau phase of intracellular bacterial growth and is postulated to play a role in controlling infection. Similar patterns may exist during *B. pseudomallei* infection however, due to the known intracellular persistence of the organism over time, it is reasonable to speculate that normal TLR9 expression is potentially compromised in infected hosts. Indications from septic melioidosis patients and murine models of infection have shown little to no detectable TLR9 expression in *B. pseudomallei* infected leukocytes (Wiersinga *et al.*, 2007c; Wiersinga *et al.*, 2008a). Enhancement of *B. pseudomallei* uptake by murine RAW 264.7 macrophages exposed to CpG ODN prior to infection has been observed (Utaisincharoen *et al.*, 2003b), however TLR9 expression was not assessed. The efficacy of CpG ODN motifs have been examined in protection studies against acute *B. pseudomallei* infection. Immunisation of mice two days prior to infection significantly reduced bacterial loads and mortality up to 15 days, but failed to control inflated pro-inflammatory cytokine production. In addition, the benefits of CpG ODN stimulation had no effect in mice vaccinated following bacterial challenge. The authors contend that CpG ODN may be used as a potential immunostimulatory agent for protection against acute *B. pseudomallei* infection and suppression of developing chronic infection. However, implementation of this protection strategy seems improbable, as patients would have to vaccinate themselves prior to the infectious event.

To date, it is not known how *B. pseudomallei* associates with TLR9 within the host phagolysosome. Several studies have determined that *B. pseudomallei* inhibits the production of nitric oxide synthase (iNOS) as well as NADPH within the phagolysosome, consequently inhibiting the release of reactive oxygen species and avoiding degradation (Utaisincharoen *et al.*, 2000; Utaisincharoen, 2001; Utaisincharoen *et al.*, 2003b). Ineffective bactericidal mechanisms may affect the breakdown of *B. pseudomallei* and the availability of its corresponding CpG DNA ligand to the TLR9 receptor. Effective TLR9 signalling is dependant on maturation of the phagolysosome (Parviz Ahmad-Nejad *et al.*, 2002). Intracellular persistence and evasion of TLR9 recognition may be a mechanism of *B. pseudomallei* survival intracellularly.

2.11 ADAPTOR MOLECULE RECRUITMENT IN TOLL-LIKE RECEPTOR SIGNALLING PATHWAYS

A detailed analysis of TLR directed intracellular signalling is beyond the scope of this review. In this section the highlights of TLR signalling and the associated intracellular adaptor signalling molecules are discussed and the reader should consult the literature for further information.

The recognition of microbial structures by TLRs initiates signal transduction pathways and the expression of pro-inflammatory genes (Figure 2.7). These gene products are critical for directing the innate response and further development of antigen-specific acquired immunity. Signalling through Toll-like receptor pathways are coordinated by Toll/IL-1 receptor (TIR) domain containing adaptor proteins (section 2.10.1). To date four adaptor proteins have been characterised: myeloid differentiation factor (MyD88); TIR domain-containing adaptor protein (TIRAP), also known MyD88 adaptor like (Mal); TIR domain-containing inducing interferon (TRIF), also known as TIR domain-containing adaptor molecule-1 (TICAM-1); and TIR-containing protein (TIRP), also known as TRIF-related adapter molecule (TRAM) or (TICAM-2) (Medzhitov *et al.*, 1998; Horng *et al.*, 2001; Yamamoto *et al.*, 2002; Fitzgerald *et al.*, 2003). Another mammalian ortholog of Drosophila and *C. elegans* proteins known as sterile alpha and HEAT/Armadillo motif protein (SARM) has also been characterised (O'Neill *et al.*, 2003). These adaptor molecules provide a platform for the recruitment of kinases, transcription factors, and other

adaptor molecules to form intricate signalling complexes at the cytoplasmic terminus of the TLR receptor. Various combinations of these TIR containing adaptors elicit specific PAMP/TLR directed responses.

2.11.1 MyD88 Function and Recruitment in Toll-like Receptor Signalling

Intracellular signalling mechanisms were not investigated in this thesis, therefore, a brief detail of MyD88 directed signalling during TLR4 stimulation will be discussed below as MyD88 is involved in the regulation of TLR signalling in melioidosis (Wiersinga *et al.*, 2008e). MyD88 is recruited to the IL-1R C terminal domain of all TLRs except TLR3, where it mediates downstream signalling via the recruitment of the IL-1R associated kinase (IRAK) (Wesche *et al.*, 1997). When TLR4 is bound to LPS this coordinates the association of MyD88 with Mal (Lu *et al.*, 2008). The association of MyD88/Mal and IRAK by another adaptor molecule, TRAF6, initiates the dissociation of the complex. Once dissociation occurs the IκB kinase complex is subsequently engaged which leads to the activation of NF-κB and the transcription of inflammatory genes (Verstrepen *et al.*, 2008).

Two distinct signalling pathways have been described following TLR4 activation. A MyD88 dependant and a MyD88 independent pathway (Feng *et al.*, 2003; Oshiumi *et al.*, 2003). PAMP directed stimulation of MyD88 dependant pathways results in rapid activation of NF-κB and release of several pro-inflammatory cytokines (Feng *et al.*, 2003). In contrast, MyD88 independent signalling results in the delayed activation of NF-κB, rapid activation of interferon regulatory factor 3 (IRF3), and the subsequent release of type I interferons (IFN-α/ß) (Kawai and Akira, 2006). IFN-ß binds to the IFN-α/ß receptor, which leads to the release of IFN-α inducible protein (IP-10). IP-10 plays a central role in the maturation of dendritic cells thus providing a critical link between the innate and adaptive immune systems

Numerous studies have investigated the role of accessory extracellular signalling proteins in TLR4/LPS recognition and signalling (Bosisio *et al.*, 2002a; O'Neill, 2006). Alterations in the association of these proteins with the TLR receptor at the N terminus can modulate intracellular signalling protein associations and vary cellular responses. Vogel and colleagues contend that specific gene signalling profiles in the host are directed by the unique association of adaptor molecules directed by

TLR/PAMP associations (Vogel *et al.*, 2003). The diversity and relative flexibility of the TLR pathway permits recognition of various types of pathogens. This is an effective PAMP directed response that is capable of neutralising and eradicating a potentially infectious agent. Modification of Toll-like receptor signalling pathways could illicit production of signalling events that alter host function. Analysis of pro-inflammatory cytokine secretion and TLR mediated responses during *B. pseudomallei* infection are still limited. Data regarding this phenomenon would help determine characteristic cytokine profiles in *B. pseudomallei* infected patients and the resultant immune response directed by the TLR family of receptors.

2.12 NF-KB: TARGET OF TLR MEDIATED SIGNALLING

Nuclear factor kappa B is a pivotal transcription factor responsible for the expression of multiple inflammatory genes including IL-1, IL-6, IL-8, IL-12, and TNF-α (Baldwin, 1996). Stimulation of TLRs and the subsequent activation of the MyD88 dependant pathway culminates in translocation of NF-κB to the nucleus to begin transcription. The NF-κB family of transcription factors is involved in a diverse array of immunological responses (Vallabhapurapu and Karin, 2009). Five different NF-κB proteins have been identified, and these proteins form a mixture of homo- and hetero-dimer complexes that associate with other accessory proteins to control gene transcription. Activation of these various NF-κB dimers is facilitated through a complex process of phosphorylation and ubiquitination. Prior to activation, NF-κB core proteins are bound by I-kappa-B or NF-κB inhibitor proteins which are relegated to the cytoplasm. Following TLR stimulation, I-kappa-B is phosphorylated, then degraded through ubiquination, leading to the release of active NF-κB (Vallabhapurapu and Karin, 2009). Upon release from inhibitor proteins NF-κB translocates to the nucleus to begin transcription of target inflammatory genes such as IL-1β, IL-6, IL-12 and TNF-α (Latimer *et al.*, 1998). Several recent studies have demonstrated *B. pseudomallei* induction of NF-κB by TLR2, TLR4, and TLR5 in transfected HEK293 cells (Wiersinga *et al.*, 2007c; Hii *et al.*, 2008; West *et al.*, 2008a; Novem *et al.*, 2009). The recently identified virulence factor TssM that is expressed in *B. pseudomallei* was shown to inhibit NF-κB activation by deubiquination of critical signalling intermediates including TRAF-6, TRAF-3 and IκBα (Tan *et al.*, 2010).

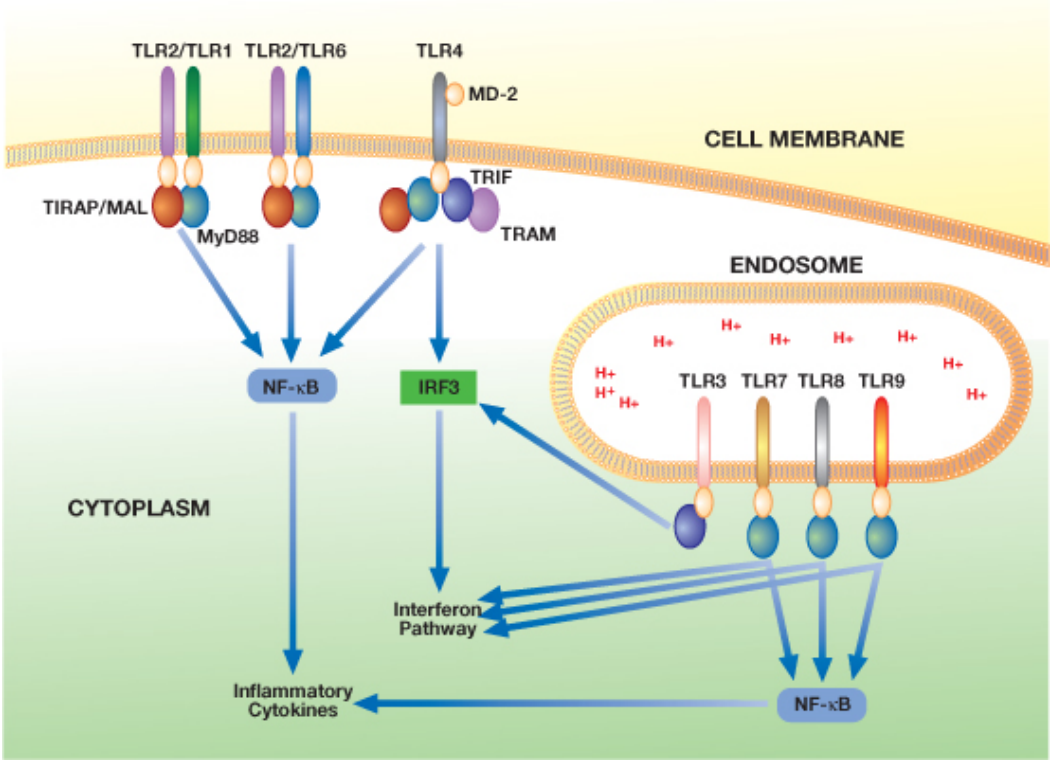

Figure 2.7 Signalling cascades of TLR2 and TLR4
Binding of TLR1/TLR2, TLR2/TLR6 and TLR4 receptor elicits a cascade of signalling events that leads to NF-κB release and the production of pro-inflammatory cytokines (MacKichan *et al*. 2005).

2.13 TOLL-LIKE RECEPTORS AND *BURKHOLDERIA PSEUDOMALLEI*

Studies regarding the interaction of *B. pseudomallei* with the Toll-like receptor family did not surface until mid 2007. Since then, a substantial body of work has been published using various *in vitro* and *in vivo* models of infection, yet the role of TLRs during *B. pseudomallei* infection remains unresolved.

Certainly the TLR family plays a significant role in innate immune signalling during melioidosis as MyD88 signalling provides a protective effect during *B. pseudomallei* infection (Wiersinga *et al.*, 2008e). However, the recognition of *B. pseudomallei* by TLR2 and TLR4 are under intense study. Currently in the literature there are reports that demonstrate that TLR2 is the primary receptor for *B. pseudomallei* recognition and the closely related species *B. thailandensis* (Wiersinga *et al.*, 2007c; Hii *et al.*, 2008; West *et al.*, 2008a; West *et al.*, 2009; Morici *et al.*). The first large scale analysis of TLR transcription in septic melioidosis patients found a significant increase in multiple TLRs including TLR1, TLR2, TLR4 and TLR5 in peripheral

blood leukocytes and granulocytes using multiplex probe amplification (Wiersinga *et al.*, 2007c). *In vitro* infection assays included in the same study indicate that *B. pseudomallei* is capable of activating TLR2 and TLR4. Other studies have shown TLR2 and TLR4 activation in similar *in vitro* assays using *B. pseudomallei* and the closely related organism *B. thailandensis*. In transfected HEK293 cells, *B. thailandensis* was shown to mediate NF-κB activation via both TLR2 and TLR4 in a dose dependant manner (West *et al.*, 2009). Similar findings for *B. pseudomallei* were also reported, where both TLR2 and TLR4 mediated NF-κB activation in similar co-reporter assays (Hii *et al.*, 2008; West *et al.*, 2008a). Preparations of heat killed *B. pseudomallei* stimulated TLR2 and TLR4, however purified lipid A and LPS signalled in a TLR4 dependent manner (West *et al.*, 2008a).

However, Wiersinga and colleagues contend that only TLR2 contributes to the host response *in vivo*, as TLR2 and not TLR4 is deleterious to the host response in survival studies using TLR knockout mice (Wiersinga *et al.*, 2007c). Similar findings were reported in a separate *in vivo* study assessing TLR4 involvement of the closely related organism *B. thailandensis* using a panel of mouse strains (Morici *et al.*, 2010). Interestingly, the detrimental effects of CD14 during murine melioidosis have demonstrated in knockout mice, which shared a similar outcome to mice with the TLR2 deficient phenotype. CD14 is capable of presenting bacterial components to TLR2 in addition to TLR4 however, this finding further complicates the issue of TLR recognition of *B. pseudomallei* by the host (Maria *et al.*, 2005; Akira *et al.*, 2006).

A recent study have been demonstrated a primary role for TLR4 recognition during the host response to *B. pseudomallei*. In a study comparing the structural and antigenic diversity of *B. pseudomallei* and *B. thailandensis* LPS, it was found that *B. pseudomallei* LPS stimulated less TNF-α, IL-6 and IL-10 production in both human THP-1 and murine RAW 264.7 macrophage cell lines in comparison to *B. thailandensis* LPS (Novem *et al.*, 2009). In addition, Novem and colleagues evaluated TLR receptor specificity from *B. pseudomallei* and *B. thailandensis* LPS preparations using NF-κB reporter assays for TLR2 and TLR4 in transfected HEK293 cells. In contrast to results reported by Wiersinga e*t al.* 2007, they found *B. pseudomallei* and *B. thailandensis* LPS activated NF-κB exclusively via TLR4. The observed variations in immunological activity between the two species were

thought to be attributed to their unique lipid A moieties. Another study using the same *B. pseudomallei* isolate found that live bacterial stimulations were capable of activating NF-κB in cells transfected with TLR2, TLR4 and TLR5 (Hii *et al.*, 2008). Given the disparity in the literature further investigations regarding TLR interactions with *B. pseudomallei* are necessary to help further understand pathogenesis and the development of a protective immune response.

2.14 NOD-LIKE RECEPTORS: CYTOSOLIC DETECTION OF PAMPs

Toll-like receptors are not the only pattern recognition receptor family responsible for detection of microbial motifs in host cells. The NOD-like receptor (NLR) family are cytosolic receptors that are capable of inducing signal transduction pathways that shape protective responses in the presence of PAMPs and intracellular pathogens. There are 23 NOD-like receptors in the human genome which are divided into subfamilies based on their N-terminal signalling domains (Takeuchi and Akira). Each NLR receptor is comprised of three domains: a C-terminal leucine rich repeat domain involved in the recognition of PAMPs and endogenous molecules, a central NACHT domain which is induced by ligand binding to activate downstream signalling, and a N terminal domain which has multiple functions including interactions with downstream signalling proteins(Fritz *et al.*, 2006; Carneiro *et al.*, 2008). Following activation NLR proteins can induce several signalling cascades involving NF-κB, MAP kinases, caspase-1 inflammasome pathways, cells death, and the activation of authophagy (Fritz *et al.*, 2006; Suzuki *et al.*, 2007; Carneiro *et al.*, 2008).

In the past few years specific PAMPs and pathogens detected by NLR subsets have been investigated. Only a few NLRs will be highlighted below as this thesis primarily focuses on the TLR family of receptors. The NOD1 receptor (also known as CARD4) recognises bacterial peptidoglycan, a major component of the cell wall, and has been shown to recognise multiple Gram-negative intracellular pathogens including *E. coli*, *L. monocytogenes*, *Campylobacter jejuni* and *Salmonella* species (Fritz *et al.*, 2006; Opitz *et al.*, 2006). The NOD2 (also known as CARD15) receptor also recognises bacterial peptidoglycan as well as providing defense against pathogenic protozoal parasites, such as *Toxoplasma gondii* (Shaw *et al.*, 2009). Recognition of PAMPs by NOD1 and NOD2 inititates oligomerization of these proteins and the recruitment of

the adaptor protein RIP2 which culminates in the activation of NF-κB, MAP kinases and the transcription of pro-inflammatory cytokines (Barnich *et al.*, 2005; Kufer *et al.*, 2008).

Few studies have been conducted assessing NLR involvement during *B. pseudomallei* infection. Hii and colleagues (2008) found that induction of IL-8 by HEK293 cells required entry of *B. pseudomallei* into the host cell, and that B. pseudomallei was capable of inducing NF-κB independently of TLR activation. Based on this observation they believed that NLR receptors may be likely candidates for *B. pseudomallei* recognition. NOD1 and NOD2 made no contribution to the production of IL-8 induced by entry of *B. pseudomallei* in HEK293 cells according to siRNA transfection and knockdown analysis (Hii *et al.*, 2008). In the same study MAPK inhibitors reduced IL-8 induction and *B. pseudomallei* entry indicating involvement of the MAPK pathway. Independent TLR induction of IL-8 in this system required a T3SS. These observations indicate a role for the inflammasome during *B. pseudomallei* infection which is a multiplex protein complex comprised of NLRs and others that lead to the activation of the caspase-1 pathway and the production of pro-inflammatory cytokines including IL-1β, IL-18 and IL-33 (Yu and Finlay, 2008).

2.15 CYTOKINE RESPONSES IN *BURKHOLDERIA PSEUDOMALLEI* INFECTION

Cytokines play a pivotal role in the induction of almost every aspect of the inflammatory response. The complexity of the cytokine network is vast and the resultant orchestrations of their effects are not ubiquitous and vary from pathogen to pathogen. Despite their importance for the host, there are instances when the production of cytokines is detrimental.

Systemic shock is initiated by the massive production of pro-inflammatory cytokines which culminates in impaired perfusion and multiple organ failure. One of the primary cytokines responsible is TNF-α, an inflammatory mediator that is released from LPS stimulated macrophages (Rice and Bernard, 2005). Tumor necrosis factor-α plays an important role in controlling intracellular pathogens, as innate immune responses are thought to contribute to resistance in murine models of

B. pseudomallei and *Listeria spp*. infection (Ulett *et al.*, 2000c; Grivennikov *et al.*, 2005; Barnes and Ketheesan, 2007). However, hyperproduction is harmful to the host. Clinical studies have indicated a correlation between septicaemic melioidosis patients and high levels of TNF-α. Severity of disease and mortality are associated with high TNF-α serum levels during acute *B. pseudomallei* infection (Suputtamongkol *et al.*, 1992). The importance of several chemokines and cytokines have been described in murine models of melioidosis (Lauw *et al.*, 1999; Ulett *et al.*, 2000b; Barnes *et al.*, 2001a; Tan *et al.*, 2008; Wiersinga *et al.*, 2008a). Differential cytokine profiles are produced in mouse strains with different susceptibilities to *B. pseudomallei* (Ulett *et al.*, 2000b; Tan *et al.*, 2008). In susceptible BALB/c mice a marked increase in IFN-γ, IL-1β and TNF-α were observed in moribound animals (Ulett *et al.*, 2000b). In a similar study BALB/c mice were ten to one hundred fold more susceptible to aerosol *B. pseudomallei* challenge in comparison to C57BL/6 mice, which was accompanied by increases in IFN-γ, IL-6, KC, MIG, and MIP-1β production in systemic and pulmonary compartments (Tan *et al.*, 2008). Increases in transcriptional profiles and total protein of several inflammatory mediators were assessed in the pulmonary compartment of C57BL/6 mice, where increases were also noted (Wiersinga *et al.*, 2008a). Disparate transcription profiles for several chemokines and cytokines have been observed between the C57BL/6 and BALB/c models, where these differences are associated with bacterial growth and cellular infiltrate composition (Ulett *et al.*, 2000c; Barnes *et al.*, 2001a). These findings have been further expanded upon regarding Toll-like receptor involvement in these two hosts in Chapter 6.

2.16 CONCLUSIONS

Melioidosis is considered an emerging infection, and its etiological agent *Burkholderia pseudomallei*, is listed as a category B bioterrorism agent by the Centers for Disease Control and Prevention (www.cdc.gov/nczved). The bacterium is highly resistant to antimicrobial therapy and research into the development of novel medical interventions including new antimicrobials, vaccines and immunomodulator targets are a strong area of interest. Understanding the pathogenesis of *B. pseudomallei*, its persistence in the host, and the subsequent inflammatory responses elicited by the organism will help identify new targets for therapy.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 BACTERIAL ISOLATES

3.1.1 Origin of *Burkholderia pseudomallei* strains

All clinical *B. pseudomallei* isolates were obtained from the Townsville Culture Collection located at Townsville General Hospital, Queensland. The identity of the isolates was determined by colonial morphology on Ashdown agar and API 20NE (bioMerieux, La Balme, France). Bacteria were grown in brain heart infusion broth (Oxford, Hampshire, England) at 37°C for 18 h and stored at -80°C in 40% glycerol until use. Detailed descriptions of isolates in relation to patient age, sex, disease presentation, and LD_{50} values in BALB/c mice for isolates 1-26 (Ulett *et al.* 2001).

3.1.2 Bacterial Isolate Preparations

All clinical isolates were aliquoted in 30 µl volumes onto 5% sheep blood agar and incubated for 24 h at 37°C. One to three colonies were selected and inoculated into Trytone soy broth at incubated for 18 h at 37°C. Three ml of inoculum was added to 5 ml of fresh TSB and incubated for a further 3 h with shaking (100 rpm) to bring bacteria into log phase proliferation. Bacteria were centrifuged (500 × g, 10 min) and the supernatant was discarded. The bacterial pellet was resuspended in 10 ml of PBS (pH 7.2, Appendix I) and centrifuged (500 × g, 10 min). The wash step was repeated three times. Bacterial suspensions were made up to 0.15 McFarland at an OD reading of 650nm, corresponding to ~ 1 x 10^8 cells per ml. One serial dilution was made in PBS and cells were challenged with 1 x 10^7 cfu/ml at an MOI of 10:1.

3.2 ANIMAL ETHICS APPROVAL

Experiments requiring the use of laboratory animals were carried out under Ethics Approval number A1069 approved by the JCU ethics subcommittee.

Table 3.1 Origin of clinical *B. pseudomallei* isolates and LD_{50} values in BALB/c mice. Data adapted from Ulett *et al.* 2001.

Isolate	Age	Sex	Presentation	Risk Factors	LD_{50} (cfu)*
CL01	46	m	unknown	unknown	1.42×10^6
CL02	66	m	PN	CST, ALC	1.26×10^6
CL03	30	m	SEP, PN	DBT	7.49×10^5
CL04	49	m	SEP	NR	6.67×10^4
CL05	45	m	PN	ALC, CST	1.96×10^4
CL06	26	m	SEP, RTI	CST, RF, IMS	1.68×10^4
CL07*** (NCTC 13179)	54	m	SEP, PN	DBT, ALC	1.52×10^4
CL08	63	m	LU, prophyria	DBT	1.42×10^4
CL09	55	m	SEP, PN	ALC, RF	1.26×10^4
CL10	28	m	SEP, PN, UTI	RF, IMS, CST, DBT	1.11×10^4
CL11	59	f	PN	ALC	7.33×10^3
CL12	57	m	Leg abscess	SLE, RF, CST, DBT	3.15×10^3
CL13	44	m	SEP, RTI	IMS, DBT	2.53×10^3
CL14	76	f	PN	NR	2.32×10^3
CL15	70	f	SEP, PN, LU	DBT	2.28×10^3
CL16	39	f	SEP, PN	DBT	2.00×10^3
CL17	31	m	SEP, PN	NR	1.36×10^3
CL18	59	m	PN	NR	1.00×10^3
CL19	31	m	PRA, UTI	NR	3.76×10^2
CL20	59	m	PN, lung lesion	IMS, CST	2.72×10^2
CL21	23	m	SEP, LA	NR	1.26×10^2
CL22	38	m	SEP, PN	DBT, ALC	4.00×10^1
CL23	52	f	PN	DBT, ALC	1.50×10^1
CL24*** (NCTC 13178)	6	m	BRSE	NR	1.00×10^1
CL25	unknown		SEP	NR	4.00×10^0
CL26	49	m	PN, MM	NR	3×10^0

***Clinical isolates (CL07 and CL24) are also known as NCTC 13179 and NCTC 13178 are the two prominent isolates investigated in this thesis. LD_{50} values were determined by *i.v* dose in BALB/c mice (Ulett *et al.*, 2001a).

ALC, alcoholism; BRSE, brain stem encephalitis; CST, corticosteroids; DBT, diabetic; IMS, immunosuppressed; LA, lumbar abcess; LU, leg ulcer, MM, mediastinal mass; NR, none recognised; PN, pneumonia; RF, renal failure; RTI, respiratory tract infection; SEP, septicaemia; SLE, systemic lupus erythematosis.

CHAPTER 4
THE EFFECT OF DIFFERENT *BURKHOLDERIA PSEUDOMALLEI* ISOLATES OF VARYING LEVELS OF VIRULENCE ON TOLL-LIKE RECEPTOR EXPRESSION *IN VITRO*

4.1 INTRODUCTION

Burkholderia pseudomallei is an intracellular pathogen that is able to persist and replicate within both phagocytic and non phagocytic cells (Jones *et al.*, 1996). Invasion into host cells is facilitated by a number of virulence factors including a type III secretion system (Stevens *et al.*, 2002b). One of the mechanisms activated macrophages use to eliminate intracellular bacteria such as *B. pseudomallei* is through the production of toxic free radicals. Reactive nitrogen intermediates (RNI) released from macrophages are important for controlling intracellular *B. pseudomallei* (Miyagi *et al.*, 1997; Barnes *et al.*, 2008a; Barnes *et al.*, 2008b). Following internalisation the organism can escape from endocytic vesicles, in addition to slowing the rate of phagosome-lysosome fusion thereby negating the production of harmful RNI species (Jones *et al.*, 1996; Puthucheary *et al.*, 2006). Evasion of host lytic mechanisms is thought to contribute to the intracellular survival of the organism.

While several investigations elucidating the intrinsic virulence factors of *B. pseudomallei* have been conducted, little is known about host cell interactions with the pathogen at the cell surface. Activation of the innate immune response by invading pathogens occurs via recognition of conserved microbial structures known as pathogen associated molecular patterns (PAMPs) (Medzhitov *et al.*, 1997). Recognition of PAMPs is facilitated by the Toll-like receptor (TLR) family. Stimulation initiates recruitment of adaptor molecules that facilitate downstream signalling events (O'Neill, 2006). Toll-like receptor 2 possesses the broadest range of specificity and has been implicated in recognition and signal transduction induced by lipoproteins, lipopeptides, and peptidoglycan in Gram-positive and Gram-negative bacteria, as well as LPS in certain bacterial species(Brightbill *et al.*, 1999; Hirschfeld

et al., 2001; Akamine *et al.*, 2005). Toll-like receptor 4, in conjunction with CD14 and MD-2 comprise the primary receptor complex for LPS and is the major contributor to Gram-negative bacterial recognition (Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998; Takeuchi *et al.*, 1999). Toll-like receptor 5 and TLR9 recognise bacterial flagellin and bacterial nucleic acids (CpG DNA), respectively (Hayashi *et al.*, 2001; Hemmi *et al.*, 2003).

Townsville, which is located in northern Queensland, Australia, is a region where several cases of melioidosis occur each year. As a result, several clinical isolates have been cultured from patients presenting with the disease. Several of these isolates (n = 26) have been assessed for virulence in our laboratory using a murine model of melioidosis and corresponding LD_{50} values have been determined (Ulett *et al.*, 2001b). It was our intention to assess TLR interactions with these isolates using *in vitro* models of infection. The work described in this chapter was carried out to assess early host pathogen interactions by assessing the cytotoxic activity and the degree which specific TLRs are expressed in macrophages exposed to *B. pseudomallei* isolates with varying levels of virulence.

The specific aims of the work described in this chapter were:

1) To determine the phagocytic and cytotoxic capabilities of a murine macrophage cell line and a primary murine macrophage cell line exposed to a panel of clinical *B. pseudomallei* isolates of different virulence using internalisation assays and Griess assays as measures of cytotoxic activity.

2) Measure pro-inflammatory cytokine secretion (TNF-α) in a murine macrophage cell line and a primary murine macrophage cell line exposed to a panel of clinical *B. pseudomallei* isolates of different virulence using ELISA.

3) To determine the expression of multiple TLRs (TLR2, TLR4, TLR5, TLR9) in a murine macrophage cell line and a primary murine macrophage cell line following *in vitro* exposure to a panel of clinical *B. pseudomallei* isolates using reverse transcriptase PCR.

The significance of these findings will help determine macrophage activation by assessing the effects on macrophage TLR transcription and inflammatory responses. This is the first large scale analysis of TLR transcription in the presence of *B. pseudomallei* and will provide a framework to understand which TLRs are integral to innate immune signalling during *B. pseudomallei* infection.

4.2 MATERIALS AND METHODS

4.2.1 Origin of *Bukholderia pseudomallei* Strains

All clinical *B. pseudomallei* isolates were obtained from the Townsville Culture Collection and are listed in Table 4.2. Identification of the isolates have been previously determined by colonial morphology on Ashdown agar and API 20NE (Ulett *et al.*, 2001a). Bacteria were grown in brain heart infusion broth (Oxoid, SA, Australia) at 37°C for 18 h and stored at -80°C until use. The LD_{50} values on these isolates were determined in BALB/c mice according to i.v. inoculation and not other routes of infection. *Escherichia coli* ACTC 25922, *Salmonella typhimurium* ACTC 14028 strains, and purified *Pseudomonas aeruginosa* LPS (Sigma Aldrich, Castle Hill, New South Wales, Australia) were used as positive controls.

4.2.2 Bacterial Isolate Preparations

Bacterial isolates, *B. pseudomallei* and controls, were cultured on 5% sheep blood agar (Oxoid, SA, Australia) and selected colonies were inoculated into Tryptone soya broth (Oxoid, SA, Australia) and incubated for 18 h at 37°C. Bacteria were brought to log phase and centrifuged (500 × g, 10 min). Log phase correlates with exponential proliferation and activation of metabolic machinery, and is utilised in this experiment to mimic the rapid proliferation of *B. pseudomallei in vivo*. The bacterial pellet was washed in PBS (pH 7.2, Appendix I) and adjusted to 1 x 10^8 cells per ml. Cell cultures were infected with 1 x 10^7 cfu/ml at a multiplicity of infection (MOI) of 10:1. Previous experiments in our laboratory assessing MOI ratios (MOI 1:1, MOI 10:1, MOI 50:1) during internalisation assays determined an MOI 10:1 ratio as optimal.

4.2.3 Cell Line and Culture Conditions

RAW 264.7 macrophages are of BALB/c lineage and were obtained from the American Type Culture Collection (ATCC; MD, USA). This cell line was selected as BALB/c mice are highly susceptible to *B. pseudomallei* infection (Leakey *et al.*, 1998) and these cells provide an appropriate model to study acute infection *in vitro*. Cells were cultured in RPMI-1640 (Invitrogen, Victoria, Australia) supplemented with 10% fetal bovine serum (FBS), with 20mM of L-glutamine at 37°C with 5% CO_2.

Infection studies in RAW 264.7 macrophages were initially carried out to assess TLR transcription in an established cell line. We intended to further assess TLR activation in *ex vivo* studies using primary cell culture, and selected elicited peritoneal exudate cells (PEC) derived from BALB/c mice as our primary cell culture source for these experiments. PECs were harvested from BALB/c mice (n=3) and pooled, three days after *i.p* injections of 2.5 ml of Brewers thioglycollate medium (Becton, Dickinson and Company, New South Wales, Australia). PECs were then incubated overnight under the same culture conditions as described above. Thioglycollate was used to activate peritoneal macrophages (Baker *et al*. 1980). Cells were seeded at 1×10^6 cells/ml into 24 well plates (Thermo Fischer Scientific, Victoria, Australia) and incubated overnight at 37°C with 5% CO_2. Cell monolayers were washed twice with PBS (pH 7.2) (Appendix I) to remove nonadherent cells prior to protection assays. All animal related procedures were carried out under Ethics Approval number A1069, provided by the James Cook University Animal Ethics Sub-Committee.

4.2.4 Determination of Internalisation and Killing of *B. pseudomallei* by RAW 264.7 Macrophages

Standard antibiotic protection assays were used to determine *B. pseudomallei* internalisation and killing by RAW 264.7 macrophages according to methods previously described using a 2 h infection period (Utaisincharoen, 2001). The numbers of bacteria internalised and killed by macrophages were assessed following 1 h and 2 h incubation periods. RAW 264.7 macrophages were infected with different

B. pseudomallei isolates (1-26) at a MOI of 10:1. BALB/c derived PEC were infected with either NCTC 13178 or NCTC 13179 at a MOI of 10:1. Internalisation and killing of bacteria following 1 h incubation was determined following lysis of macrophages with 0.1 % Triton X (BDH Chemicals, Kilsyth, VIC, Australia). Serial dilutions of cell lysates were carried out using PBS (pH 7.2) and bacterial colony forming units (cfu) were determined on Ashdown agar (Appendix I).

A second series of internalisation assays were run in parallel to determine the number of viable bacteria following 2 h incubation. One hour following co-culture, cells were washed three times with PBS (Appendix I) to remove extracellular bacteria. One ml of culture medium with 250 μg/ml kanamycin (Sigma, Sydney, Australia) was added to wells. The optimal concentration of kanamycin used to eliminate extracellular *B. pseudomallei* from culture supernatant was determined prior to experimental trials using 250 μg/ml, 500 μg/ml and 650 μg/ml. After 1 h of further incubation cells were lysed and cfu were determined as described above. All internalisation experiments were carried out in triplicate.

Percent uptake, percent killing and killing efficiency were calculated from bacterial cfu from 1 h and 2 h lysates. Bacterial counts from 1 h lysate groups were determined as the number of bacteria internalised by RAW 264.7 macrophages. The difference in cfu between 1 h and 2 h lysates was reported as the number of bacteria eliminated by macrophages as determined by the following equations:

$$\% \text{ Uptake} = \frac{\text{No. of bacteria in 1h lysate}}{\text{inoculating dose}} \times 100$$

$$\% \text{ Killing} = \frac{\text{No. of bacteria in 1h lysate} - \text{No. of bacteria in 2h lysate}}{\text{No. of bacteria in 1 h lysate}} \times 100$$

$$\text{Killing Efficiency} = \frac{\text{No. of bacteria in 1 h lysate} - \text{No. of bacteria in 2 h lysate}}{\text{No. macrophage per well} = (10^6)}$$

4.2.5 Determination of Internalisation and Killing of *B. pseudomallei* by BALB/c PECs

In order to determine internalisation and killing of *B. pseudomallei* in primary cell culture, standard antibiotic protection assays using BALB/c PECs were carried out as outlined in section 4.2.4. Percent uptake, percent killing and killing efficiency were calculated.

4.2.6 Primer Optimisation

Forward and reverse primers for TLR2, TLR4, TLR5, TLR9 and β-actin for use in RT PCR trials were designed using Oligo Primer Analysis Software version 6.0 (Wojcieh Rychlik, CO, USA). Primers were designed to span intron junctions to eliminate amplification of potential DNA contaminants. Prior to experimental trials, RT PCR reactions were carried out on control cDNA constructs to test efficiency of TLR2, TLR4, TLR5, TLR9 and β-actin primers to amplify target genes. Annealing temperatures for TLR and β-actin primers were recommended at 60°C according to primer specifications provided by the manufacturer (Sigma Aldrich, New South Wales, Australia). Initial trials using RT PCR cycling conditions with an annealing temperature of 60°C were unsuccessful for TLR2, TLR4 and TLR5 primers (data not shown). As a result, RT PCR reactions for the aforementioned primers were run in subsequent trials using a temperature gradient to ascertain the optimal annealing temperature for TLR2, TLR4 and TLR5 primer sets. Isolation of cDNA and cycling parameters for RT PCR reactions are outlined in section 4.2.10. Agarose gels were used to visualise RT PCR gene products. An example of temperature gradient analysis for optimisation of TLR2 forward and reverse primers is provided in Figure 4.1. Examples for TLR4 and TLR5 are provided in Appendix II. Experimental trials were carried out following optimisation. Primer sequences and annealing temperatures are provided in Table 4.1.

Table 4.1 Primer sequences used for amplification of TLRs in RAW 264.7 macrophages and BALB/c derived PEC cells

Primer	Primer Sequence	Tm (°C)	Product Size (bp)
TLR2 (for)	ATCAGTCCCAAAGTCTAA	54	787
TLR2 (rev)	TCCAACACCTCCAGCGTCT		
TLR4 (for)	ATTGTATCGCCTTCTTAGCAG	62	141
TLR4 (rev)	GGTCCAAGTTGCCGTTTCT		
TLR5 (for)	AACGTCACCCTGTTCGGCTCTC	62	523
TLR5 (rev)	CGGCTCTGGGCATACCTGA		
TLR9 (for)	TCTTCCGCTCGCTCAACA	60	356
TLR9 (rev)	ACACTGGAGGCGTGAGAGATTGAC		
β-actin (for)	TCATGAAGTGTGACGTTGACATCCGT	60	234
β-actin (rev)	CCTAGAAGCATTTGCGGTGCACGATG		

4.2.7 Cloning of PCR Products

In order to ensure target TLR gene products generated during optimisation trials using the primers outlined in Table 4.1 were of the correct sequence, a general cloning strategy was carried out. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, New South Wales, Australia) according to the manufacturer's instructions. Cloning of TLR2, TLR4, TLR5, TLR9 and β-actin PCR products were carried out using the pGEM®-T Easy Vector System (Promega, New South Wales, Australia). Briefly, PCR product ligations were carried out in 0.5 ml PCR tubes using 5 µl of 2 x Rapid Ligation Buffer, 1 µl of pGEM-T Vector (50 ng), 3 µl of purified PCR product (TLR2, TLR4, TLR5, TLR9, β-actin) and 1 µl of T4 DNA Ligase (3 Weiss units/ul) were added for a total reaction volume of 10 µl. Ligation reactions were mixed by pippetting and incubated for 3 h at 12°C.

JM109 competent cells (Fermentas, New South Wales, Australia) were removed from -80°C storage and placed into an ice bath until just thawed. JM109 cells were mixed by gentle flicking of the tube. Competent cells (50 µl) were transferred into 1.5 ml tubes containing 3 µl of ligation reactions and were mixed by flicking the tube. Control reactions were placed into another tube with 0.1 ng uncut plasmid to determine transformation efficiency of JM109 competent cells. Cell and ligation reaction mixtures were placed on ice for 20 min. Cell and ligation mixtures were then heat shocked for 45-50 seconds in a water bath at 42°C. Reactions were then

immediately placed on ice for 2 min. SOC medium (Appendix I) (950 µl) at room temperature were then added to tubes containing JM109 cells transformed with ligation reactions. Transformation cultures (100 µl) were plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. Culture plates were incubated overnight at 37°C for 24 h. Plates were then placed in 4°C for 2 h to facilitate blue color development of non transformed colonies. Two to three white colonies from LB/ampicillin/IPTG/X-Gal plates containing TLR or cytokine inserts were inoculated into 5 ml LB broth (Appendix I) and incubated at 37°C for 24 h.

4.2.8 Isolation of Plasmids Containing TLR Inserts

High copy plasmid DNA containing TLR inserts was purified using the QIAprep Spin Miniprep Kit (Qiagen, New South, Wales, Australia) according to the manufacturer's instructions. Briefly, 3 ml of overnight TLR transformant culture from section 4.2.6 was centrifuged at 8,000 × g for 5 min. The bacterial pellet was resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. A volume of 250µl of Buffer P2 was then added and the tube was mixed by gentle inversion. A volume of 350 µl of Buffer N3 was added and then mixed by gentle inversion. The mixture was then centrifuged at 18,000 × g for 10 min. Supernatants were then pipetted into QIAprep Spin Columns and centrifuged for 8,000 × g for 1 min. The flow through was then discarded. QIAprep Spin Columns were then washed with 0.5 ml Buffer PB and centrifuged at 8,000 × g for 1 min. The flow through was then discarded. Columns were washed again by adding 0.75 ml Buffer PE and centrifuged at 8,000 × g for 1 min. The flow through was again discarded and an additional centrifuge step at 8,000 × g for 1 min was carried out. The QIAspin column was then placed in a clean 1.5 ml microcentrifuge tube. A volume of 50 µl of Buffer EB was added to the column and allowed to stand for 1 min prior to a final centrifuge at 8,000 × g for 1 min.

4.2.9 Restriction Digest of TLR Plasmid and Insert

Restriction enzyme digests were carried out on eluted DNA samples using the FastDigest SalI Kit (Fermentas, New South Wales, Austalia) according to the manufacturer's instructions. Briefly, restriction digests were carried out in a 30 µl

total volume containing 17 µl nuclease-free water, 2 µl 10 × FastDigest Buffer, 10 µl (~0.2 µg) purified PCR product, and 1 µl FastDigest enzyme. Reactions were incubated at 37°C in a heat block for 60 min. Termination of reactions was then carried out for 10 min at 65°C. Sequencing of TLR inserts for verification of correct gene targets was outsourced and completed by Macrogen Corporation (Tokyo, Japan).

4.2.10 Sequencing of TLR2, TLR4, TLR5, TLR9 and β-actin Products Using M13/pUC Primers

Sequencing primers are single oligonucleotides with 5'-hydroxyl and 3'-hydroxyl ends. The M13/pUC sequencing primers anneal to the region in the 5'-terminus of the *lacZ* gene and were used to sequence plasmids containing TLR inserts. M13/pUC forward primer 5'-GTAAAACGACGGCCAGT-3' (S0100), M13/pUC reverse primer 5'-CAGGAAACAGCTATGAC-3' (S0101; Fermentas, New South Wales, Australia). A single white colony was selected from overnight LB/ampicillin/IPTG/X-Gal plates inoculated with transformed JM109 cells from section 4.2.6. The colony was resuspended in 20 µl of the following PCR reaction: 0.6 µl M13 forward primer (10 µM), 0.6 µl M13 reverse primer (10 µM), 2 µl 10x Taq Buffer, 1.2 µl $MgCl_2$ (25mM), 2 µl dNTPs (2mM), 0.1 µl Taq polymerase (0.5 units/ µl), nuclease free water up to 20 µl. A standard PCR reaction was carried out for 30 cycles under the following conditions: 94°C for 3 min, 94°C for 30 sec, 45°C for 30 sec, 72°C for 1 min, held at 15°C. PCR products were visualised on agarose gels to verify TLR gene inserts into pGEM®-T Easy vectors (Appendix III).

4.2.11 Reverse Transcriptase Polymerase Chain Reaction

RAW 264.7 macrophages were infected with *B. pseudomallei* isolates (1-26), and BALB/c PEC were infected with either NCTC 13178 or NCTC 13179 according to standard antibiotic protection assay protocols (section 4.4.5). Following 2 h incubation, cells were lysed using the QIAshredder column (Qiagen, New South Wales, Australia) as this technique generated higher yields of RNA (Figure 4.2). Following cell lysis, mRNA was isolated from duplicate sets of treatment groups

using the Qiagen RNeasy Mini Kit (Qiagen, New South Wales, Australia) according to the manufacturer's instructions. cDNA was constructed from extracted mRNA (100 ng) using the Superscript III First-Strand Synthesis System (Invitrogen, New South Wales, Australia). PCR reactions were setup as follows: a master mix (ten reactions/tube) consisting of: 168.5 ul H_20, 25 µl 1x Reaction Buffer, 25 µl $MgCl_2$ (2.5mM), 5 µl dNTPs (200uMea), 7.5 µl forward primer (300nM), 7.5 µl reverse primer (300nM), 1.5 µl Taq polymerase (0.03 units/ µl). Primer sequences are listed in Table 4.1. Master mixes (24 µl) were aliquoted into PCR reaction tubes, and 1 µl of the appropriate cDNA template was added. Cycles were repeated 35 times for all reactions under the following conditions, 94°C for 15 sec, 60°C* for 30 sec, 72°C for 1 min (*Annealing temperatures vary, Table 4.1).

4.2.12 Densitometry Analysis of TLR Expression

PCR product bands for TLRs were visualised on agarose gels. Briefly, 100 ml TAE buffer (Appendix I) was added to 1g of agarose (Astral Scientific, New South Wales, Australia) and brought to a boil using a conventional microwave oven. Two hundred µl of ethidium bromide was added after 2 to 5 min, and gel mixture was poured into Liberty tanks. PCR products (10 µl) were added to wells with the addition of 5 µl loading dye (Promega, New South Wales, Australia). Electrophoresis of PCR products were carried out at 200 V for 15 min. Expression of TLRs was determined as a percentage of the TLR gene product density over β-actin density from the same treatment group using GeneTools Software (Syngene, Cambridge, United Kingdom). TLR expression was determined in comparision to non infection controls. Ranges of TLR expression were considered moderate if intensity equalled ± 20 percent of *S. typhmurium* controls, while intensity below or above the aforemenetioned range were considered low and high respectively. TLR gene expression was reported according to the following equation and an example is provided in Figure 4.3:

$$\text{Percent (\%) Intensity} = \frac{\text{Gene of Interest Band Intensity}}{\text{β-actin Control Band Intensity}} \times 100$$

4.2.13 Determination of Cytokine Production

Tumor necrosis factor-α, secretion from infected RAW 264.7 macrophages and BALB/c PEC was determined using the eBioscience Ready-Set-Go! ELISA kit (Jomar Diagnostics, South Australia, Australia). A Multiskan EX plate reader (Labsystems, Victoria, Australia) was used to read the optical density (OD_{450}) of ELISA results.

4.2.14 Assessment of Nitric Oxide Production

The production of NO from infected cells was measured using a standard Griess reaction to determine nitrite levels from culture supernatants (Green *et al.*, 1982). The Griess assay is one of the most commonly used methods to determine NO secretory activity from culture supernatants (Green *et al.*, 1982).

A standard curve was generated using five-fold dilutions of 10mM sodium nitrite ($NaNO_2$, Oxoid, SA, Australia) in triplicate. Briefly, 50 µl of culture supernatants or standard was added to individual wells of a 96-well plate, followed by 50 µl of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4 in sterile dH_2O). After incubation for 10 min at RT, the absorbance was read at 540 nm on a Multiskan EX plate reader (Labsystems, Victoria, Australia). (Multiskan EX; Labsystems).

4.2.15 Statistical Analysis

Linear regression analysis was used to determine correlations between the levels of uptake, killing, and isolate virulence (R^2 values are reported). Analyses of uptake, killing, TLR expression, cytokine and NO secretion by RAW 264.7 macrophages and BALB/c PEC were done using a post hoc univariate analysis (SPSS software version 12.0.0) of means ± standard deviation (SD). Values reported with $p < 0.05$ were considered significant.

4.3 RESULTS

4.3.1 The Effect of *Burkholderia pseudomallei* on RAW 264.7 Macrophage Internalisation and Killing

Percent uptake of *B. pseudomallei* isolates by RAW 264.7 macrophages ranged from 2.5% to 21.1 % 2 h post infection (Table 4.2). No correlations were observed between virulence of the infecting strain (range 10^0 cfu - 10^6 cfu) and entry of the bacteria into macrophages ($R^2 = 0.0005$). No correlations were found between killing of phagocytosed bacteria and bacterial virulence ($R^2 = 0.031$).

4.3.2 The Effect of *Burkholderia pseudomallei* on BALB/c PEC Internalisation and Killing

Percent uptake of *B. pseudomallei* ranged from 0.04 % to 2.8 % in BALB/c PEC. No correlation between uptake and virulence of the infecting *B. pseudomallei* strain for BALB/c PEC was observed ($R^2 = 0.0032$). Similarly, no correlation between virulence and killing was observed ($R^2 = 0.620$). Analysis of mean cfu from 1 h and 2 h lysates between BALB/c PEC demonstrated no significant difference between uptake or killing of the two isolates tested (Table 4.3).

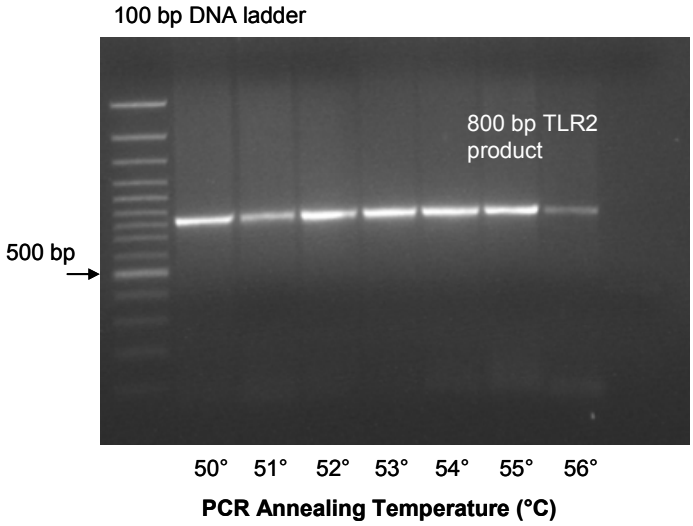

Figure 4.1 Temperature gradient for optimising binding of TLR2 primers
Standard PCR conditions using 100 ng RAW 264.7 RNA templates were carried out as described in section 4.2.5, with the exception of varied annealing temperatures as indicated. DNA size markers are shown on the far left column and negative template control is in the far right lane.

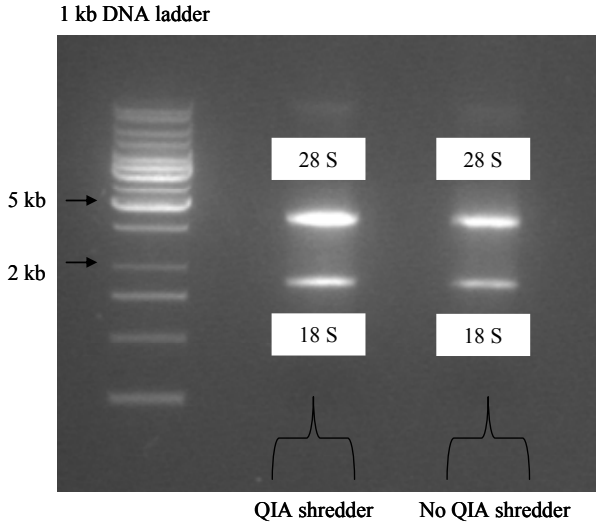

Figure 4.2 Isolation of total RNA from RAW 264.7 macrophages
28S and 18S ribosomal RNA (10 µg) bands were visualised at 4.7 kb and 1.9 kb respectively on agarose gels. Sharp bands indicate intact RNA. A larger yield of 28S and 18S RNA was achieved using the QIA shredder during mRNA isolation (middle column). DNA size markers are shown on the far left column.

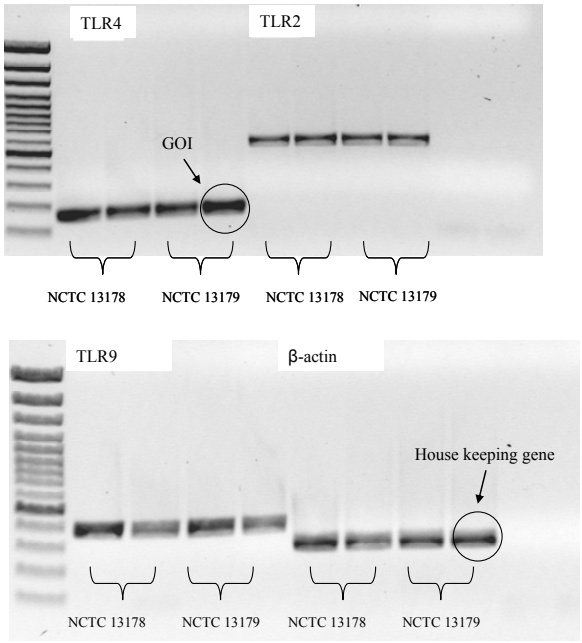

Figure 4.3 Example of densitometry analysis of TLR expression in BALB/c PEC infected with *B. pseudomallei*

TLR PCR products were electrophoresed and visualised on agarose gels. DNA size markers are shown on the far left column with TLR4, TLR2, TLR9 and β-actin products to the right as indicated. The expression of TLR2, TLR4, and TLR9 in BALB/c PEC infected with either *B. pseudomallei* NCTC 13178 or NCTC 13179 were determined by calculating the percent expression of the TLR gene of interest (GOI) to the β-actin house keeping gene.

Table 4.2 Uptake and killing of *B. pseudomallei* isolates of different virulence by RAW 264.7 macrophages after 2 h

Isolate	LD_{50} (cfu)*	Mean %Uptake	Mean %Killing	Killing Efficiency (10^{-2}) **
S. typhimurium	1×10^{1}	6.26	8.29	25
CL01	1.42×10^{6}	18.2	8	27
CL02	1.26×10^{6}	13	12.7	20
CL03	7.49×10^{5}	21.1	50	83
CL04	6.67×10^{4}	17.5	26.3	51
CL05	1.96×10^{4}	16.8	58.9	91
CL06	1.68×10^{4}	9.8	7.7	12
CL07*** (NCTC 13179)	1.52×10^{4}	1.97	3.85	75
CL08	1.42×10^{4}	2.96	51.7	29
CL09	1.26×10^{4}	8.9	14.3	20
CL10	1.11×10^{4}	6.3	52.3	75
CL11	7.33×10^{3}	17.9	55.5	94
CL12	3.15×10^{3}	9.6	61.8	62
CL13	2.53×10^{3}	10.9	41	90
CL14	2.32×10^{3}	6.9	73.4	126
CL15	2.28×10^{3}	15.29	52	125
CL16	2.00×10^{3}	20.5	1.86	37
CL17	1.36×10^{3}	17.3	87.2	139
CL18	1.00×10^{3}	2.5	9.7	37
CL19	3.76×10^{2}	19	39.1	76
CL20	2.72×10^{2}	10.6	49.1	83
CL21	1.26×10^{2}	20.9	8.56	35
CL22	4.00×10^{1}	8.8	12.8	26
CL23	1.50×10^{1}	10.6	76.2	143
CL24*** (NCTC 13178)	1.00×10^{1}	9.8	16.7	27
CL25	4.00×10^{0}	14.2	43.2	69
CL26	3×10^{0}	15.3	51.92	89

* Determined by Ulett *et al* .2001

**Killing efficiency was calculated as the mean number of bacteria killed per RAW 264.7 macrophage

***NCTC 13178 and NCTC 13179 are also known as CL24 and CL07

All mean values are reported from triplicate samples

Table 4.3 Uptake and killing of *B. pseudomallei* isolates of different virulence and *S. typhimurium* by elicited peritoneal macrophages derived from BALB/c mice.

Isolate	LD_{50} (cfu)	% Uptake	% Killing	Killing Efficiency *
S.typhimurium	1×10^1	0.04	21	0.36
NCTC 13178	1×10^1	2.8	65	0.3
NCTC 13179	1.52×10^4	3	70	0.15

*Killing efficiency was calculated as the mean number of bacteria killed per macrophage

4.3.3 The Effect of *Burkholderia pseudomallei* on RAW 264.7 Macrophage TLR Expression

There were no correlations between isolate virulence and TLR2 ($R^2 = 0.029$), TLR4 ($R^2 = 0.0092$), TLR5 ($R^2 = 0.279$), or TLR9 ($R^2 = 0.042$) expression. Comparatively high levels of TLR2 expression were observed in RAW 264.7 macrophages infected with 23 of 26 *B. pseudomallei* isolates (range 75% to 158%) (Figure 4.4A). TLR4 expression was moderate (range 22% to 57%) in RAW 264.7 macrophages infected with 25 of 26 isolates (Figure 4.4B). A trend of increased TLR5 expression was observed in RAW 264.7 macrophages infected with lower virulence isolates (Figure 4.4C). RAW 264.7 macrophages infected with the high virulence isolates (10^2 cfu to 10^0 cfu) demonstrated the lowest expression of TLR9 (Figure 4.4D).

4.3.4 The Effect of *Burkholderia pseudomallei* on BALB/c PEC TLR Expression

In BALB/c PEC, TLR2 and TLR4 expression were moderately increased (range 21% to 39%, range 14% to 31%) 2 h post infection in cells challenged with both the high and low virulence isolates (Figure 4.5A and 4.5B). Increased TLR2 and TLR4 expression in BALB/c PEC did not exceed that observed in RAW 264.7 macrophages. In addition, TLR5 and TLR9 expression were low in both cell types infected with the high virulent NCTC 13178 strain, and the low virulence NCTC 13179 isolate (Figure 4.5C and 4.5D).

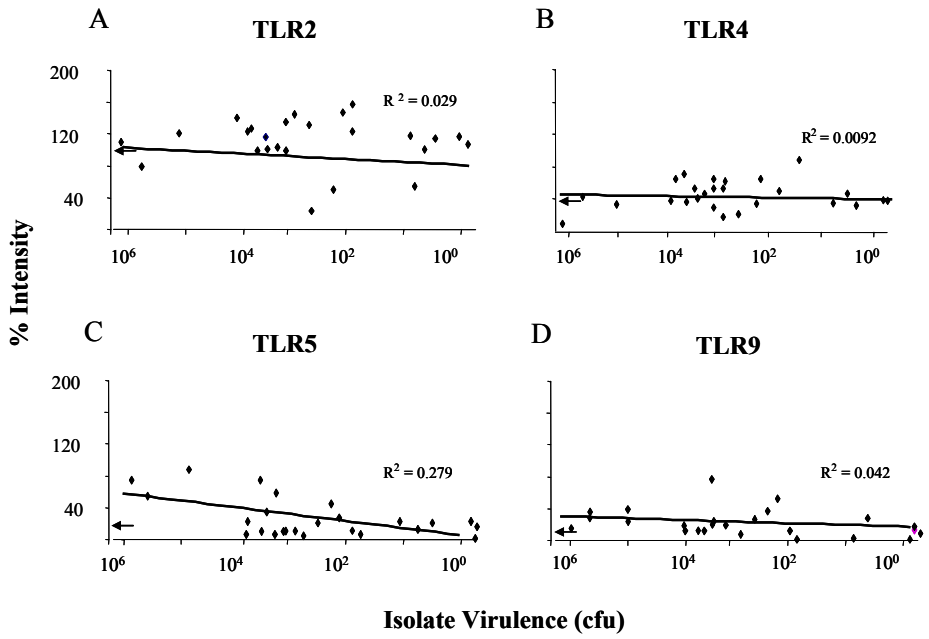

Figure 4.4 Expression of TLRs in RAW 264.7 macrophages following infection with *B. pseudomallei*

Intensity of TLR2, TLR4, TLR5, and TLR9 gene products amplified from RAW 264.7 macrophages following 2 h co-culture with *B. pseudomallei* isolates (1-26). Expression is reported as a percentage of β-actin. *S. typhimurium* (←) was used as a positive control.

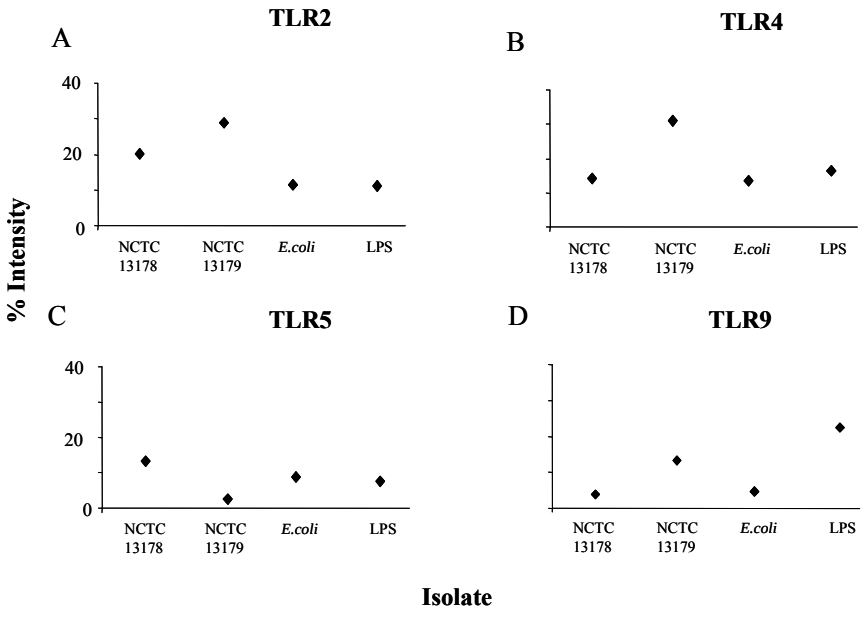

Figure 4.5 Expression of TLRs in BALB/c derived PEC following infection with *B. pseudomallei*

Intensity of TLR2, TLR4, TLR5, and TLR9 gene products amplified from BALB/c derived PEC infected with either high virulent (NCTC 13178) or low virulent (NCTC 13179) *B. pseudomallei*. Expression is reported as a percentage of β-actin. *E. coli* and LPS were used as positive controls.

4.3.5 The Effect of *Burkholderia pseudomallei* Virulence on TNF-α and Nitric Oxide Production in RAW 264.7 Macrophages and BALB/c PEC

TNF-α secretion was below 200 pg/ml in RAW 264.7 macrophages infected with 24 of 26 isolates in comparison to non infected controls (Figure 4.6). In the presence of two isolates of low and high virulence (CLO1 and CL19 respectively), TNF-α production was significantly greater in comparison to other isolates ($p < 0.01$). Nitrite production in both RAW 264.7 macrophages and BALB/c PEC was below 4 μM, 2 h post infection (Figure 4.7A and 4.7B).

TNF-α secretion by BALB/c PEC infected with high virulent NCTC 13178 and low virulent NCTC 13179 was significantly lower than *E. coli*, *S. typhimurium* and LPS controls ($p < 0.05$) (Figure 4.7). Nitrite production was significantly greater ($p < 0.05$) in BALB/c PECs infected with NCTC 13179 in comparison to NCTC 13178 (Figure 4.9).

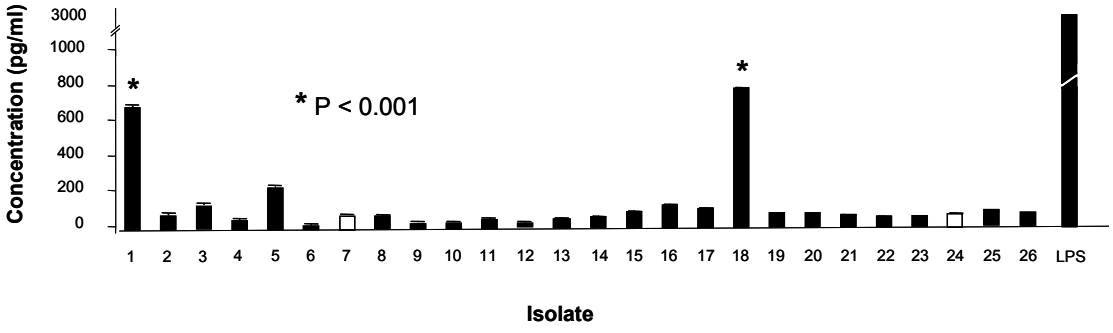

Figure 4.6 Production of TNF-α from RAW 264.7 macrophages following infection with *B. pseudomallei*

Two isolates (CL01 and CL18) were significantly different at inducing TNF-α production in comparison to uninfected controls ($p < 0.001$). CL07 (NCTC 13179) and CL24 (NCTC 13178) are represented by empty columns (□). Results are reported as the concentration (pg/ml) ± SEM.

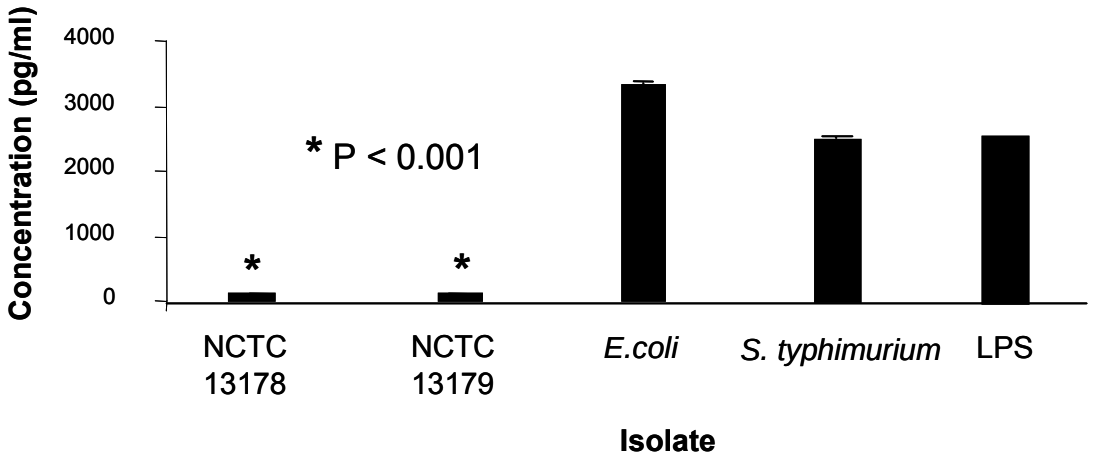

Figure 4.7 Production of TNF-α from BALB/c PEC following infection with *B. pseudomallei*

TNF-α production was assessed in PEC cells infected with NCTC 13178 and NCTC 13179 isolates was significantly less than that produced by *E. coli*, *S. typhimurium* and LPS controls ($p < 0.001$). Results are reported as the concentration (pg/ml) ± SEM.

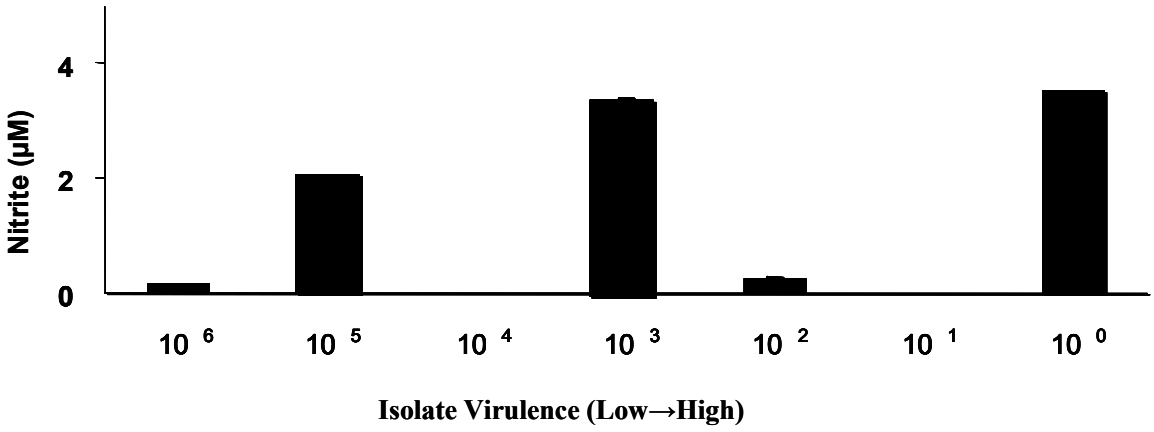

Figure 4.8 Production of nitric oxide from RAW 264.7 macrophages following infection with *B. pseudomallei*

Nitrite production of RAW 264.7 macrophages infected with *B. pseudomallei* 2 h post infection. Isolates (n=26) on the x-axis are grouped according to virulence in BALB/c mice and are listed in Table 4.2. Results are reported as the nitrite (μM) ± SEM.

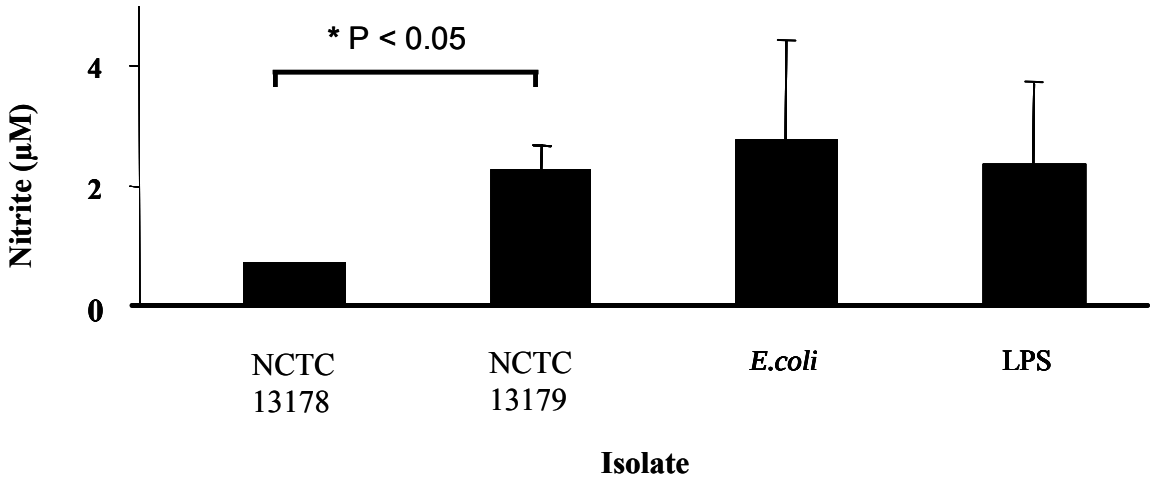

Figure 4.9 Production of nitric oxide from BALB/c PEC following infection with *B. pseudomallei*

Nitrite production of BALB/c derived PEC infected with *B. pseudomallei* isolates 2 h post infection. Nitrite production was greater in BALB/c PECs infected with low virulent NCTC 13179 in comparison to high virulent NCTC 13178. Results are reported as nitrite (µM) ± SEM.

4.4 DISCUSSION

In this chapter we aimed to determine the effect of *B. pseudomallei* isolates of varying virulence on the expression of selected TLRs by RAW 264.7 macrophages and PEC derived from BALB/c mice at 2 h post infection. This work was carried out to determine the activation of macrophages by *B. pseudomallei* via analysis of TLR transcription and the production of TNF-α, an important inflammatory cytokine in melioidosis.

Following co-culture with *B. pseudomallei* increased TLR2 expression was observed in infected RAW 264.7 macrophages irrespective of isolate virulence. Although TLR2 is predominantly regarded as a receptor for Gram positive bacteria, some Gram negative bacterial species have been shown to signal via TLR2 (Akamine *et al.*, 2005). Wiesinga *et al.* 2007 investigated the interaction between *B. pseudomallei* and TLRs in macrophages of human and murine origin. In that study, elevated mRNA levels encoding several TLRs were observed in peripheral blood leukocytes isolated from septic melioidosis patients. They also found that TLR2 knockout mice demonstrated a greater survival advantage than TLR4 knockouts infected with

B. pseudomallei up to six weeks post infection. The function of TLR2 in *B. pseudomallei* infection may play an integral role, however further investigation is warranted.

Two hours post infection it was found that *B. pseudomallei* is a relatively poor inducer of TLR4 in both RAW 264.7 macrophages and BALB/c PEC irrespective of isolate virulence (Figure 4.4 and Figure 4.5). It is possible that the low level expression of TLR4 observed in this investigation is due to the unique LPS configuration of *B. pseudomallei,* which may lack the acylation pattern necessary for effective TLR4 stimulation. In contrast, the related species *B. mallei* has been shown to activate TLR4 substantially (Brett *et al*. 2007). Future studies analysing downstream TLR signalling molecules in the presence of the *B. pseudomallei* isolates used in this study would illustrate whether low level expression of TLRs actively affects transcription and cytokine output. In addition, kinetic studies have shown a time lag in the production of cytokines and nitric oxide in cells stimulated with *B. pseudomallei* LPS. In addition, the pyrogenic activity of the molecule in mice was 30 times less in comparison to *Salmonella* species (Kawahara *et al.*, 1992).

Specific amino acids composing bacterial flagellin structure are responsible for protofilament formation and motility, and are recognized by the cell surface receptor TLR5 (Smith *et al.*, 2003). The role of flagellin as a virulence factor in *B. pseudomallei* infection is not fully understood as conflicting results have been reported (DeShazer *et al.*, 1997; Chua *et al.*, 2003). In this study TLR5 expression was increased in RAW 264.7 macrophages infected with low virulence *B. pseudomallei* isolates (Figure 4.4C). However, in BALB/c PEC, TLR5 expression was low in cells infected with both high and low virulence strains (Figure 4.5C). Hii *et al.* 2008 have reported that TLR5 stimulated with live *B. pseudomallei* and recombinant *B. pseudomallei* flagellin was capable of stimulating transfected HEK293T cells seven hours post infection. While TLR5 expression varied for isolates in our study, the results suggest that TLR5 may contribute to recognition of *B. pseudomallei* flagellin in isolates of lesser virulence and play a role in cytokine production, however studies assessing NF-κB activation by these isolates must be conducted to confirm this.

Recognition of bacterial nucleic acids (CpG-DNA) occurs in the intracellular environment via TLR9 (Latz *et al.*, 2004). In this investigation, low levels of TLR9 expression were observed in both RAW 264.7 macrophages and BALB/c PEC. In RAW 264.7 macrophages, TLR9 expression was particularly low when infected with the high virulent NCTC 13178 isolate. Previous studies have shown that TLR9 expression was absent in human and murine macrophages challenged with *B. pseudomallei* which is consistent with the observations in this study (Hii *et al.*, 2008). It is possible that the lack of TLR9 mRNA expression seen at the early stages of infection is a result of the ability of *B. pseudomallei* to avoid host lytic mechanisms. Analysis of intracellular trafficking of *B. pseudomallei* and TLR9 to host phagolysosomes would be necessary to confirm this.

Burkholderia pseudomallei is known to interfere with iNOS expression in RAW 264.7 macrophages and abrogate NO production during the early stages of infection (Utaisincharoen *et al.* 2001). This phenomenon is thought to contribute to the intracellular survival of the bacteria. We found that nitrate production two hours post infection was low in RAW 264.7 macrophages and BALB/c PEC infected with all *B. pseudomallei* isolates (Figure 4.8, Figure 4.9). These results are consistent with those reported previously (Utaisincharoen *et al.* 2001; Breitbach *et al.* 2006).

Low concentrations of TNF-α were detected in cultured supernatants from both RAW 264.7 macrophages and BALB/c PEC (Figure 4.6). In PEC cell supernatants TNF-α was significantly less than in co-cultures containing *E. coli* and LPS. Similar findings were reported elsewhere (Utaisincharoen *et al.* 2001).

In this study it was found that at 2 h post infection, there is no correlation between TLR expression and cytokine signalling in macrophages co-incubated with multiple *B. pseudomallei* isolates of different virulence. It is possible that longer incubations periods to allow for increased ligand receptor interactions are necessary to observe any definitive change in expression and should be conducted in the future. In addition, more detailed analysis of mechanisms involved in downstream TLR signalling such as NF-κB activation are warranted to determine if low TLR expression correlate to low level pro-inflammatory cytokine production.
Burkholderia. pseudomallei induced low level production of nitrate indicating a

reduction in the killing capacity of infected cells. This phenomenon may possibly be due to the intracellular nature of the organism, and the unique composition of *B. pseudomallei*. However further investigations are warranted in determining the effect of *B. pseudomallei* on macrophage function following co-culture for extended periods of time.

CHAPTER 5

TOLL-LIKE RECEPTOR ACTIVATION IN HUMAN MACROPHAGES INFECTED WITH A HIGH OR LOW VIRULENCE *BURKHOLDERIA PSEUDOMALLEI* ISOLATE

5.1 INTRODUCTION

In 2004 Holden and colleagues sequenced the genome of a clinical *B. pseudomallei* isolate (Holden *et al.*, 2004). The genome is comprised of two chromosomes of 4.07 and 3.17 megabase pairs (chromosomes 1 and 2) respectively, and a functional partitioning of genes exists between the two. Chromosome 1 is conserved across related species and is responsible for gene products essential for cell growth. While Chromosome 2 contains some coding sequences for essential functions it harbours more accessory genes in comparison to Chromosome 1 which are housed on genomic islands that enable survival in various environments. Soil and invasive isolates contain various groupings of these genomic islands that are absent in the most closely related organism *B. mallei*. The authors contend that the genetic evolution of *B. pseudomallei* and its pathogenic characteristics are acquired via horizontal gene transfer to the small chromosome. It has been shown that *B. pseudomallei* contains an open genome that recombines at high frequency and this variation leads to intra species variation (Kim *et al.*, 2005). In a study comparing the genomes of five reference strains of *B. pseudomallei* isolated from patients with variable disease outcomes it was found that horizontal acquisition of up to 71 genomic islands involved in pathogenicity and metabolism varied between strains (Tuanyok *et al.*, 2008).

While not a natural human pathogen, *B. pseudomallei* has the capacity to transcend its natural soil habitat and elicit disease in humans and animals via opportunistic infection (White, 2003). Primarily, infection occurs through direct entry into abraded skin or by ingestion or inhalation of contaminated soil and surface water (Dance, 2002). Whether or not an individual develops symptomatic disease following exposure is thought to be dependant on several known factors, including: inoculum

size, pathogen virulence, iron bioavailability, host immunocompetence, and potentially, host genetic variation (Barnes *et al.*, 2001a; Ulett *et al.*, 2001). Investigation into the microbial diversity and evolution of virulence determinants in *B. pseudomallei* is important to understanding its pathogenicity. Pathogenicity can be defined as the ability of a microorganism to cause disease and is determined by that pathogen's ability to gain entry into the host organism, evade host defence mechanisms, colonise, and cause damage to infected tissues (Dance, 2002; Liu *et al.*, 2002). Numerous putative virulence factors have been described for *B. pseudomallei* including type III secretion systems, capsular polysaccharide, flagella and lipopolysaccharide (Cheng and Currie, 2005; Wiersinga *et al.*, 2006).

Several Gram-negative bacteria, including *S. typhimurium*, *E. coli*, *Shigella flexneri* and *P. aeruginosa* encode a type III secretion system (T3SS). These virulence determinants help facilitate entry into host cells via injection of effector proteins into the host cytosol (Stevens *et al.*, 2002; Stevens *et al.*, 2004). Once *B. pseudomallei* enters the host cell actin rearrangements are induced at one pole of the bacteria, leading to actin tail formation and the generation of host cellular protrusions which contribute to cell to cell spreading (Kespichayawattana *et al.*, 2000; Katrin *et al.*, 2003). Mutations in the T3SS apparatus of *B. pseudomallei*, identified as Bsa, reduce intracellular growth and evasion of host lytic mechanisms (Stevens *et al.*, 2002).

Several cell surface associated antigens contribute to the pathogenesis and virulence of *B. pseudomallei*. A capsular polysaccharide characterised by Masoud and colleagues, was recognised in sera of 13 patients with different clinical manifestations of melioidosis. Mutant strains of *B. pseudomallei* lacking capsular polysaccharide are less virulent and more readily cleared by human polymorphonuclear cells and macrophages (Reckseidler-Zenteno *et al.*, 2005; Chanthiwa *et al.*, 2009). The addition of purified capsular proteins to human serum challenged with mutant *B. pseudomallei* was shown to abrogate host opsonisation by diminishing complement deposition on the bacterial cell wall similar to wild type strains (Reckseidler-Zenteno *et al.*, 2005). In addition, *B. pseudomallei* expresses a unique LPS structure comprised of two distinct O-polysaccharide moieties: type I and type II O-PS (Perry *et al.*, 1995). *In vitro* assays of infection demonstrated that type II O-PS biosynthesis was essential for *B .pseudomallei* serum resistance and virulence (DeShazer *et al.*,

1997). Mutant *B. pseudomallei* lacking type II O-PS were more efficiently cleared in guinea-pig, rat and human serum in comparison to wild type and *E. coli* controls. These studies indicate the importance of these virulence factors for *B. pseudomallei* to establish infection yet how host cells recognise these structures is still poorly understood. The innate immune systems recognition of pathogens is facilitated by TLRs which recognise conserved microbial structures, some of which are virulence determinants such as LPS (Medzhitov, 2001).

Of the 10 TLRs discovered in humans TLR2 and TLR4 are the most extensively studied. Recently, several studies have indicated a critical role for TLR2 and TLR4 during innate immune signalling in melioidosis and are the focus of this Chapter. These studies indicate that both are involved, however certain strains elicit either a strong TLR2 or TLR4 dependent response in both *in vitro* and *in vivo* models of infection (Wiersinga *et al.*, 2007c; West *et al.*, 2008a; Novem *et al.*, 2009). Similar reports for TLR2 and TLR4 dependant activation have been shown for the closely related *Burkholderia thailandensis* species (West *et al.*, 2008c; Novem *et al.*, 2009; West *et al.*, 2009). Certainly these differences can be attributed to the relative plasticity of the *B. pseudomallei* genome and suggest that TLR mediated recognition of individual isolates may not be universal, but rather tailored to the specific isolate encountered. In the work described in Chapter 4 we found no correlation between *B. pseudomallei* isolate virulence (as determined in mice, Table 3.1), TLR expression and selected pro-inflammatory cytokine production at 2 h post infection suggesting universal TLR expression amongst murine macrophages co-cultured with this panel of *B. pseudomallei* isolates. We intended to assess longer periods of infection to determine if this trend of TLR and pro-inflammatory cytokine expression continued using two *B. pseudomallei* isolates of high and low virulence. Determining the activation of TLRs in the presence of *B. pseudomallei* will assist in understanding pathogenesis and host response.

In the work described in Chapter 4 we assessed a panel of *B. pseudomallei* isolates and found no correlation between TLR expression and the virulence of the infecting isolate in murine macrophages after 2 h co-culture. Based on these results the work in this Chapter focuses on two *B. pseudomallei* clinical isolates, the highly virulent NCTC 13178 and low virulent NCTC 13179 (Table 4.2). These isolates were selected

as they are NCTC reference strains and have been extensively studied in both *in vitro* and *in vivo* systems in our laboratory. Originally, NCTC 13178 was cultured from a fatal case of melioidosis in a child, while NCTC 13179 was cultured from a chronic leg abscess in a patient with diabetes. Studies using these two isolates have demonstrated differing immunological responses and disease outcomes in BALB/c and C57BL/6 mouse models of *B. pseudomallei* infection (Leakey *et al.*, 1998; Ulett *et al.*, 2000b; Ulett *et al.*, 2000d; Barnes *et al.*, 2001b; Ulett *et al.*, 2001; Barnes and Ketheesan, 2005). The virulence of NCTC 13178 and NCTC 13179 were determined in BALB/c and C57BL/6 mice (Table 3.1) according to ID_{50} values (Leakey *et al.*, 1998; Ulett *et al.*, 2001; Barnes and Ketheesan, 2005). The NCTC 13178 isolate is highly virulent in BALB/c mice with an ID_{50} value of >10 cfu following *i.v.* injection. In BALB/c mice infected with the NCTC 13179 isolate the ID_{50} value increases 100-fold to 1×10^3 cfu. In C57BL/6 mice ID_{50} values for NCTC 13178 and NCTC 13179 are 5×10^3 cfu and 6×10^6 cfu respectively via *i.v.* challenge (Barnes and Ketheesan, 2005).

To determine if human macrophage activation is ubiquitous in the presence of these two *B. pseudomallei* isolates, we examined TLR2, TLR4 and selected pro-inflammatory cytokine profiles in infection assays. In addition, we have extended the co-culture period of macrophages with *B. pseudomallei* up to 24 h to determine if changes in TLR expression profiles differ by increasing the time of PAMP/TLR interactions.

The specific aims of the work described in this Chapter were to:

1) Assess the efficiency of human macrophages to phagocytose and kill a high virulent (NCTC 13178) and low virulent (NCTC 13179) clinical *B. pseudomallei* isolate.

2) Determine transcriptional profiles of TLR2 and TLR4 in human macrophages co-cultured with a high virulent (NCTC 13178) and low virulent (NCTC 13179) clinical *B. pseudomallei* isolate using qRT PCR analysis.

3) Determine transcriptional profiles of selected pro-inflammatory cytokines (IL-1β, IL-12p40 and TNF-α) induced by a high virulent (NCTC 13178) and low virulent (NCTC 13179) clinical *B. pseudomallei* isolate using qRT PCR analysis.

4) Quantify selected pro-inflammatory cytokine secretion (IL-1β, IL-12p40 and TNF-α) by human macrophages co-cultured with *B. pseudomallei* using ELISA.

Determination of *B. pseudomallei* interactions with TLR2 and TLR4 will further our understanding of innate immune recognition of the pathogen. Furthermore, it will demonstrate whether transcriptional profiles of TLR2 and TLR4 and the subsequent inflammatory responses elicited by the selected isolates are distinct.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture Conditions

Human monocytic THP-1 cells were originally derived from a one year old patient with acute monocytic leukaemia in the United States (Shigeru *et al.*, 1980), and were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). It is a widely used cell line that displays characteristic monocytic-derived macrophage properties. THP-1 cells were selected as they are a model cell line used for investigations into intracellular pathogen affects on human macrophages (Herzyk *et al.*, 1992; Wei-Mei, 2008). Cells were cultured in RPMI medium (Gibco Labs, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS), (Gibco Labs, Grand Island, NY, USA), with 20mM of L-glutamine (Sigma Aldrich, Sydney, NSW, Australia), and 12.5 mM HEPES buffer at 37°C with 5% CO_2.

5.2.2 Internalisation Assay

Internalisation assays to determine TLR2 and TLR4 transcription were carried out according to methods previously described with the following modifications (Feterl *et al.*, 2008). Prior to co-culture with *B. pseudomallei* THP-1 cells were seeded into 24 well plates (1×10^6 cells/well) and incubated at 37°C with 5% CO_2 for 24 h. Cells

were washed three times with fresh medium and 20 ng/ml phorbol 12-myristate 13-acetate (PMA), (Invitrogen, New South Wales, Australia) was added for 18 h to induce THP-1 cell differentiation to macrophage-like cells. Cells were washed three times with fresh RPMI medium (without supplements) and stimulated with 300 U/ml purified human IFN-γ (Prospec-Tany TechnoGene, Rehovot, Israel) for 18 h. IFN-γ was utilised to activate THP-1 cells, as this cytokine has been shown to play an important role in innate resistance to *B. pseudomallei* and has been used in similar models of infection (Santanirand *et al.*, 1999b; Koo and Gan, 2006). Cells were washed with fresh RPMI medium and co-cultured with either NCTC 13178 or NCTC 13179 at a multiplicity of infection (MOI) of 10:1 (1×10^7 cfu/ml), or *E. coli* 0111:B4 LPS (10 ng/ml) (Sigma Aldrich, New South Wales, Australia) for 2 h, 6 h or 24 h. Co-cultures were incubated for 2 h prior to addition of kanamycin (250 μg/ml) to eliminate extracellular bacteria. The efficacy of kanamycin to clear extracellular bacteria at this concentration was determined by culture of supernatants on Ashdown agar prior to experimental trials.

5.2.3 Internalisation and Killing of *Burkholderia pseudomallei* by THP-1 Cells

Assays were performed to determine *B. pseudomallei* internalisation and killing by THP-1 cells according to methods previously described using a 2 h infection period (Utaisincharoen *et al.* 2001). The number of internalised and killed bacteria by THP-1 cells was assessed following 1 h and 2 h incubation periods. THP-1 cells were infected with NCTC 13178 or NCTC 13179 at an MOI 10:1 as described above (section 5.5.2). Internalisation of bacteria following 1 h incubation was determined following lysis of THP-1 cells with 0.1 % Triton X, and bacterial colony forming units (cfu) were determined following plating on Ashdown agar.

A second series of internalisation assays were carried out in parallel to determine the number of viable bacteria following 2 h incubation. After 1 h co-culture, cells were washed three times with PBS (pH 7.2) (Appendix I) to remove extracellular bacteria. One ml of culture medium supplemented with 250 μg/ml kanamycin was added to wells. After 1 h of further incubation cells were lysed and cfu were determined as described above.

Percent uptake and percent survival were calculated from bacterial cfu from 1 h and 2 h lysates. Bacterial counts from 1 h lysate groups were determined as the number of bacteria internalised by THP-1 cells. The difference in cfu between 1 h and 2 h lysates was reported as the number of bacteria eliminated by macrophages as determined by the equations outlined in section 4.2.5.

5.2.4 PCR Product Purification

Standard reverse transcriptase PCR was used to detect TLR2, TLR4, IL-1β, IL-12p40, TNF-α and GAPDH gene products to construct standard curves for quantitative real time polymerase chain reaction (qRT PCR) analysis. Methods for standard RT PCR are described in Chapter 4, section 4.2.10. PCR products (10 µl) were electrophoresed on 1% agarose gels at 200V. PCR product bands were visualised under UV light and excised with a scalpel blade and placed into 1.5 ml microfuge tubes. PCR products were recovered from agarose gels using the HiYield Gel/PCR DNA Fragment Extraction Kit (Real Biotech Corporation, Banqiao City, Taiwan). Up to 300 mg of gel slice were placed into tubes. DF Buffer (500 µl) was added to the sample and vortexed. The gel and buffer mixture was then incubated at 55°C for 15 min until gel slice was completely dissolved. During incubation, the tube was inverted every 2 to 3 min. Following incubation, 800 µl of the sample mixture was placed into a DF column. The sample was then centrifuged at 6,000 × g for 30 s. Samples were then washed with RBC wash buffer and centrifuged at 6,000 × g for 30 s. The flowthrough of the tube was discarded and the tubes were centrifuged for 2 min at 8,000 × g to dry the column matrix. Purified PCR reactions were then eluted in 30 µl, 70°C DEPC water by centrifugation for 2 min at 8,000 × g. Products were stored at -80°C until use.

5.2.5 Protocol for PCR Product Ligation and Transformation

A general cloning and transformation scheme was carried out on TLR and cytokine PCR products as outlined in section 4.2.6 with the following modifications using the pGEM®-T Easy Vector System (Promega, New South Wales, Australia). Briefly, PCR product ligations were carried out in 0.5 ml PCR tubes using 5 µl of 2 x Rapid Ligation Buffer, 1 µl of pGEM-T Vector (50 ng), 3 µl of purified PCR product

(TLR2, TLR4, TLR5, TLR9, IL-1β, IL-12p40, TNF-α or GAPDH) and 1 µl of T4 DNA Ligase (3 Weiss units/µl) were added for a total reaction volume of 10 µl. Reactions were mixed by pippetting and incubated for 3 h at 12°C for ligation.

5.2.6 Isolation of Plasmids Containing TLR and Cytokine Inserts

High copy number plasmid isolations were carried out according to the manufacturer's instructions using the HiYield Plasmid Mini Kit (Real Biotech Corporation, Banqiao City, Taiwan). Briefly, 1.5 ml of bacterial culture containing either TLR or cytokine inserts were transferred to a 1.5 ml microfuge tube. Bacteria were centrifuged, resuspended in lysis buffer, neutralised and bound to DNA binding columns. DNA (50 µl) was eluted in 70°C DEPC treated water.

5.2.7 Restriction Digest of TLR Plasmid and Insert

Restriction enzyme digests were carried out on eluted DNA samples using the FastDigest SalI Kit (Fermentas, New South Wales, Australia) according to the manufacturer's instructions. Briefly, restriction digests were carried out in a 30 µl total volume containing 17µl nuclease-free water, 2 µl 10 × FastDigest Buffer, 10 µl (~0.2 µg) purified PCR product, and 1 µl FastDigest enzyme. Reactions were incubated at 37°C in a heat block for 60 min. Termination of reactions was then carried out for 10 min at 65°C.

5.2.8 Creation of Product Standards for Quantification of TLR and Cytokines Using Quantitative Real Time Polymerase Chain Reaction

The concentration of the purified product insert was determined using a nanophotometer (Implen, Munich, Germany). The copy number of TLR and pro-inflammatory cytokine gene products was calculated using the equation detailed in Appendix II. qRT PCR standards were created by running quadruple samples of TLR and selected pro-inflammatory cytokine primers (Table 5.1) on control THP-1 cDNA constructs using cycling conditions outlined in section 5.2.9. An example of a standard curve used for quantitation of TLR and selected pro-inflammatory gene products is provided in Figure 5.1. The remaining standard curves are provided in Appendix III.

5.2.9 RNA Extraction and Real Time Polymerase Chain Reaction

Following 2 h, 6 h, and 24 h co-culture of THP-1 cells with *B. pseudomallei* and controls, mRNA was isolated from triplicate culture wells using the innuPrep Mini Kit (Analytikjena, Jena, Germany). Extracted RNA (100 ng) from infected THP-1 cells was converted to cDNA using the MMLV High Performance Reverse Transcriptase Kit (Epicentre Biotechnologies, Wisconsin, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for TLR2, TLR4, IL-1β, IL-12p40, TNF-α and GAPDH using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Cycling parameters for each trial were run as follows: 95°C for 10 sec, 60°C for 10 sec, 72°C for 20 sec, 72° to 95°C for 1 min. A list of primers and primer sequences is listed in Table 5.1. Quantification of TLRs and cytokines was obtained using the two-standard curve method within the Rotor-Gene 6000 software. Means ± standard error of the means (SEM) were reported for TLR data and are subtracted from baseline transcription values.

Table 5.1 TLR and cytokine primers used for qRT PCR analysis of THP-1 cells

Primer	Primer Sequence	GenBank Accession #	Product Size (bp)
TLR2 (for)	GGTAGTTGTGGGTTGAAG	NM_003264.3	83
TLR2 (rev)	TTGGAGAGGCTGATGATG		
TLR4 (for)	TGGAAGTTGAACGAATGGAATG	NM_138554.2	131
TLR4 (rev)	AGATACTACAAGCACACTGAGG		
IL-1β (for)	TGGCTTATTACAGTGGCAATG	NM_000576.2	134
IL-1β (rev)	GTGGTGGTCGGAGATTCG		
IL-12p40 (for)	CCTCCTCCTTGTGGCTAC	NM_000882.2	88
IL-12p40 (rev)	GAACATTCCTGGGTCTGG		
TNFα (for)	TCAGCAAGGACAGCAGAG	NM_000594.2	124
TNFα (rev)	GTATGTGAGAGGAAGAGAACC		
GAPDH (for)	TGCACCACCAACTGCTTAGC	NM_002046	87
GAPDH (rev)	GGCATGGACTGTGGTCATGAG		

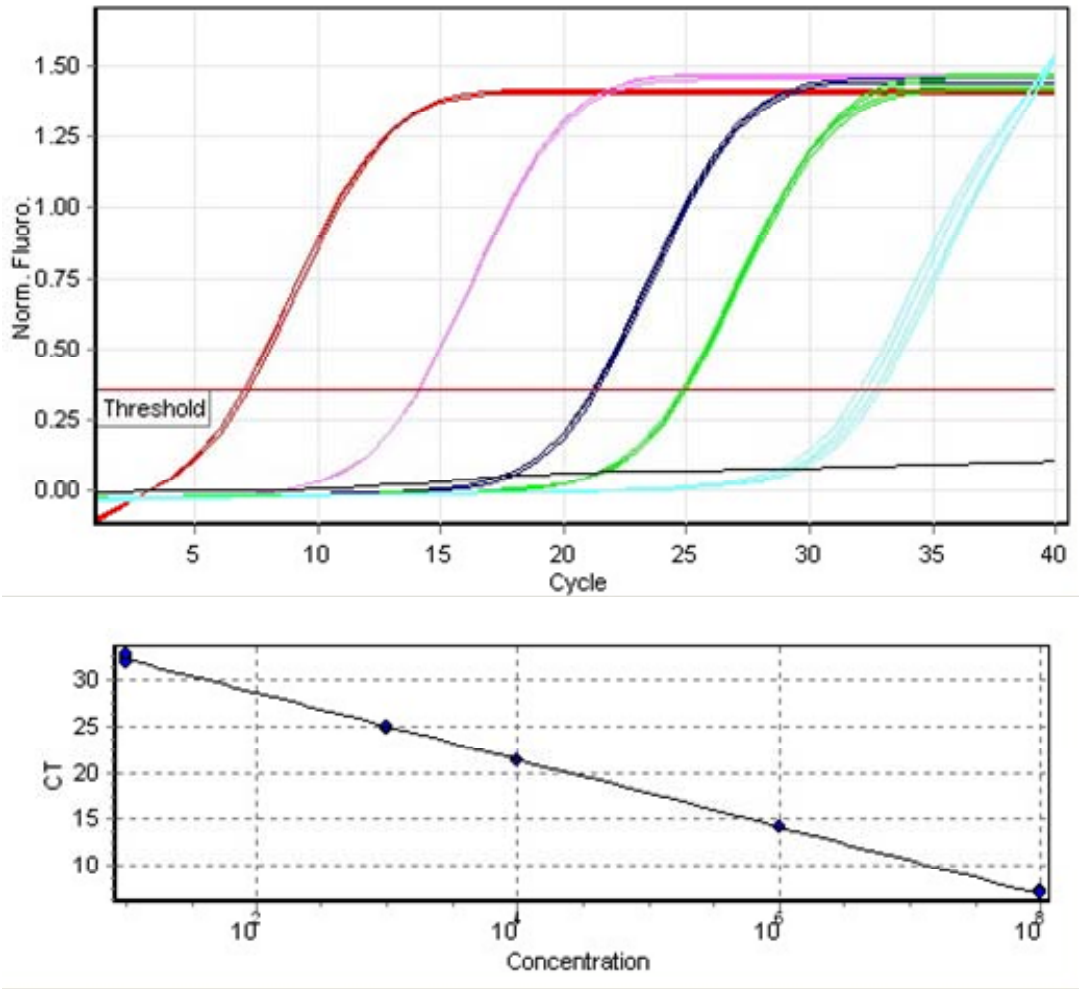

Figure 5.1 Standard curve values for quantitation of TLR4 transcription in *B. pseudomallei* infected THP-1 cells

Standard curve mean values are reported as copy number of TLR4 transcripts (Red) 9.47×10^7, (Purple) 1.04×10^6, (Blue) 1.08×10^4, (Green) 1.07×10^3, (Light Blue) 1.1×10^1, (Black) non-template controls and were calculated according to the equation detailed in Appendix II. $R^2 = 0.99$ for standard curve.

5.2.10 Determination of Cytokine Secretion by THP-1 Cells Co-Cultured with *Burkholderia pseudomallei*

Co-culture supernatants were assessed for IL-1β, IL-12p40 and TNF-α at 2 h. 6 h and 24 h using the Quantikine ELISA kit (R&D Systems, New South Wales, Australia). Concentration of selected pro-inflammatory cytokines were reported as the means ± standard error of the means (SEM).

5.2.11 Statistical Analysis

Univariate repeated measures ANOVA were performed on qRT PCR results for TLR and cytokine transcription as well as ELISA results assessing cytokine concentration from co-culture supernatants using Statistica software (StatSoft Inc, Victoria, Australia). Differences with a value of $p < 0.05$ were considered significant.

5.3 RESULTS

5.3.1 Internalisation and Survival of *Burkholderia pseudomallei* in THP-1 cells

Internalisation of the highly virulent NCTC 13178 isolate was significantly greater ($p < 0.05$) than the low virulent NCTC 13179 isolate in THP-1 cells following 2 h co-culture (Figure 5.2A). Percent survival of NCTC 13178 and NCTC 13179 in THP-1 cells decreased over 24 h. Percent survival of the highly virulent NCTC 13178 at 2 h, 6 h and 24 h in comparison to the low virulent NCTC 13179 isolate was not significant (Figure 5.2B).

5.3.2 Comparative Transcription of TLR2 and TLR4 in THP-1 Cells Co-Cultured with *Burkholderia pseudomallei*

In order to examine the comparative transcription of TLR2 and TLR4 induced by co-culture with *B. pseudomallei* isolates of different virulence, we performed qRT PCR analysis on THP-1 cells at several time points. qRT PCR analysis revealed that TLR2 and TLR4 were maximally transcribed in the presence of *B. pseudomallei* NCTC 13179 by 24 h (Figure 5.3A, Figure 5.3B). TLR2 and TLR4 transcription between THP-1 cells co-cultured with NCTC 13178 and NCTC 13179 were similar at 2 h and 6 h (Figure 5.3A). Interestingly, we found that TLR2 transcription with NCTC 13179 was three times greater than NCTC 13178 (NCTC 13178: 34.6 ± 8.9 vs. NCTC 13179; 108 ± 15.2, $p < 0.05$) at 24 h (Figure 5.3A)A similar pattern was noted for TLR4 transcription, where THP-1 cells co-cultured with NCTC 13179 exhibited a 22.5 times greater increase (NCTC 13178: 47.4 ± 13.2 vs. NCTC 13179: 1,070 ± 38.8, $p < 0.05$) at 24 h (Figure 5.3B).

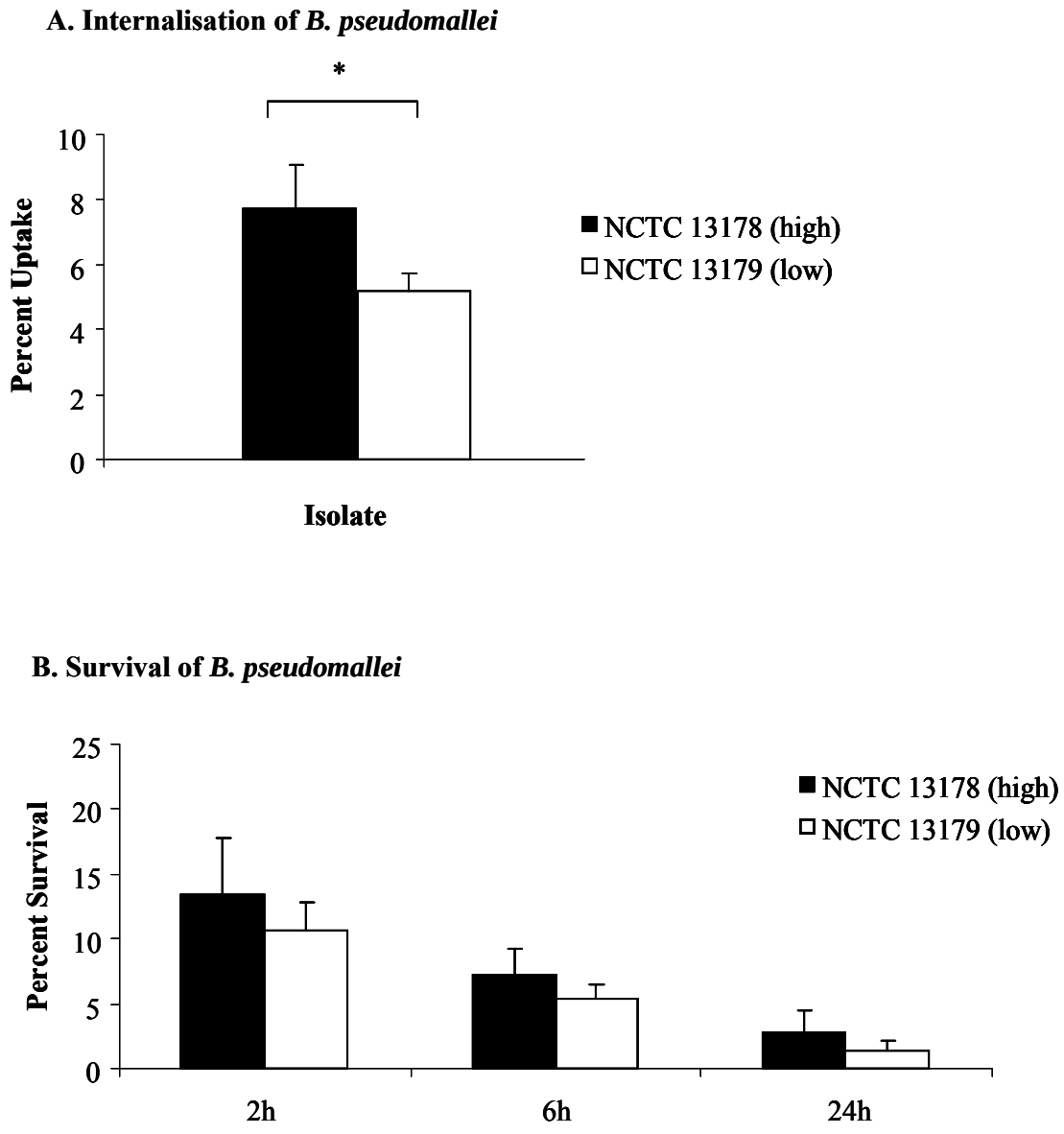

Figure 5.2 Internalisation and survival of *B. pseudomallei* isolates co-cultured with THP-1 cells

(A) Internalisation of *B. pseudomallei* isolates NCTC 13178 and NCTC 13179 were determined after 2 h. (B) Survival was determined following 2 h, 6 h, and 24 h co-culture with NCTC 13178 and NCTC 13179. Internalisation of the highly virulent NCTC 13178 isolate was significantly greater than the low virulent NCTC 13179 isolate at 2 h (A). Means ± SEM are reported.
* $p < 0.01$.

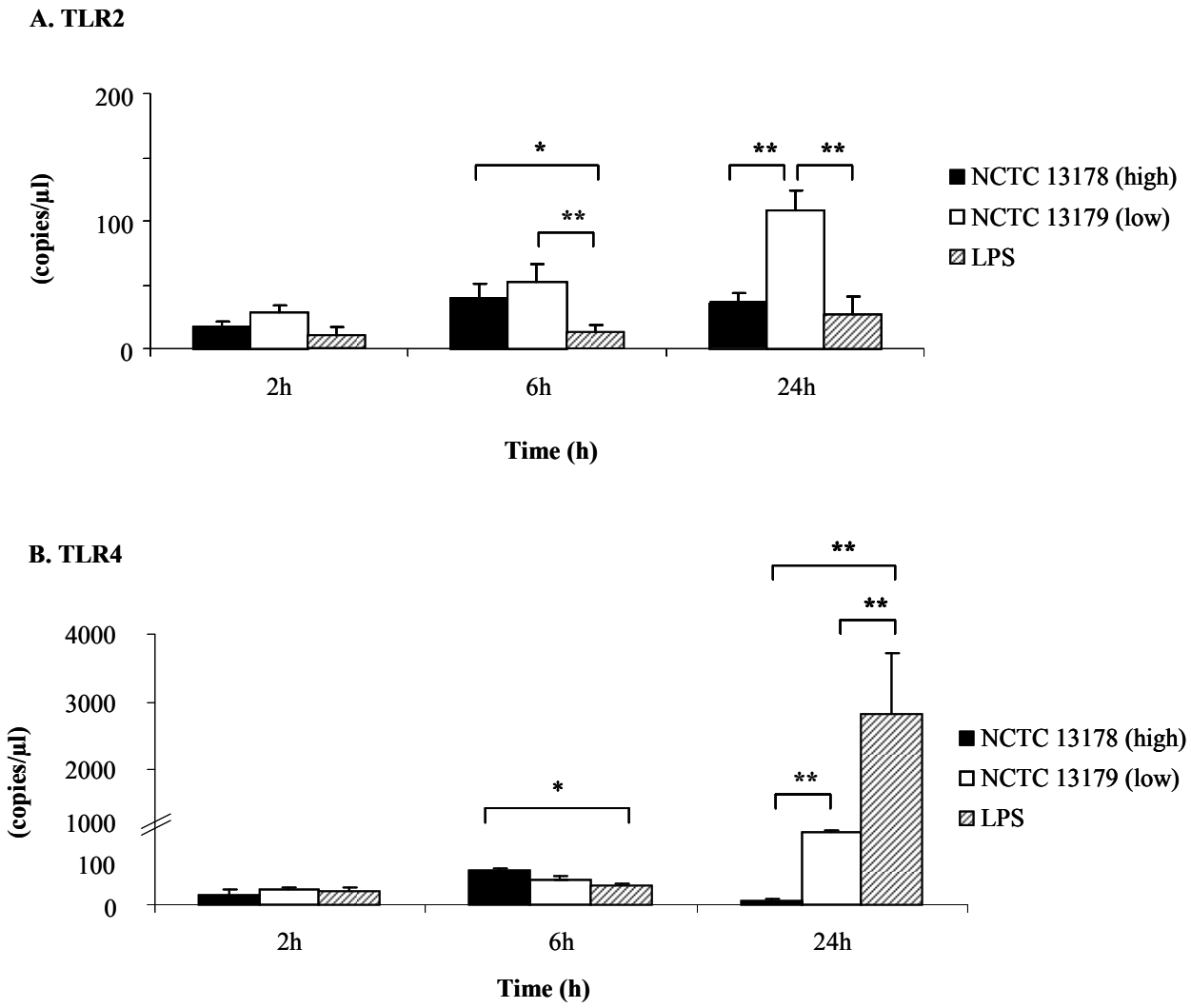

Figure 5.3 Comparative expression of TLR2 and TLR4 in THP-1 cells co-cultured with *B. pseudomallei* isolates

(A) TLR2 and (B) TLR4 transcription in THP-1 cells co-cultured with *B. pseudomallei* isolates (NCTC 13178 and NCTC 13179; 4×10^6 cfu/ml) or *E .coli* 0111:B4 LPS (10 ng/ml), were determined using qRT PCR. Means ± SEM are reported. (*) $p < 0.01$; (**) $p < 0.05$.

5.3.3 Comparative Transcription of Selected Pro-Inflammatory Cytokines by THP-1 Cells Co-Cultured with *Burkholderia pseudomallei*

Comparative transcription of IL-1β, IL-12p40 and TNF-α in THP-1 cells induced by co-culture with the two *B. pseudomallei* isolates was performed using qRT PCR analysis at serial time points following co-culture. IL-1β transcription was significantly greater in THP-1 cells co-cultured with low virulent NCTC 13179 in comparison to high virulent NCTC 13178 at 2 h (NCTC 13178: 42.7 ± 5.7 vs.

NCTC 13179: 4910 ± 511.4, $p < 0.01$), (Figure 5.4A). However, at 6 h co-culture this trend was reversed, as the high virulent NCTC 13178 isolate elicited an increase in IL-1β transcription (NCTC 13178: 15801 ± 4486 vs. NCTC 13179: 75.4 ± 2.6, $p < 0.01$) in comparison to the low virulent NCTC 13179 isolate (Figure 5.4A). No significant difference in transcription of IL-1β was observed between isolates at 24 h, however, transcription decreased in THP-1 cells co-cultured with NCTC 13178. Transcription of IL-1β was significantly lower for both isolates in comparison to LPS controls at 24 h ($p < 0.05$).

IL-12p40 transcription was significantly greater in THP-1 cells co-cultured with the low virulent NCTC 13179 isolate in comparison to the high virulent NCTC 13178 isolate and LPS controls at both 6 h (NCTC 13178: 156.6 ± 3.2 vs. NCTC 13179: 279.9 ± 2.7, $p < 0.01$) and 24 h (NCTC 13178: 25 ± 27.3 vs. NCTC 13179: 205 ± 28.4, $p < 0.05$).

TNF-α transcription was significantly greater in THP-1 cells co-cultured with both NCTC 13178 and NCTC 13179 isolates (NCTC 13178: 109 ± 10.3 vs. NCTC 13179: 101 ± 5.1, $p < 0.05$) at 2 h in comparison to LPS controls (Figure 5.4C). At 6 h TNF-α was maximally transcribed in THP-1 cells co-cultured with the highly virulent NCTC 13178 isolate (NCTC 13178: 153.8 ± 38.8 vs. NCTC 13179: 21.4 ± 6.6, $p < 0.01$), and transcription values were significantly greater than those induced by the low virulent NCTC 13179 isolate and LPS controls (Figure 5.4C). Maximal transcription of TNF-α at 24 h was observed in THP-1 cells co-cultured with the low virulent NCTC 13179 isolate and LPS controls. At 24 h TNF-α transcription induced by NCTC 13179 and LPS were significantly greater than those induced by NCTC 13178 (Figure 5.4C).

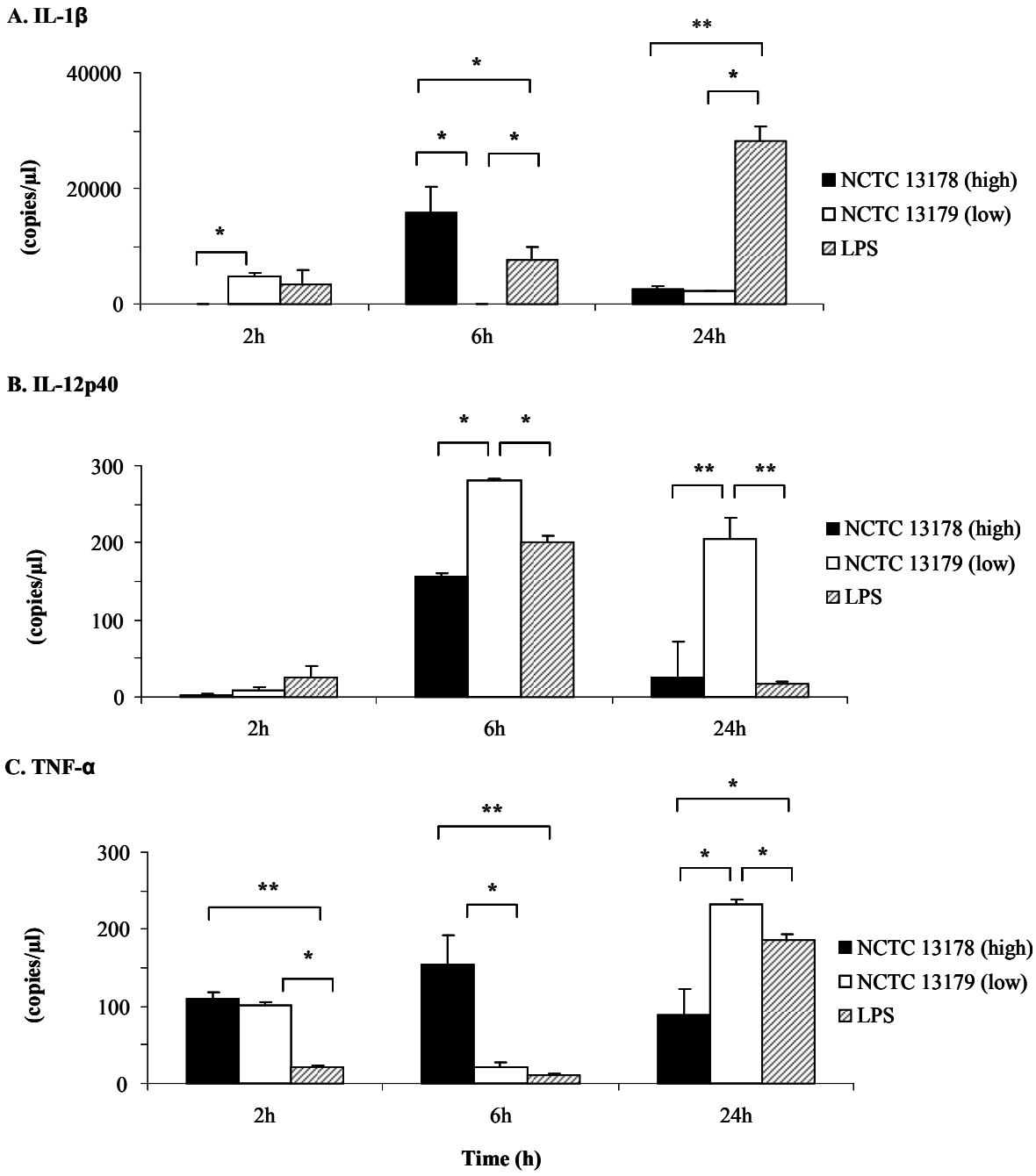

Figure 5.4 Selected pro-inflammatory cytokine expression profiles of THP-1 cells co-cultured with *B. pseudomallei* isolates

THP-1 cells were co-cultured with two *B. pseudomallei* isolates (the highly virulent NCTC 13178 and the low virulent NCTC 13179) for 2 h, 6 h and 24 h. RNA was extracted from infected cells and (A) IL-1β, (B) IL-12p40 and (C) TNF-α mRNA expression were determined using qRT-PCR. Means ± SEM are reported. * $p < 0.01$; ** $p < 0.05$.

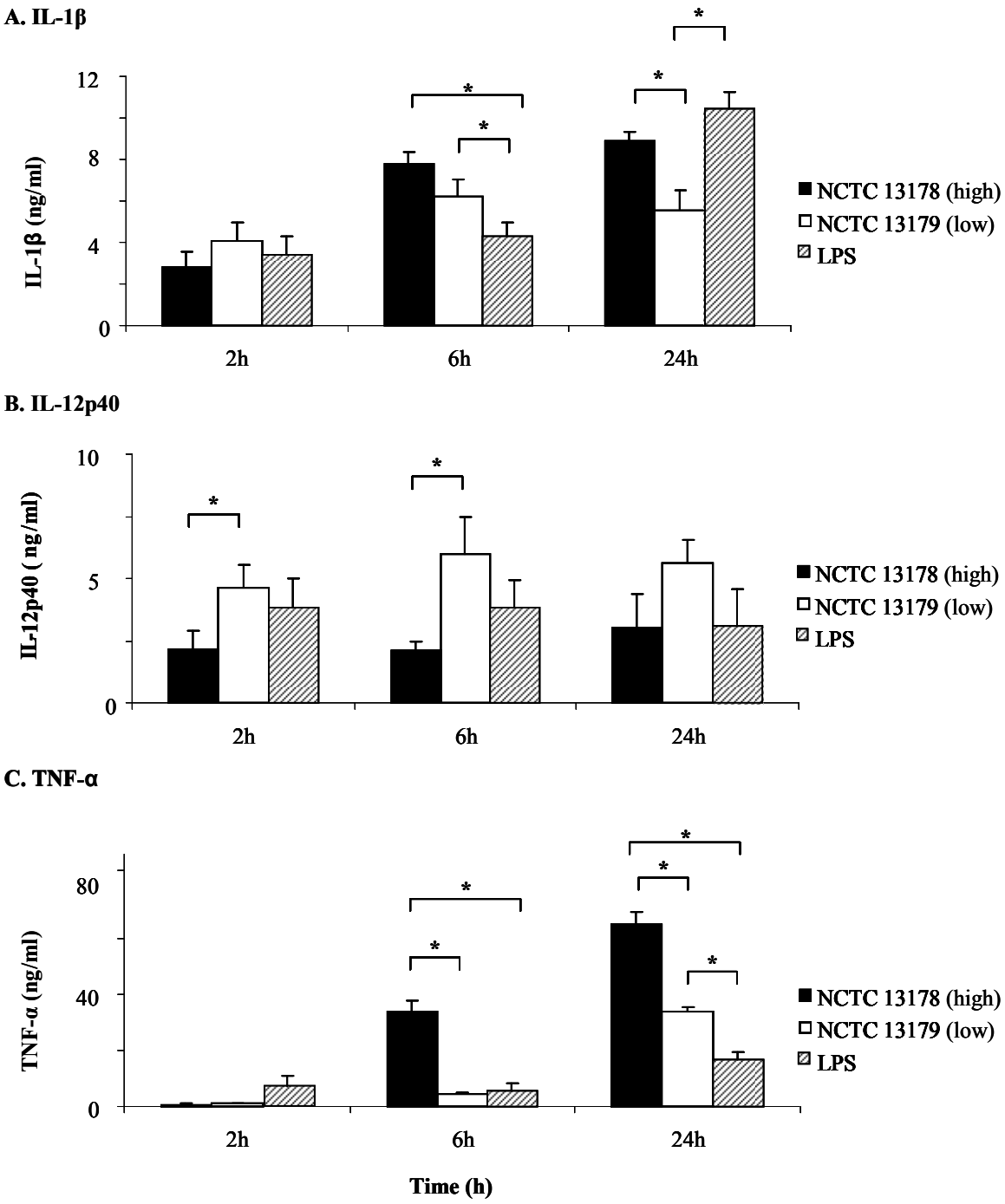

Figure 5.5 Selected pro-inflammatory cytokine secretion in THP-1 cells co-cultured with *B. pseudomallei* isolates

THP-1 cells were co-cultured with two *B. pseudomallei* isolates (the highly virulent NCTC 13178 and the low virulent NCTC 13179) for 2 h, 6 h and 24 h. Co-culture supernatants were assessed for (A) IL-1β (B) IL-12p40 and (C) TNF-α using ELISA. Means ± SEM are reported. * $p < 0.05$.

5.3.4 Determination of Cytokine Secretion by THP-1 Cells Co-Cultured with *Burkholderia pseudomallei*

To evaluate whether TLR-mediated secretion of cytokines varies in response to infection with two isolates, THP-1 cells were co-cultured with NCTC 13178 or NCTC 13179 and IL-1β, TNF-α, and IL-12p40 protein levels were measured in the supernatants. IL-12p40 secretion was significantly greater in co-cultures with the NCTC 13179 isolate at 6 h ($p < 0.05$; Figure 5.5B) however, secretion was not significantly different at 24 h. IL-1β secretion was significantly elevated in co-cultures at 24 h with NCTC 13178 in comparison to NCTC 13179 ($p < 0.05$; Figure 5.5A). Secretion of TNF-α ($p < 0.05$; Figure 5.5C) was also significantly greater from co-cultures with NCTC 13178 in comparison to NCTC 13179 at both 6 h (NCTC 13178: 34 ng/µl ± 3.5 vs. NCTC 13179: 4.2 ng/µl ± 0.6) and 24 h (NCTC 13178: 65 ng/µl ± 4.3 vs. NCTC 13179: 34 ng/µl / ± 1.3).

5.4 DISCUSSION

The current study focused on the activation of TLR2 and TLR4 in macrophages challenged with two *B. pseudomallei* isolates taken from patients with different clinical outcomes. These isolates (the highly virulent NCTC 13178 and the low virulent NCTC 13179) are extensively studied by our research group. Infection with NCTC 13178 and NCTC 13179 have been shown to establish disparate outcomes in murine models of *B. pseudomallei* infection where experimental disease outcome and severity are influenced by route of infection, bacterial strain, and infectious dose (Ulett *et al.*, 2000a; Barnes, 2005). It was our intention to assess these isolates in infection studies using human cell lines to determine if the severity of the inflammatory response elicited by these isolates could potentially be attributed to variations in TLR2 and TLR4 expression. The work described in this Chapter focused on TLR2 and TLR4 expression as these two receptors have both been shown to recognise *B. pseudomallei* and have generated some debate on which plays the more dominant role in host recognition.

Over the past few years several studies have indicated a critical role for TLRs during innate immune signalling in melioidosis (Wiersinga *et al.*, 2007c; Hii *et al.*, 2008;

West *et al.*, 2008a; Wiersinga *et al.*, 2008e). Conflicting results have emerged regarding the roles of TLR2 and TLR4 during *B. pseudomallei* infection (Wiersinga *et al.*, 2007c; Novem *et al.*, 2009). Several studies indicate that both are involved, however certain strains elicit a stronger TLR2 or TLR4 dependent response. Using *in vivo* assays of *B. pseudomallei* infection Wiersinga and colleagues have demonstrated that TLR2 plays the preeminent role as TLR2-deficiency extends survival compared to wild-type controls in knockout mice. They found reduced injury, bacterial burden and pro-inflammatory cytokine levels in distant organs of TLR2 deficient mice (TLR2 -/-), while TLR4 -/- mice were similar to wild-type controls. Similar findings for TLR2 involvement have been reported for *L. pneumophila*, another Gram negative intracellular pathogen (Archer and Roy, 2006). Several groups have used the closely related and avirulent species *B. thailandensis* as a surrogate to study *B. pseudomallei* pathogenesis. One such study investigating aerosolised *B. thailandensis* infection found that both TLR4 competent C3H/HeN and TLR4 -/- C3H/HeJ mice to be highly resistant to infection and were asymptomatic following a high dose challenge (Morici *et al.*, 2010). West and colleagues (2008) observed a modest TLR4-dependence in early pulmonary and systemic containment of low dose *B. thailandensis* aerosol infection, but survival was not TLR4-dependent. At a higher inoculum, similar to the *B. pseudomallei* model, TLR2-deficiency extended survival. Interestingly, in bone marrow derived macrophages TNF-α production induced by *B. thailandensis* required TLR4 and not TLR2 (West *et al.*, 2009). In another investigation, heat killed *B. pseudomallei*, lipid A and LPS preparations from *B. pseudomallei* signalled in a TLR4 dependant manner (West *et al.*, 2008a). Combined, these results suggest that TLR4 signalling may be relatively redundant *in vivo* whereas TLR2 activation is deleterious.

In a study comparing the structural and immunological activity of *B. pseudomallei* and *B. thailandensis* LPS, it was found that LPS derived from *B. pseudomallei* stimulated less TNF-α, IL-6 and IL-10 in both human and murine macrophage cell lines in comparison to *B. thailandensis* (Novem *et al.*, 2009). The observed variations in immunological activity between these two species have been attributed to their unique lipid A structure. In addition, both *B. pseudomallei* and *B. thailandensis* LPS mediated NF-κB activation via TLR4 rather than TLR2. The authors contend that the

attenuated cytokine production induced by *B. pseudomallei* is thought to be a potential mechanism of host evasion.

In the work described in this Chapter we found the uptake of the high virulent NCTC 13178 isolate was significantly greater in THP-1 cells than the low virulent NCTC 13179 isolate. Similar findings of increased uptake of NCTC 13178 in murine bone marrow derived dendritic cells have also been reported (Williams *et al.*, 2008). The aforementioned study and the current observations in this Chapter are consistent with those reported in Chapter 4 where the uptake of NCTC 13178 was greater than NCTC 13179 at 2 h in RAW 264.7 macrophages and BALB/c derived PECs. Of the internalised bacteria we found reduced intracellular survival of both isolates up to 24 h with no difference in survival between isolates at this time (Figure 5.5B). In the BALB/c and C57BL/6 models of *B. pseudomallei* infection histological analysis and bacteria enumeration in infected organs consistently shows increased NCTC 13178 survival over NCTC 13179 (Leakey *et al.*, 1998; Barnes and Ketheesan, 2005). The variation between NCTC 13178 and NCTC 13179 survival in THP-1 cells observed here may be due to analysis of a single cell type rather whole organs. The THP-1 cells used in this study were stimulated with IFN-γ prior to co-culture and this may account for the effective reduction in intracellular numbers of both isolates as IFN-γ is important for host cell priming and clearance of *B. pseudomallei* (Miyagi *et al.*, 1997; Santanirand *et al.*, 1999a; Koo and Gan, 2006).

Among the TLRs, TLR2 recognises the most diverse array of pathogens including bacteria, viruses, fungi and parasites (Akira *et al.*, 2006). While TLR2 primarily recognises Gram-positive bacterial surface structures such as peptidoglycan and lipoproteins, TLR2 can recognise LPS from some Gram negative pathogens including *L. pneumophila* and certain isolates of *B. pseudomallei* (Takeuchi *et al.*, 1999; Wiersinga *et al.*, 2007b; Hii *et al.*, 2008; West *et al.*, 2008a; Wiersinga *et al.*, 2008d; Yang and Joyee, 2008). TLR4 is the conical receptor for LPS and is also activated by *B. pseudomallei* isolates *in vitro* (Wiersinga *et al.*, 2007b; Hii *et al.*, 2008; Novem *et al.*, 2009). In the current study, TLR2 and TLR4 were differentially expressed in THP-1 cells co-cultured with two *B. pseudomallei* isolates. The low virulent NCTC 13179 isolate induced a substantially increased transcription of TLR2 and TLR4 in comparison to high virulent NCTC 13178 at 24 h (Figure 5.3A,

Figure 5.3B). The increase in TLR2 and TLR4 transcription detected here is supported by others who found increased TLR2 and TLR4 transcription in monocytes and granulocytes isolated from patients with septic melioidosis (Wiersinga *et al.*, 2007c). Similarly, other studies have demonstrated increased TLR2 transcription and total surface protein in patient cohorts with Gram-negative, Gram-positive and polymicrobial infection (Armstrong *et al.*, 2004). Our findings demonstrate that that the low virulent NCTC 13179 isolate has the capacity to increase TLR2 and TLR4 transcription in THP-1 cells. However, despite the increased transcription of TLR2 and TLR4 observed by the low virulent NCTC 13179 isolate, total pro-inflammatory cytokine secretion of IL-1β and TNF-α was significantly greater in THP-1 cells co-cultured with the highly virulent NCTC 13178 isolate (Figure 5.5A, Figure 5.5C). These findings are consistent with data in the BALB/c and C57BL/6 mouse models of *B. pseudomallei* infection where NCTC 13178 consistently induces greater pro-inflammatory cytokine secretion and mortality in infected animals (Ulett *et al.*, 2000a; Ulett *et al.*, 2000c; Ulett *et al.*, 2001; Barnes and Ketheesan, 2005). Due to the discrepancy between transcription and secretion of selected pro-inflammatory cytokines, determination of TLR2 and TLR4 surface expression in addition to downstream TLR activation by these two *B. pseudomallei* isolates is warranted.

The association of TLR expression and protein production is complex and not clearly defined. It has been shown that TLR2 protein expression in human monocytes is regulated transcriptionally and that mRNA expression correlates with TLR2 surface expression (Flo *et al.*, 2001; Armstrong *et al.*, 2004). Others have found increases in TLR4 mRNA expression and a decrease in total protein levels in LPS induced mononuclear phagocytes (Bosisio *et al.*, 2002). A study examining endotoxaemia in mice found a significant increase in TLR4 protein and a reduction in mRNA expression (Williams *et al.*, 2003). Additionally, LPS stimulated murine macrophages demonstrated transiently reduced TLR4 transcription after 2.5 h followed by a return to baseline levels at 20 h (Nomura *et al.*, 2000). Total TLR4 surface protein was also reduced after 1 h in the same study. Armstrong and colleagues found that TLR4 mRNA expression in monocytes only increased in patients with Gram positive infection and found no increase in total TLR4 protein in Gram negative infected individuals. However, in monocytic cultures from these patients, LPS stimulation increased TLR4 expression *in vitro* (Armstrong *et al.*, 2004). We observed similar

increases in TLR4 expression in THP-1 cells stimulated with commercial *E. coli* LPS preparations (Figure 5.3B). The biological relevance of TLR expression and actual TLR surface protein expression is difficult to determine when quantifying only one of these parameters given the large variation noted in the literature. Therefore, further work quantifying total TLR2 and TLR4 surface proteins using monoclonal antibodies in THP-1 cells co-cultured with our high and low virulent isolates is needed to clarify the relationship between transcription and translation of TLR2 and TLR4 during *B. pseudomallei* infection in this cell type.

The mechanism of downstream TLR activation by *B. pseudomallei* is also quite complex. Several Gram negative pathogens produce modified lipid A structures that nominally activate TLR4 (Darveau *et al.*, 2004; Kawasaki *et al.*, 2004; Portnoy, 2005). Certain *B. pseudomallei* isolates have been reported to activate TLR4 while others do not (Wiersinga *et al.*, 2007b; Novem *et al.*, 2009). Further, *B. pseudomallei* is capable of activating NF-κB and secretion of IL-8 independently of TLRs, as poor activation of TLRs may be a mechanism for *B. pseudomallei* survival in host cells (Hii *et al.*, 2008). We observed low level transcription of TLR2 and TLR4 by NCTC 13178, yet this isolate induced the greatest amount of pro-inflammatory cytokine production in co-culture supernatants (Figure 5.5A, Figure 5.5C). However, determination of NF-κB activation in cells co-cultured with these two isolates (Chapter 7) are needed to clarify this and help determine if the transcriptional differences reported here have any association to TLR2 and TLR4 mediated signalling.

In the current study we observed low level transcription of IL-1β in THP-1 cells co-cultured with the low virulent NCTC 13179 isolate and transcription was greater than the high virulent NCTC 13178 isolate at 2 h (Figure 5.4A). IL-1β transcription reached maximal levels at 6 h in THP-1 cells co-cultured with the high virulent NCTC 13178 isolate then decreased at 24 h (Figure 5.4A). These results are similar to others reported for *B. pseudomallei* where IL-1β transcription in monocytes infected with *B. pseudomallei* and *B. thailandensis* were relatively low over 24 h but also reached peak levels at 6 h (Pongcharoen *et al.*, 2008). Increased levels of IL-1β have been observed in leukocytes isolated from patients with septic melioidosis,

however IL-1β transcription was not increased in monocytes from these patients (Wiersinga *et al.*, 2007a).

At 24 h secretion of IL-1β was significantly increased by the high virulent NCTC 13178 isolate in comparison to low virulent NCTC 13179 (Figure 5.5A) with concentrations similar to those reported by others (Pongcharoen *et al.*, 2008). However, in the current study, transcription of IL-1β did not associate with total protein production (Figure 5.4A, Figure 5.5A). Large stores of IL-1β protein are know to persist in the cytosol of endotoxin stimulated macrophages and this could potentially explain the discrepancy between IL-1β transcription and total protein observed here, however we can cannot confirm this (Wewers and Herzyk, 1989; Hogquist *et al.*, 1991). Studies within our laboratory have correlated high levels of IL-1β mRNA in livers of susceptible BALB/c mice and relatively resistant C57BL/6 mice following acute infection with the same isolates (Ulett *et al.*, 2000c; Ulett *et al.*, 2000a). The discrepancy from the results presented here could potentially be attributed to assessment of cell line mRNA as opposed to whole organ analysis. *In vitro* matured monocytic cells have been shown to have a decreased capacity to produce both IL-1β and TNF-α (Wewers and Herzyk, 1989; Hogquist *et al.*, 1991). In the work described in Chapter 4 and in this Chapter we found that *B. pseudomallei* is a relatively poor inducer of selected pro-inflammatory cytokines at the early stages of infection and that a decreased immune response may lead to establishment of intracellular persistence initially as indicated by *B. pseudomallei* survival in RAW 264.7 macrophages (Table 4.2), BALB/c PECs (Table 4.3) and THP-1 cell co-culture (Figure 5.2B).

When stimulated by intracellular organisms macrophages secrete IL-12, an important cytokine whose central role involves the link between innate resistance and the development of adaptive immunity (Trinchieri, 1995). IL-12 is a 70kDa heterodimer consisting of two subunits, p40 and p35 which are encoded by separate genes. Production and secretion of both subunits are necessary to produce the bioactive p70 heterodimer (Gibney *et al.*, 2008). The p35 subunit is secreted in association with the p40 subunit and is expressed in lower abundance in activated cells making it the limiting factor in active IL-12p70 heterodimer formation (Wolf *et al.*, 1991; Snijders *et al.*, 1996). While p35 and p40 are independently regulated both are needed to

generate the bioactive IL-12p70 heterodimer (Babik *et al.*, 1999). Besides dimerisation with IL-12p35, the p40 subunit can dimerise with itself forming an IL-12p80 dimer (Ling *et al.*, 1995). In this study IL-12p40 was selected as a measure of IL-12 transcription in THP-1 cells and was assessed previously for the NCTC 13178 and NCTC 13179 isolates in murine models of *B. pseudomallei* infection (Ulett *et al.*, 2000a). Further studies should be conducted to determine expression of total functional IL-12p70 expression and production in THP-1 cells challenged with these two isolates.

IL-12 stimulates IFN-γ production by NK and T cells and initiates a Th1 type response which drives macrophage activation and host resistance to intracellular infection (Trinchieri, 1995). In patients with culture-proven melioidosis, IFN-γ inducing cytokines, including IL-12, demonstrated a positive correlation with increased IFN-γ plasma levels (Lauw *et al.*, 1999). IFN-γ has been shown to increase macrophage resistance to *B. pseudomallei* in murine macrophages by increasing production of radical oxygen and nitrogen species (Miyagi *et al.*, 1997; Santanirand *et al.*, 1999b). In this study, IL-12p40 transcription was significantly greater in THP-1 cells co-cultured with the low virulent NCTC 13179 isolate at 6 h and 24 h in comparison to the high virulent NCTC 13178 isolate and LPS controls (Figure 5.4B). Similarly, IL-12p40 secretion was elevated in THP-1 cells co-cultured with NCTC 13179 however this was only significantly different at 2 h and 6 h (Figure 5.5B). Nominal increases in levels of IL-12p40 expression have been observed in spleen and liver homogenates isolated from BALB/c and C57BL/6 mice infected with NCTC 13178 and NCTC 13179 in our laboratory and are consistent with the results in this study (Ulett *et al.*, 2000a). Others have found increased IL-12p40 levels from whole blood stimulated with heat killed *B. pseudomallei* at 24 h (Lauw *et al.*, 1999). In the same study IL-12p40 levels were also elevated in blood taken from 31 patients admitted with septic melioidosis (Lauw *et al.*, 1999). Our findings are consistent with those studies and we have demonstrated that IL-12p40 secretion elicited by low virulent NCTC 13179 in this *in vitro* system is greater than the high virulent NCTC 13178 isolate.

TNF-α is secreted by macrophages and neutrophils and possesses a vast spectrum of activity. These functions include up-regulation of adhesion molecules, increased

vascular permeability and recruitment of leukocytes to infected tissues. TNF-α is expressed in soluble and membrane bound forms by various cell types, and this variation in form dictates either local or systemic effects (Sedgwick *et al.*, 2000). TNF-α plays an important role in controlling intracellular pathogens as innate immune responses are thought to contribute to resistance in murine models of *B. pseudomallei* and *Listeria spp*. infection (Ulett *et al.*, 2000c; Grivennikov *et al.*, 2005; Barnes and Ketheesan, 2007). In support of this, Barnes and colleagues have shown that TNF-α and its associated cell surface receptors TNFR1 and TNFR2 are essential to modulate inflammatory cell infiltrate composition, contain rapid dissemination and minimise necrosis in infected tissue in mice (Barnes *et al.*, 2008). While lower concentrations of TNF-α are beneficial, hyperproduction of TNF-α is deleterious and contributes to the pathogenesis of septic shock. Clinically, high levels of TNF-α correlate with disease severity and early mortality in patients with acute melioidosis and this phenomenon is also observed in animal models of infection (Suputtamongkol *et al.*, 1992; Ulett *et al.*, 2000c).

The high virulent NCTC 13178 isolate used in this study has been shown to elicit a severe inflammatory response in experimental melioidosis (Ulett *et al.*, 2000a), where acute infection was lethal in mice within 48 h (Leakey *et al.*, 1998). In the current study we observed a significant increase in TNF-α secretion induced by the high virulent NCTC 13178 isolate from 6 h to 24 h in comparison to the low virulent NCTC 13179 isolate and LPS controls (Figure 5.5C). This finding supports earlier work in our laboratory and others whereby high levels of TNF-α transcription and secretion correlate with the development of acute *B. pseudomallei* infection in mice (Ulett *et al.*, 2000a; Wiersinga *et al.*, 2008b). Others have found that *B. pseudomallei* is a poor inducer of TNF-α in RAW 264.7 macrophages using low infectious doses and detectable levels are only observed when bacterial challenge is high (MOI 10:1). This would support the levels of TNF-α observed here as an MOI 10:1 was used. High bacterial numbers correlate with increased pro-inflammatory secretion in both mice and humans and would help explain the results here as bacteria were not completely cleared at 24 h (Lauw *et al.*, 1999; Simpson *et al.*, 2000; Ulett *et al.*, 2002; Wiersinga *et al.*, 2007b). While previous *in vitro* studies have found that *B. pseudomallei* is a less potent activator of macrophages in comparison to other Gram negative species (Utaisincharoen, 2001), our data suggest that under these

conditions *B. pseudomallei* is capable of inducing an increased inflammatory response from 6 h of co-culture particularly for the high virulent NCTC 13178 isolate (Figure 5.3, Figure 5.5). Prior to 6 h selected pro-inflammatory cytokine secretion is minimal and this could be a possible mechanism of host evasion and establishment of infection as evidence by survival of *B. pseudomallei* in THP-1 cells (Figure 5.2B). An essential aspect is to determine whether the degree of TNF-α expression and secretion is a function of TLR2 and TLR4 activation. Quantifying TLR2 and TLR4 surface expression and TLR activation via NF-κB would further our understanding of their roles in *B. pseudomallei* induced signalling in macrophages.

This study evaluated TLR2 and TLR4 mediated signalling in THP-1 cells and focused on TLR2, TLR4 and selected pro-inflammatory cytokine mRNA expression and secretion in the presence of two *B. pseudomallei* isolates, one of high and one of low virulence. A different TLR2 and TLR4 expression pattern was observed in THP-1 cells co-cultured with the high virulent NCTC 13178 and low virulent NCTC 13179 isolates with TLR2 and TLR4 expression increased in cells co-cultured with NCTC 13179. Despite lower level expression of TLR2 and TLR4, the high virulent isolate induced significantly greater concentrations of IL-1β and TNF-α and demonstrate different THP-1 cell responses to these two isolates. Further studies need to be conducted on the panel of *B. pseudomallei* isolates analysed in the work described in Chapter 4 (Table 3.1) to determine if THP-1 cell response to high virulence isolates (as determined in mice) is consistent with the observations in the current study. The mechanistic details of downstream TLR activation were not determined here and further work assessing the activation of NF-kB by NCTC 13178 and NCTC 13179 are needed to clarify the relationship between TLR expression and activation of TLR pathways in THP-1 cells co-cultured with these isolates. Activation of NF-kB by these isolates is the focus of the work described in Chapter 7. Furthermore, studies assessing survival of TLR2 and TLR4 knockout mice infected with NCTC 13178 and NCTC 13179 would provide *in vivo* data on the progression of infection in the host by these isolates.

CHAPTER 6
TOLL-LIKE RECEPTOR ACTIVATION IN SUSCEPTIBLE AND PARTIALLY RESISTANT MICE FOLLOWING *BURKHOLDERIA PSEUDOMALLEI* INFECTION WITH A HIGHLY VIRULENT ISOLATE

6.1 INTRODUCTION

Host genetic factors and associated co-morbidities influence disease outcome in individuals with melioidosis. In 20% to 30% of patients there are no evident risk factors, with apparently healthy individuals contracting the disease (Currie, 2003). Infection in healthy individuals is often cleared provided early diagnosis and appropriate antibiotic therapy is provided. Patients who present with no underlying risk factors are more likely to survive (Currie *et al.*, 2010). There is a marked heterogeneity amongst clinical presentations and disease outcome in patients with melioidosis. The clinical manifestations are varied, ranging from an asymptomatic state to a potentially fatal acute septicaemia (White, 2003). Even if a patient survives an acute episode, a chronic infection can develop where the onset of clinical symptoms can range from several months to many years (Chaowagul *et al.*, 1989). Chronic melioidosis is characterised by a latent, localised foci of infection within almost any organ with symptoms of the disease persisting for over two months (Dance, 1991; Currie *et al.*, 2010). Recrudescence or reactivation of infection is correlated with immunosuppressive co-morbidities (Chaowagul *et al.*, 1993; White, 2003; Peacock, 2006).

Determination of the pathogenic mechanisms of *B. pseudomallei* infection relies on the use of animal models. BALB/c and C57BL/6 mice have been used extensively to investigate the pathogenesis of multiple intracellular pathogens including *S. typhimurium* (Jabado *et al.*, 2003), *L. major* (Louis *et al.*, 2003) and mycobacterium infection (Rosas-Taraco *et al.*, 2010). In our laboratory the pathogenesis of acute and chronic *B. pseudomallei* infection have been investigated

using these murine models. BALB/c mice represent highly susceptible hosts and the progression of disease in these animals is rapid and mimics the symptoms observed clinically in acute melioidosis cases (Leakey *et al.*, 1998). Substantial bacterial loads are present in the blood, lung, spleen and liver following *i.v.* challenge with virulent *B. pseudomallei* in these mice which succumb to sepsis within 72 h to 96 h (Leakey *et al.*, 1998). In contrast, C57BL/6 mice are relatively resistant to infection, often remaining asymptomatic for up to six weeks before fatal outcomes were observed (Leakey *et al.*, 1998). Others have confirmed these results (Hoppe *et al.*, 1999). In a recent study, Conejero and colleagues characterised chronic infection in C57BL/6 mice up to 100 days where low dose infection induced histopathology and pro-inflammatory cytokine production (IFN-γ, IL-6, MCP-1, TNF-α) consistent with human melioidosis (Conejero *et al.*, 2011). Therefore, C57BL/6 mice provide an appropriate model to study immune responses to *B. pseudomallei* in a partially resistant host. The exact mechanism of disease progression is not fully understood in these hosts, however, genetic variation are known to influence susceptibility to *B. pseudomallei* (Ulett *et al.*, 2000c; Ulett *et al.*, 2000a). Using these models the establishment of both acute and chronic infection can be investigated to assist in the understanding of disease progression in hosts with different susceptibilities.

The TLR receptor family plays a pivotal role in pathogen recognition. Toll-like receptors are located on several immune cells types including dendritic cells, monocytes and macrophages which recognise microbial structures termed pathogen associated molecular patterns (PAMPs). Several of these PAMPs include bacterial components such as LPS, lipoteichoic acid, lipoproteins, flagella, and CpG DNA (Akira *et al.*, 2006). Ligand specific binding of PAMPs to TLRs initiates distinct patterns of pro-inflammatory gene expression which help shape the host response (O'Neill, 2006). *Burkholderia pseudomallei* has the capacity to up-regulate host cell gene transcription of pro-inflammatory cytokines in both infected human and murine hosts (Ulett *et al.*, 2000c; Ulett *et al.*, 2001; Wiersinga *et al.*, 2008b). The initiation of an efficient inflammatory response and production of several of these cytokines (IFN-γ, TNF-α) are critical for clearance of *B. pseudomallei* (Ulett *et al.*, 2000c; Justin *et al.*, 2007; Barnes *et al.*, 2008; Tan *et al.*, 2008). However, prolonged or dysregulated immune responses to *B. pseudomallei* can lead to hyperproduction of pro-inflammatory cytokines resulting in fatal septicaemias as seen in patients with

acute melioidosis (Currie *et al.*, 2010). Mice deficient in TLR4 are hyporesponsive to LPS and mice with this particular TLR4 phenotype are more susceptible to Gram negative infection (Hoshino *et al.*, 1999; van Westerloo *et al.*, 2005; Knapp *et al.*, 2006). In a recent study in northern Thailand, an area of high endemicity for *B. pseudomallei*, individuals with the $TLR4_{1196C>T}$ polymorphisms demonstrated protection against infection while those with the rs960312 variant were more susceptible (West *et al.*, 2011). In mice, TLR2 deficiency in a *P. aeruginosa* pneumonia model demonstrated that TLR2 serves a counter regulatory role and is deleterious to the host (Skerrett *et al.*, 2007). In that same study, both TLR4 and TLR5 mediated signalling provided protection. These data imply that TLR activation and the immune response during infection varies depending on the host genetic profile.

Toll-like receptor and pro-inflammatory cytokine profiles in cells and tissues infected with *B. pseudomallei*, using both *in vitro* and *in vivo* assays have been reported. On the surface of peripheral blood monocytes and granulocytes of patients with septic melioidosis, several TLRs (TLR1, TLR2, TLR4) and the TLR-associated protein CD14 were up-regulated (Wiersinga *et al.*, 2007c). In the same study, mRNA levels of TLR1, TLR2, TLR4, TLR5, TLR8, CD14 and MD-2 were also increased in unfractioned leukocytes as determined by multiplex ligation probe dependant amplification (Wiersinga *et al.*, 2007c). A comprehensive study of the pulmonary and systemic compartments of *B. pseudomallei* infected C57BL/6 mice, demonstrated marked increases in transcription of multiple TLR (TLR1-TLR10) and pro-inflammatory genes (Wiersinga *et al.*, 2008a; Wiersinga *et al.*, 2008b). Additionally, elevated transcriptional levels of multiple cytokines were reported in spleens and livers from BALB/c and C57BL/6 mice at several time points of infection using standard reverse transcriptase PCR (Ulett *et al.*, 2000a; Ulett *et al.*, 2000c). Determining the effect of *B. pseudomallei* on TLR expression, pro-inflammatory cytokine transcription and secretion is important to gaining a more comprehensive understanding of some of the key functions of phagocytic cell recognition of *B. pseudomallei* in hosts with different genetic backgrounds.

In this study the highly virulent NCTC 13178 isolate was selected for infection studies in PECs derived from BALB/c and C57BL/6 mice. Detailed cytokine profiles have

been established in these hosts using the NCTC 13178 isolate up to 96 h (Ulett *et al.*, 2000c; Ulett *et al.*, 2000a; Ulett *et al.*, 2001). The pathogenesis of the disease using the NCTC 13178 isolate has been compared using different infection routes (*iv*, *ip*, intranasal, subcutaneous and oral) in BALB/c and C57BL/6 mice. C57BL/6 mice demonstrated up to a 3 fold increase in bacterial cfu in infected organs in LD_{50} studies in comparison to BALB/c mice (Barnes and Ketheesan, 2005). The results in this Chapter build on the knowledge we have about the immune responses of these murine hosts by assessing TLR and selected pro-inflammatory cytokine expression in hosts with differing susceptibilities at several time points during the first 24 h of infection. This follows from the results described in Chapter 5 where we observed changes in TLR2 and TLR4 expression in human THP-1 macrophages up to 24 h which provided the template for the work described in this Chapter. To our knowledge this is the first time comparisons in TLR expression and TLR mediated responses have been assessed in primary cells derived from susceptible and partially resistant hosts following exposure to live *B. pseudomallei*.

The specific aims of the work described in this Chapter were to:

1) Compare the uptake and killing of a highly virulent *B. pseudomallei* isolate by elicited peritoneal exudate cells (PECs) harvested from susceptible (BALB/c) and partially resistant (C57BL/6) hosts during the first 24 h of infection.

2) Determine changes in TLR2, TLR4, TLR5, and TLR9 expression in PECs harvested from susceptible (BALB/c) and partially resistant (C57BL/6) hosts during the first 24 h of *B. pseudomallei* infection using reverse transcriptase polymerase chain reaction.

3) Determine changes in selected pro-inflammatory cytokine (IL-1β, IL-12p40, TNF-α) expression in PECs harvested from susceptible (BALB/c) and partially resistant (C57BL/6) hosts during the first 24 h of infection using reverse transcriptase polymerase chain reaction.

4) Compare selected levels of pro-inflammatory cytokine (IL-1β, IL-12p40, TNF-α) secretion by PECs harvested from susceptible (BALB/c) and partially resistant (C57BL/6) hosts during the first 24 h of *B. pseudomallei* infection using ELISA.

6.2 MATERIALS AND METHODS

6.2.1 Origin of Bacterial Isolate

A highly virulent clinical *B. pseudomallei* isolate (NCTC 13178) was used in this study. Bacteria were grown in brain heart infusion broth (Oxoid, SA, Australia) at 37°C for 18 h and stored at -80°C with 40% glycerol until use. The LD_{50} values for this isolate in BALB/c and C57BL/6 mice have been previously described (Barnes, 2005). *Salmonella typhimurium* ACTC 14028 was selected as a control for internalisation assays as it represents another Gram negative intracellular pathogen. *Escherichia coli* ACTC 25922 and purified *P. aeruginosa* LPS (Sigma-Aldrich, New South Wales, Australia) were used as controls for TLR and cytokine transcription analyses. Bacterial isolate preparations have been previously described in Chapter 3, section 3.1. PECs were seeded at 1×10^8 cells per ml and infected at a MOI 10:1 with NCTC 13178.

6.2.2 Culture of Peritoneal Exudate Cells

PECs were induced and isolated as described in Chapter 4, section 4.2.3. PEC culture conditions of BALB/c and C57BL/6 mice (mixed sex, 6-8 weeks of age) were carried out according to methods described in Chapter 4, section 4.2.3. All animal related procedures were carried out under Ethics Approval A1069, provided by the James Cook University Animal Ethics Sub-Committee.

6.2.3 Internalisation Assay

Antibiotic protection assays were performed on BALB/c and C57BL/6 PECs at 2 h, 6 h and 24 h according to methods previously described in Chapter 4, section 4.2.4. PECs were co-cultured with live NCTC 13178, *E. coli* , *S. typhimurium* or *P. aeruginosa* LPS (10 ng/ml). LPS concentrations (1 ng/ml, 10 ng/ml and 100ng/ml)

were optimised prior to experimental trials and a concentration of 10 ng/ml was selected and used in all subsequent assays.

6.2.4 Reverse Transcriptase Polymerase Chain Reaction

Following 2 h, 6 h, and 24 h co-culture of PECs with NCTC 13178 and *E. coli* and LPS controls, mRNA was isolated from duplicate culture wells according to methods described in Chapter 4, section 4.2.5. Briefly, cDNA was constructed and semi quantitative reverse transcriptase polymerase chain reaction (RT PCR) was performed using gene-specific primers for TLRs and cytokines (Table 6.1). Cycling parameters were repeated 35 times for all reactions under the following conditions, 94°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min.

Table 6.1 Gene specific primers used for amplification of TLRs and cytokines in BALB/c and C57BL/6 PECs co-cultured with *B. pseudomallei* isolates.

Primer	Primer Sequence	Product Size (bp)
TLR2 (for)	ATCAGTCCCAAAGTCTAA	787
TLR2 (rev)	TCCAACACCTCCAGCGTCT	
TLR4 (for)	ATTGTATCGCCTTCTTAGCAG	141
TLR4 (rev)	GGTCCAAGTTGCCGTTTCT	
TLR5 (for)	AACGTCACCCTGTTCGGCTCTC	523
TLR5 (rev)	CGGCTCTGGGCATACCTGA	
TLR9 (for)	TCTTCCGCTCGCTCAACA	356
TLR9 (rev)	ACACTGGAGGCGTGAGAGATTGAC	
IL-1β (for)	TGTGAAATGCCACCTTTTGA	123
IL-1β (rev)	GTAGCTGCCACAGCTTCTCC	
IL-12p40 (for)	AGACCCTGCCCATTGAACTG	135
IL-12p40 (rev)	CTGACCTCCACCTGTGAGTTCTT	
TNFα (for)	AAGAGGCACTCCCCAAAAG	157
TNFα (rev)	ACCGATCACCCCGAAGTTC	
β-actin (for)	TCATGAAGTGTGACGTTGACATCCGT	234
β-actin (rev)	CCTAGAAGCATTTGCGGTGCACGATG	

6.2.5 Densitometry Analysis of TLR Expression and Selected Pro-Inflammatory Cytokines

Image analysis was carried out on agarose gels containing TLR and cytokine gene products using Gene Tools Software (Syngene, Cambridge, UK). Intensity of TLR and cytokine mRNA products are reported as a proportion of intensity of the house keeping gene, β-actin. Densitometry analysis of gene product bands were carried out using the equation outlined in Chapter 4, section 4.2.6, and semi quantification of band intensity was determined using GeneTools Software (Syngene, Cambridge, United Kingdom). Experiments used pooled PEC preparations (n = 3) and were run in duplicate.

6.2.6 Determination of Cytokine Production

IL-1β, IL-12p40 and TNF-α secretion from BALB/c and C57BL/6 PEC co-culture supernatants were determined at 2 h, 6 h and 24 h using the eBioscience Ready-Set-Go! ELISA kit (Jomar Diagnostics, South Australia, Australia) and concentrations were determined according to the manufacturer's instructions (Appendix III). Experiments used pooled PECs preparations (n = 3) and were run in duplicate.

6.2.7 Statistical Analysis

Analysis of uptake and killing from BALB/c and C57BL/6 PEC was done using a post hoc univariate analysis (SPSS software version 12.0.0) of means ± standard deviation (SD). Univariate repeated measures ANOVA were performed on TLR and selected pro-inflammatory cytokine expression and on ELISA results assessing cytokine concentration in co-culture supernatants using Statistica software (StatSoft Inc, Victoria, Australia). Differences with a value of $p < 0.05$ were considered significant.

6.3 RESULTS

6.3.1 Internalisation and Killing of *B. pseudomallei* by BALB/c and C57BL/6 Derived PECs

C57BL/6 derived PECs were significantly more efficient at uptake of NCTC 13178 at 2 h, 6 h and 24 h co-culture in comparison to BALB/c PECs (Figure 6.1).

C57BL/6 PECs were more efficient at killing internalised NCTC 13178 at 2 h (Figure 6.1). Over 92% of all internalised bacteria were killed by both BALB/c and C57BL/6 PECs by 24 h and there was no difference in killing between hosts at this time point (Figure 6.1). However, it should be noted that C57BL/6 PECs killed more bacteria than BALB/c PECs due to a greater uptake of bacteria at 2 h, 6 h and 24 h.

6.3.2 TLR Expression Following Co-Culture with *B. pseudomallei*

Elevated TLR2 expression was observed in BALB/c PECs and was significantly greater than C57BL/6 PECs at 24 h (Figure 6.2A). In BALB/c PECs co-cultured with NCTC 13178 TLR4 expression was low from 2 h to 6 h followed by rapidly increased expression from 6 h to 24 h (Figure 6.2B). Of the TLRs examined, TLR4 expression was the highest observed in both hosts. NCTC 13178 induced expression of TLR4 was similar to *E. coli* and LPS controls. TLR5 expression in BALB/c PECs decreased over 24 h co-culture and was significantly greater than C57BL/6 PECs at 2 h (Figure 6.2C). TLR9 expression increased in BALB/c PECs at 6 h and was significantly greater than C57BL/6 PECs at this time followed by decreased expression at 24 h (Figure 6.2D).

Elevated TLR2 expression was observed in C57BL/6 PECs and remained approximately the same intensity from 2 h to 24 h (Figure 6.2A). Increased TLR4 expression was also observed between 6 h and 24 h co-culture (Figure 6.2B). TLR4 expression was significantly lower in C57BL/6 PECs (123%) in comparison to BALB/c PECs (295%) at 24 h. TLR5 expression was detected at very low levels in C57BL/6 PECs co-cultured with NCTC 13178 from 2 h to 24 h (Figure 6.2C). In contrast to BALB/c PECs (range 6%-9.1%) TLR9 expression increased in C57BL/6 PECs (range 34.2%-40.2%) at 24 h (Figure 6.2D).

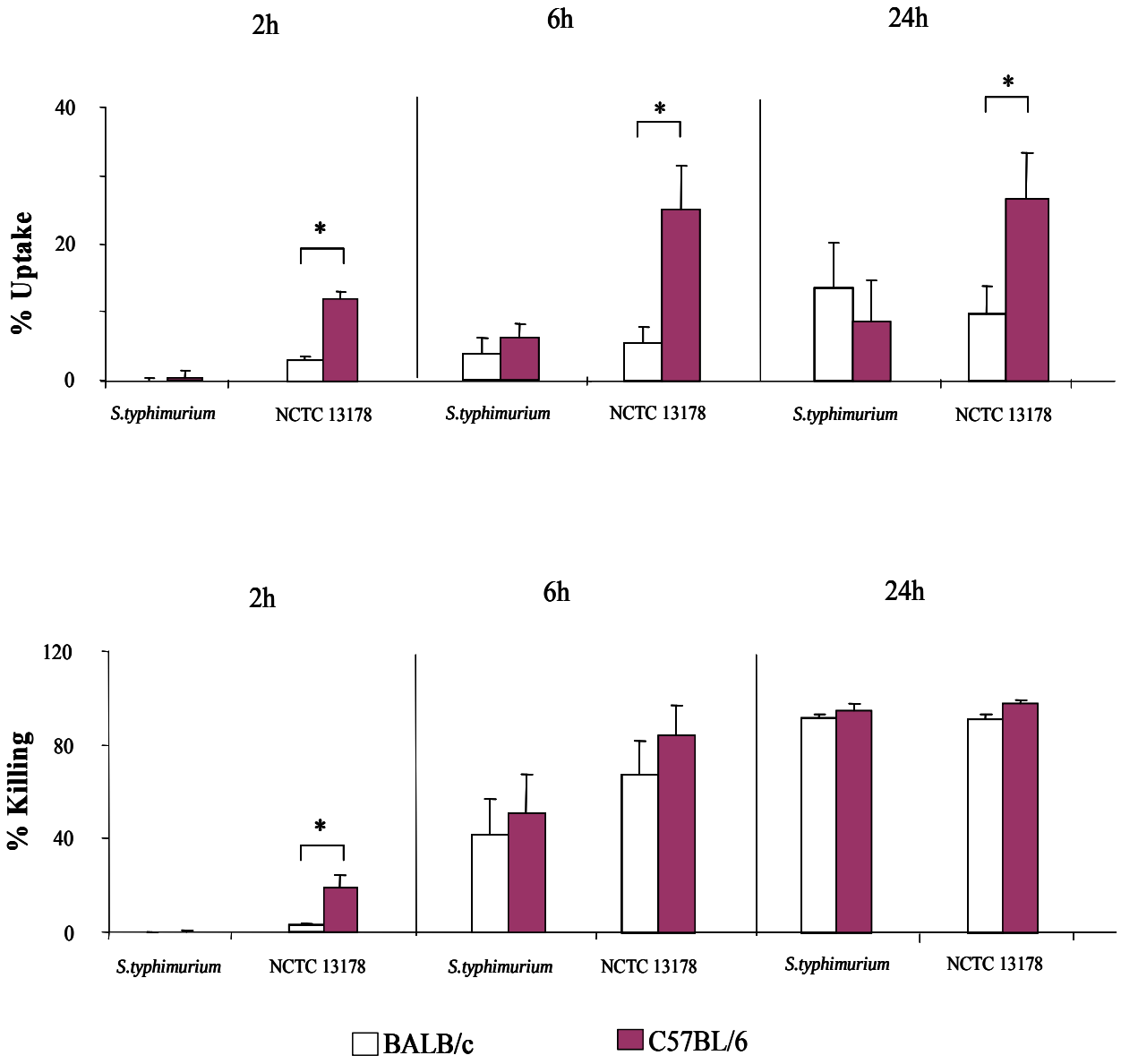

Figure 6.1 Uptake and killing of a highly virulent *B. pseudomallei* isolate by BALB/c and C57BL/6 derived PEC cells

A significant increase in the uptake of the highly virulent NCTC 13178 *B. pseudomallei* isolate by C57BL/6 PECs was observed at 2 h, 6 h and 24 h in comparison to BALB/c PECs. C57BL/6 PECs were more efficient at killing internalised NCTC 13178 at 2 h in comparison to BALB/c PECs. *S. typhimurium* was used as control. Means ± SEM are reported. Differences with a value of $p < 0.05$ were considered significant.

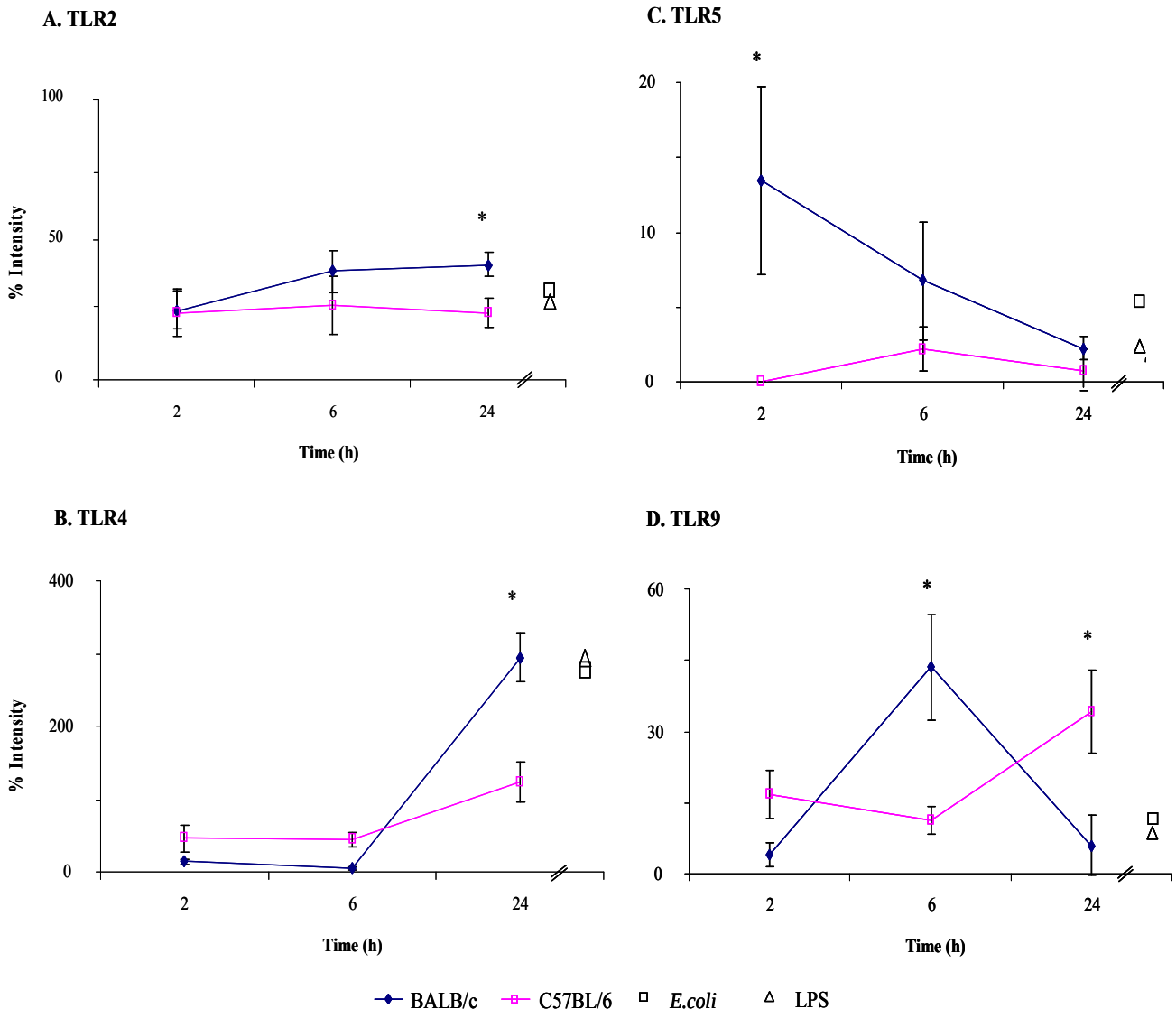

Figure 6.2 RT PCR analysis of TLR mRNA expression in PECs derived from BALB/c and C57BL/6 co-cultured with a highly virulent *B. pseudomallei* isolate

(A) Elevated TLR2 expression was observed in both BALB/c and C57BL/6 PEC over 24 h. (B) An increase in TLR4 expression was observed in BALB/c and C57BL/6 PECs over 24 h. TLR4 expression was greater in BALB/c PEC cells in comparison to C57BL/6 at 24 h. (C) TLR5 expression was greater in BALB/c PECs in comparison to C57BL/6 PEC at expression was low in both BALB/c and C57BL/6 PECs over 24 h. (D) A significant increase in TLR9 expression in BALB/c PECs in comparison to C57BL/6 was significant at 6 h. At 24 h co-culture C57BL/6 PEC TLR9 expression was greater than BALB/c PEC. *E. coli* (Δ) and LPS (□) controls at 24 h are to demonstrate endpoint comparisons to *B. pseudomallei* treatments. Intensity of TLRs is reported as a percentage of β-actin. Means ± SEM are reported. Differences with a value of $p < 0.05$ were considered significant.

6.3.3 Cytokine Expression Following Co-Culture with *B. pseudomallei*

In BALB/c PECs cytokine expression increased as measured by RT PCR for IL-1β, IL-12p40 and TNF-α over 24 h (Figure 6.3). A sharp increase in expression of TNF-α expression was observed between 6 h and 24 h in BALB/c PECs (Figure 6.3C). In PECs harvested from C57BL/6 mice, increased cytokine expression was observed for IL-1β and TNF-α at 6 h and 24 h (Figure 6.3A, Figure 6.3C). Maximal expression of IL-12p40 was observed at 6 h co-culture in C57BL/6 PECs.

6.3.4 Cytokine Production Following Co-Culture with *B. pseudomallei*

Secretion of IL-1β, IL-12p40 and TNF-α production from BALB/c and C57BL/6 PECs was assessed to determine whether production correlates with increased mRNA expression. IL-1β, IL-12p40 and TNF-α expression increased in BALB/c PECs over 24 h co-culture (Figure 6.3), correlating with increased production of each cytokine up to 24 h (Figure 6.4). TNF-α production in BALB/c PECs co-cultured with NCTC 13178 increased between 2 h and 6 h with a concentration plateau from 6 h to 24 h (Figure 6.4C).

For all selected pro-inflammatory cytokines, production increased in C57BL/6 PECs over 24 h (Figure 6.4). Rapid increases in secretion of IL-1β and TNF-α were observed between 6 h and 24 h (Figure 6.4A, Figure 6.4C), while IL-12p40 secretion steadily increased up to 24 h (Figure 6.4B).

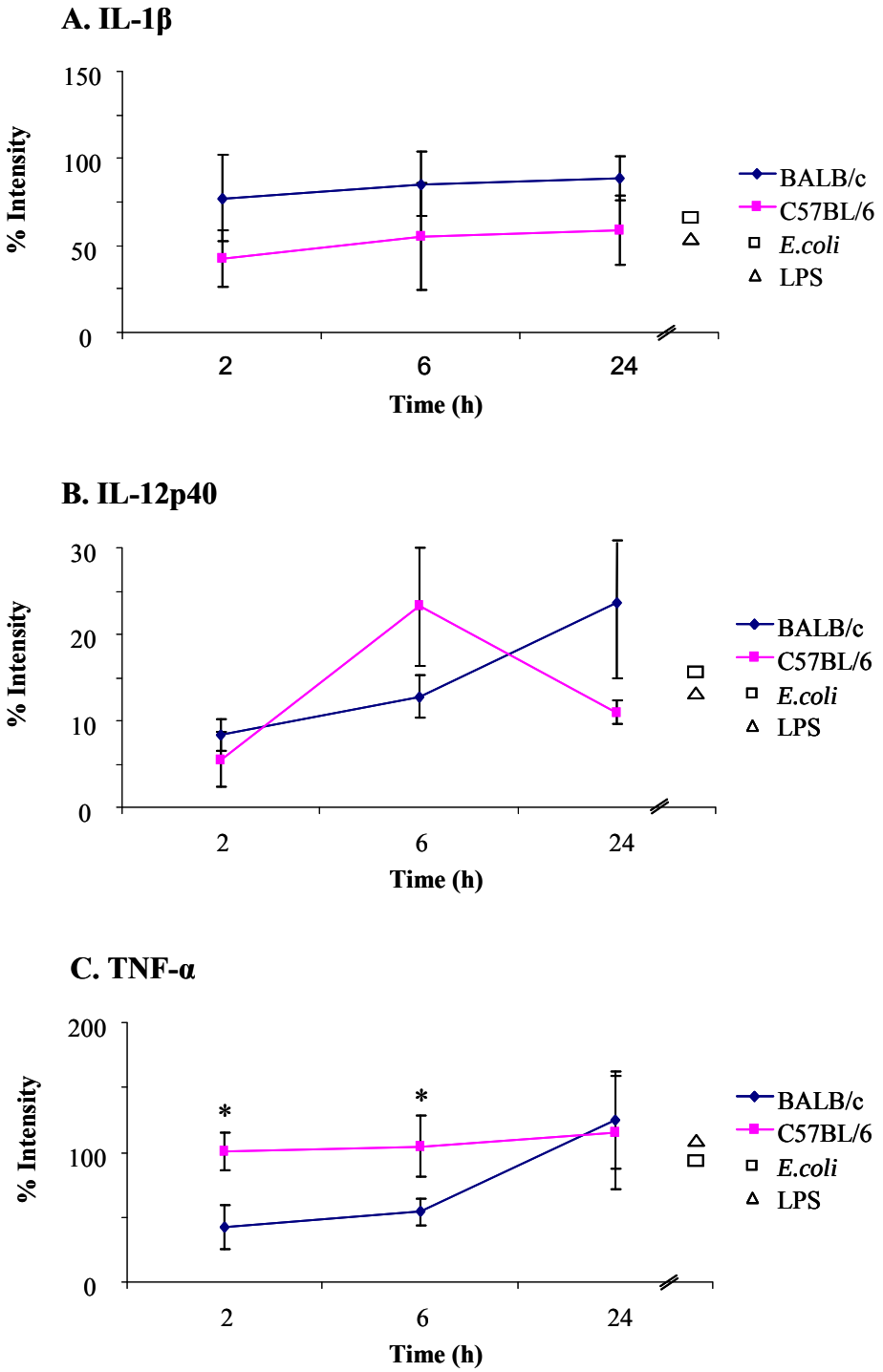

Figure 6.3 mRNA expression of selected pro-inflammatory cytokines from BALB/c and C57BL/6 PECs co-cultured with *B. pseudomallei*

With the exception of IL-12p40 transcripts in C57BL/6 PEC cells, increased mRNA expression was observed for IL-1β, IL-12p40 and TNF-α over 24 h. NCTC 13178 induced a higher overall expression of IL-1β and TNF-α over 24 h of co-culture in BALB/c PEC. *E. coli* (Δ) and LPS (□) controls at 24 h are to demonstrate endpoint comparisons to *B. pseudomallei* treatments. Means ± SEM are reported. (*) Differences with a value of $p < 0.05$ were considered significant.

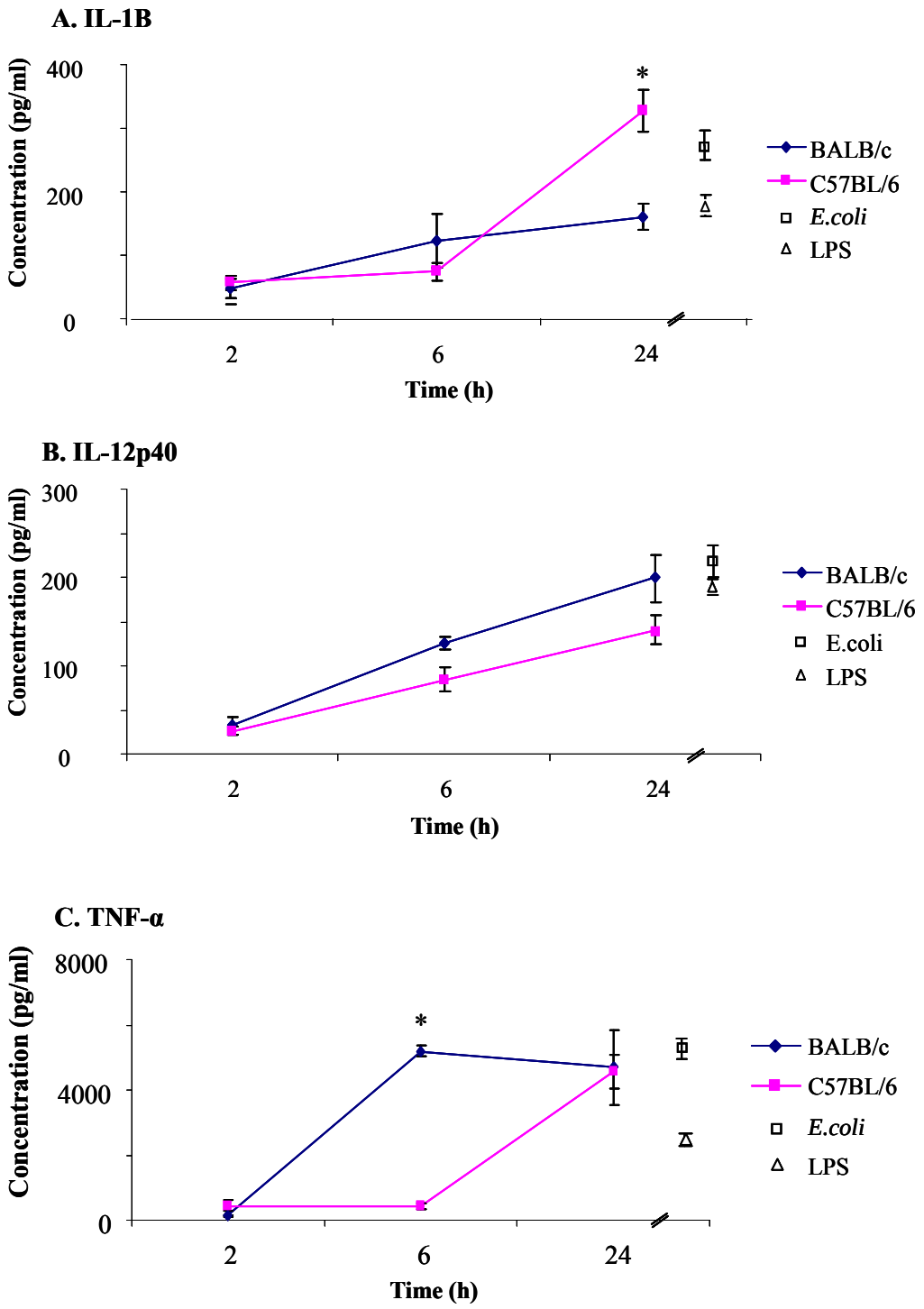

Figure 6.4 Cytokine secretion from PECs isolated from BALB/c and C57BL/6 mice co-cultured with *B. pseudomallei*

All cytokine concentrations significantly increased at 24 h in both BALB/c and C57BL/6 PEC cells for all isolates. The NCTC 13178 isolate induced greater secretion of TNF-α at 6 h in BALB/c PECs in comparison to C57BL/6. *E. coli* (Δ) and LPS (□) controls at 24 h time point to demonstrate endpoint comparisons to *B. pseudomallei* treatments. Means ± SEM are reported. (*) Differences with a value of $p < 0.05$ were considered significant.

6.4 DISCUSSION

In the current study we aimed to demonstrate the expression of selected TLRs and pro-inflammatory cytokines in susceptible and partially resistant hosts following infection with a highly virulent *B. pseudomallei* isolate. We assessed the uptake and killing of *B. pseudomallei* NCTC 13178 using phagocytic assays and determined selected TLR and pro-inflammatory transcriptional profiles using the BALB/c and C57BL/6 mouse models of *B. pseudomalle*i infection. The work carried out in this Chapter will help further understand the progression of disease and immune responses to *B. pseudomallei* in hosts with genetic variance and different susceptibilities to infection.

To determine if phagocytic ability differed between hosts, the uptake and killing of *B. pseudomallei* NCTC 13178 by BALB/c and C57BL/6 PECs was assessed. We observed an increased uptake of NCTC 13178 by C57BL/6 PECs in comparison to BALB/c at 2 h, 6 h and 24 h. Killing of *B. pseudomallei* by C57BL/6 PECs was significantly greater at 2 h in comparison to BALB/c PECs which indicates that cells from C57BL/6 hosts may potentially have greater phagocytic ability and are capable of decreasing bacterial replication and dissemination at the early stages of infection (Figure 6.1). Similar findings of reduced *B. pseudomallei* loads in lung, spleen and blood of C57BL/6 mice in comparison to BALB/c mice challenged with NCTC 13178 have been reported in our laboratory at 14 days post infection (Ulett *et al.*, 2005). There were no differences in percent killing of phagocytosed bacteria between BALB/c and C57BL/6 PECs at 6 h and 24 h, but it should be noted that C57BL/6 PECs phagocytosed a larger percentage of NCTC 13178 at these time points and hence killed more bacteria. We have demonstrated that PECs derived from partially resistant hosts phagocytose *B. pseudomallei* NCTC 13178 to a greater extent than susceptible hosts; however, both are capable of killing nearly all bacteria within 24 h. Puthucheary and colleagues have shown in *B. pseudomallei* infected macrophages that phagosome-lysosome fusion and respiratory burst are limited which leads to intracellular persistence (Puthucheary *et al.*, 2006). Further studies assessing early and late endosome formation in BALB/c and C57BL/6 PECs would provide insight into the intracellular progression of infection following uptake in these hosts.

Within inactive monocytes TLR2 is expressed on the plasma membrane, in early and late endosomes and in rab11+ compartments (Nilsen *et al.*, 2008). Upon stimulation with the appropriate ligand, TLR2 is accumulated into lipid rafts then rapidly shuttled to maturing phagosomes to continue sampling its contents and initiate an appropriate inflammatory response via the MyD88 pathway (Underhill *et al.*, 1999; Ozinsky *et al.*, 2000; Flo *et al.*, 2001). The recruitment of TLR2 along with TLR1 and TLR6 is not only limited to those compartments containing TLR2 ligands and are thus apart of a conglomeration of receptors and enzymes that associate with phagocytic vesicle membranes that serve to eliminate internalised pathogens (McGettrick and O'Neill, 2010).

In the current Chapter we observed elevated TLR2 expression for both BALB/c and C57BL/6 PECs, with BALB/c PECs demonstrating increased expression at 24 h (Figure 6.2A). In the work described in Chapter 4 we also found elevated TLR2 expression in RAW 264.7 macrophages, however, in the work described in Chapter 5 we found low level TLR2 expression in THP-1 cells co-cultured with NCTC 13178. This variation could be due to cell type differences and species variation as THP-1 cells are a human monocytic cell line derivative. Increases in TLR2 expression have also been observed in spleens and livers of *i.v.* infected BALB/c mice via microarray and qRT PCR analysis lending further support to TLR2 involvement in *B. pseudomallei* infection (Chin *et al.*, 2010). Additionally, our findings are in support of others who have found TLR2 involvement in *B. pseudomallei* recognition using *in vitro* infection models (Wiersinga *et al.*, 2007b; Hii *et al.*, 2008; West *et al.*, 2008a). Wiersinga and colleagues have demonstrated in human and murine leukocytes a strong induction of TLR2 expression by *B. pseudomallei* in both *in vitro* and *in vivo* systems and contend that TLR2 recognises *B. pseudomallei* LPS and is deleterious to the host immune response *in vivo* (Wiersinga *et al.*, 2007c; Wiersinga *et al.*, 2008a).

We did not determine TLR2 surface protein expression and it is possible that TLR2 mRNA levels do not necessarily correlate with surface expression. Expression of TLR4 mRNA in murine PECs was shown to transiently decreased in response to LPS challenge over 24 h however total surface expression decreased after only 1 h (Nomura *et al.*, 2000). We have found that there are significant differences in TLR2

expression between BALB/c and C57BL/6 hosts, but the biological relevance of this variation cannot be determined. A comparison of TLR2 surface expression and TLR2 mRNA in this system as conducted by others are warranted (Wiersinga *et al.*, 2007b).

The TLR4 receptor is a strong initiator of pro-inflammatory cytokine signalling via MyD88 dependant pathways (O'Neill, 2006). TLR4 is the primary receptor for Gram negative bacteria and works in concert with CD14 and MD-2 surface proteins to form a receptor complex capable of LPS recognition (Hoshino *et al.*, 1999). Recently studies have shown an increase in mRNA expression of TLR4, CD14 and MD-2 in patients presenting with septic melioidosis (Wiersinga *et al.*, 2007c). In CD14 knockout mice infected with *B. pseudomallei*, the absence of CD14 was found to provide a survival advantage and reduced bacterial burden in mice, indicating a detrimental role of CD14 in the host immune response in melioidosis (Wiersinga *et al.*, 2008c). Purified *B. pseudomallei* LPS as well as live and heat killed *B. pseudomallei* have been shown by others to stimulate TLR4 in transfected HEK293 cells using co-reporter assays for NF-κB (Hii *et al.*, 2008; West *et al.*, 2008b).

Previously, we had reported that TLR4 expression was low in RAW 264.7 macrophages and BALB/c PECs at 2 h co-culture (Feterl *et al.*, 2008). In the work described in Chapter 5 we found low level expression of TLR4 in the human THP-1 macrophages co-cultured with NCTC 13178 from 2 h to 24 h. The aforementioned data and recent work by others prompted us to examine TLR expression in BALB/c and C57BL/6 PECs over a 24 h period of infection to assess changes in TLR and cytokine expression profiles in hosts with different susceptibilities to infection (Wiersinga *et al.*, 2007a; Hii *et al.*, 2008; West *et al.*, 2008b). In contrast to results in Chapter 5, we observed rapid increases in TLR4 expression in BALB/c PECs and C57BL/6 PECs following co-culture with NCTC 13178 from 6 h to 24 h (Figure 6.2A). Expression of TLR4 in BALB/c PECs was 120% greater than C57BL/6 PECs at 24 h. Similar reports for increased TLR4 expression at 24 h have been reported by others in bronchoalveolar lavage fluid of intranasal infected *B. pseudomallei* C57BL/6 mice and support our findings here (Wiersinga *et al.*, 2008a). As to whether the differences in TLR4 expression observed between host PECs contributes to the progression of disease remains unclear. We did not determine surface expression of TLR4 and or determine downstream activation of TLR

signalling pathways. In patients with melioidosis an increase in expression of TLRs correlated with increased surface expression on circulating monocytes and granulocytes (Wiersinga *et al.*, 2007b). Future studies determining surface expression of TLR4 in BALB/c and C57BL/6 using similar methods are warranted.

In resting macrophages, TLR4 is located on the plasma membrane and in the Golgi (Latz *et al.*, 2002; Husebye *et al.*, 2006). The TLR4/CD14/MD-2 complex cycles between the plasma membrane and the Golgi until stimulated by LPS (Husebye *et al.*, 2006). Following stimulation, TLR4 translocates to the endosome for duel purposes, first, to activate TRAM-TRIF signalling pathways and the release of type I interferons. The second, to ubiquinate internalised TLR4 for breakdown and downregulation of TLR4 mediated signalling (McGettrick and O'Neill, 2010). In the current investigation C57BL/6 mice showed an increase in uptake of NCTC 13178 and lower levels of TLR4 expression and secretion of TNF-α in comparison to BALB/c PECs at the early stages of infection (Figure 6.1A, Figure 6.2B, Figure 6.4C). Lysosome-endosome fusion in monocytes is attenuated in patients with melioidosis and this contributes to increased bacterial numbers (Puthucheary *et al.*, 2006). Detailed studies comparing the tracking of TLR4 and MyD88 localisation to *B. pseudomallei* containing endosomes in addition to determining activation of transcription factors in BALB/c and C57BL/6 PECs would help verify this.

Host genetic influences on susceptibility to infection have been established in melioidosis albeit on a limited scale (Dharakul *et al.*, 1998; Nuntayanuwat *et al.*, 1999). Increases in the class II HLA allele DRB1*1602 variant and a decrease in the DQA1*03 variant were associated with increased clinical severity, mortality and bacterial dissemination in 79 patients in northern Thailand (Dharakul *et al.*, 1998). Further, genetic variations in the TNF-α gene of melioidosis patients are associated with more fatal outcomes in acute presentations in the same region (Nuntayanuwat *et al.*, 1999). Recently a large scale study assessing human genetic variants of TLRs and their association with melioidosis was conducted in a similar northern Thai population (West *et al.*, 2011). In that study TLR polymorphisms demonstrated variable infection rates based on patient TLR polymorphisms. The study was broad in scope analysing eight TLR pathway polymorphisms in 490 cases compared with 950 non-hospitalised controls or 458 hospitalised controls. Variations in the TLR4 gene in

this population were associated with melioidosis. Specifically, the rare $TLR4_{1196C>T}$ allele and the rs10818066 allele conferred protection against melioidosis, while the rs960312 allele was associated with susceptibility to infection (West *et al.*, 2011). In whole blood samples from individuals with the +896A/G TLR4 polymorphisms a reduction in IL-6, TNF-α and IL-10 was observed when stimulated with LPS (Balistreri *et al.*, 2011). Others have found that genetic mutations in TLR4 alter LPS responsiveness in humans (Arbour *et al.*, 2000). Polymorphisms (Asp299Gly and Thr399lle) in the TLR4 gene abrogated responsiveness to inhaled LPS in airway epithelial cells harvested from donors (Arbour *et al.*, 2000). Given the delay in TLR4 expression and TNF-α production observed in C57BL/6 mice in this study, it is possible that TLR4 may contribute to host resistance.

Toll-like receptor 5 is the conical receptor for bacterial flagellin (Aderem and Ulevitch, 2000). *Burkholderia pseudomallei* is a flagellated motile bacteria, and typically bacterial structures with this phenotype, including *S. enterica serovar Typhimurium* and *Vibrio cholerae* have the ability to cause disease (Carsiotis *et al.*, 1984; Gardel and Mekalanos, 1996). The validity of flagellin as a virulence determinant of *B. pseudomallei* has been confirmed using deletion mutants in similar *in vitro* assays of infection as described in this Chapter (Chua *et al.*, 2003; Chuaygud *et al.*, 2008; Chanthiwa *et al.*, 2009). We observed a decrease in TLR5 expression in BALB/c PECs over 24 h co-culture with significantly greater TLR5 expression in comparison to C57BL/6 PECs at 2 h (Figure 6.2C). Despite the difference in expression between hosts, TLR5 mRNA was only 13% over baseline levels in BALB/c PECs at maximum expression levels. Based on these observations and those in Chapter 4, it may be possible that NCTC 13178 flagellin is a poor inducer of TLR5 expression in PEC co-culture. In contrast, TLR5 mRNA is increased in monocytes and granulocytes in patients with melioidosis (Wiersinga *et al.*, 2007b). The discrepancy between our results and those reported from these patient samples may be cell specific however analysis of TLR5 from multiple cell types from the BALB/c and C57BL/6 hosts are needed to confirm this. Other human pathogens, including *Campylobacter jejuni* and *Heliobacter pylori* produce a modified flagellar structure that is not recognised by TLR5 (Andersen-Nissen *et al.*, 2005). However, *B. pseudomallei* is capable of NF-kB activation via TLR5 albeit at low levels (Hii *et al.*, 2008; Tan *et al.*, 2010). We did not determine total TLR5 surface protein and or

downstream activation of NF-κB. These studies are necessary to determine how the low level of TLR5 expression induced by NCTC 13178 correlates to selected pro-inflammatory cytokine responses in BALB/c and C57BL/6 hosts.

Following phagocytosis, TLR9 recognises bacterial nucleic acids (CpG DNA) released within the lysosome by the breakdown of bacterial pathogens (Latz *et al.*, 2004). Within the cell TLR9 is localised in the ER. Once CpG DNA is internalised via a clathrin-dependant endocytic pathway, it is quickly moved into early endosomes and finally localised into a tubular lysosomal compartment (Latz *et al.*, 2004). TLR9 then redistributes from the ER to the CpG DNA containing lysosome along with MyD88 adapter molecules to initiate signalling. Phagosomes contain multiple proteins, including hydrolases, ATP proton pump subunits and several ER fusion proteins (Garin *et al.*, 2001). During ER and phagosome fusion, MHC class I molecules and ER proteins are integrated into the membrane, thereby becoming an antigen presenting organelle (Garin *et al.*, 2001).

Burkholderia pseudomallei is able to avoid host lytic mechanisms and disrupt formation of phagolysosome fusion where TLR9 is localised following internalisation. Puthucheary and colleagues found that phagolysosome formation was attenuated and disrupted in macrophages isolated from patients diagnosed with melioidosis when co-cultured *B. pseudomallei* (Puthucheary *et al.*, 2006). This mechanism has also been observed in other intracellular pathogens including *M. tuberculosis* (Armstrong and Hart, 1971; Armstrong and Hart, 1975). Others have found little to no expression of TLR9 during melioidosis, as no detectable levels of TLR9 expression have been observed in peripheral blood cells harvested from patients with septic melioidosis (Wiersinga *et al.*, 2007c; Wiersinga *et al.*, 2008a) . Similar results have been reported in a C57BL/6 mouse model of melioidosis, however TLR9 was measurable in whole lung and blood leukocyte samples (Wiersinga *et al.*, 2008a). In this study we observed differences in TLR9 expression in BALB/c and C57BL/6 PECs. BALB/c PEC TLR9 expression peaked at six hours co-culture and was significantly greater than C57BL/6 PECs, and subsided by 24 h with expression at only 3% above baseline levels (Figure 6.2D). In contrast, TLR9 expression in C57BL/6 PEC peaked at 24 h and was significantly greater than BALB/c PECs. As to whether TLR9 expression profiles observed here correlate to TLR9 total protein and

functional TLR9 signalling during *B. pseudomallei* infection cannot be determined here. Host cell invasion by similar pathogens *S. enterica* and *B. cenocepacia*, use type III secretion systems to abrogate intracellular trafficking and formation of functional lysosomes (Uchiya *et al.*, 1999; Sajjan *et al.*, 2008). *Burkholderia pseudomallei* uses secretion systems in a similar manner which are essential for host cell entry and activation of MAP kinase pathways (Mark *et al.*, 2002; Hii *et al.*, 2008). Further work identifying recruitment of TLR9 and MyD88 to phagocytic vesicles containing *B. pseudomallei* in both BALB/c and C57BL/6 phagocytic cells would help clarify the role of this receptor during infection (Latz *et al.*, 2004). In addition, the use of TLR9 knockout mice challenged with NCTC 13178 would help understand the role of this receptor using an *in vivo* model.

Cytokine mRNA levels for IL-1β, IL-12p40 and TNF-α increased in BALB/c PECs and C57BL/6 PECs however a trend of delayed TNF-α production was noted in C57BL/6 PECs during the first 6 h of infection. IL-1β concentrations were low in BALB/c PECs co-cultured with NCTC 13178 at 24 h and were significantly less than C57BL/6 PECs (Figure 6.4A). This data is similar to previously reported findings regarding mRNA levels for these cytokines in the liver and spleens of acutely infected BALB/c mice where low level expression was observed up to 48 h post infection (Ulett *et al.*, 2000c). Increased expression of IL-1β, IL-12p40 and TNF-α correlated with increased protein levels in culture supernatants from both BALB/c and C57BL/6 PECs (Figure 6.3, Figure 6.4). These findings are in support of other studies which found increases in both mRNA and cytokine protein levels for TNF-α in peripheral blood leukocytes and granulocytes of humans and in the pulmonary compartment of C57BL/6 mice infected intra-nasally with *B. pseudomallei* (Wiersinga *et al.*, 2007c; Tan *et al.*, 2008; Wiersinga *et al.*, 2008a). In this study host differences in TNF-α production were observed with susceptible BALB/c PECs demonstrating increased production until 24 h. These findings are supported by TNF-α production observed for NCTC 13178 in THP-1 cells in Chapter 5 as well as in *in vivo* models conducted in our laboratory using the same isolate (Ulett *et al.*, 2001; Barnes and Ketheesan, 2005).

In this investigation we have shown host differences in TLR4, TLR5 and TLR9 expression in PEC cells co-cultured with a highly virulent *B. pseudomallei* isolate. In

addition we have shown increased production of IL-12p40 and TNF-α in PECs from susceptible hosts which support *in vitro* and *in vivo* data generated in our laboratory for this isolate. However, as to whether the TLR expression profiles observed in this investigation correlate to total functional TLR protein and induction of selected pro-inflammatory cytokine secretion cannot be ascertained. Further studies using monoclonal antibodies for quantification of surface TLRs in PECs and using NF-κB co-reporter assays as described by others would help clarify whether the transcription of TLRs observed is associated with downstream activation.

CHAPTER 7
DETERMINING THE ROLE OF TLR2/TLR4 MEDIATED INDUCTION OF NF-KB BY TWO *BURKHOLDERIA PSEUDOMALLEI* ISOLATES

7.1 INTRODUCTION

Recognition of pathogen associated molecular patterns (PAMPs) by TLRs triggers the recruitment of adaptor molecules that facilitate downstream signalling. These intracellular signalling events are tailored to elicit specific responses to a variety of microbial ligands and through various combinations of the adaptor proteins recruited on the cytoplasmic surface (Kawai and Akira, 2005). Cytoplasmic recruitment of these adaptor molecules is specific for which homo- and or hetero-dimer complex forms following TLR interactions with their respective ligands. One of these adaptor molecules, MyD88, is utilised by all TLRs except TLR3, and creates the platform for a MyD88 dependant signalling pathway. Activation of the MyD88 dependant pathway results in the expression of inflammatory genes and the secretion of several inflammatory cytokines including TNF-α, IL-1β and IL-12, via activation of the nuclear translocator protein, NF-κB (Kawai *et al.*, 1999; Akira, 2000; Akira and Takeda, 2004).

Recent literature has shown that TLR2 and TLR4 are important in recognition of many Gram-negative organisms, including *B. pseudomallei* (Chen *et al.*, 2004; Wiersinga *et al.*, 2008e; McGettrick and O'Neill). TLR2 is localised on the cell surface, and can be found in early and late endosomes (Takeuchi *et al.*, 1999; Nilsen *et al.*, 2008). TLR2 recognises diacyl- and triacylated lipopeptides, as well as LPS in certain species (Takeuchi *et al.*, 1999; Chen *et al.*, 2004; Archer and Roy, 2006; Hawn *et al.*, 2006). Signalling is dependent on heterodimer formation of TLR2 with TLR1 (diacylated lipopeptides) or TLR6 (triacylated lipopeptides), which are also localised on the cell surface and form associations with the co-receptors CD14 and CD36 (Nilsen *et al.*, 2008; Kang *et al.*, 2009). Downstream TLR2 signalling is MyD88 dependant (Yamamoto *et al.*, 2003). Like TLR2, TLR4 is also located on the cell

surface and traffic into early and late endosomes following activation with bacterial LPS. Recognition of LPS by TLR4 requires the formation of a complex with other co-receptors, including LPS binding protein (LBP), MD-2 and CD14 (Lu *et al.*, 2008). Stimulation of TLR4 by LPS triggers TLR4, MyD88 and Mal adaptor protein interactions on the cytoplasmic surface which initiates signalling via engagement with the IL-1 receptor associated kinases (IRAKs) and TRAF6, culminating in the activation of NF-κB (Verstrepen *et al.*, 2008).

The NF-κB family of transcription factors are critical regulators of pro-inflammatory responses and apoptotic gene profiles (Vallabhapurapu and Karin, 2009). A variety of stimuli, including cellular stress and TLR activation can trigger NF-κB pathways which are tightly regulated and controlled by a complex process of phosphorylation and ubiquitination. Each stimulus activates one of two distinct inhibitory kinases known as inhibitors of kinase complexes (I-κB and I-κK) (Wertz and Dixit, 2010). Two types of NF-κB, canonical and non-canonical, are activated following stimuli. Canonical NF-κB signalling pathways assemble proximal signalling complexes on polyubiquitin chain scaffolds which result in the phosphorylation of inhibitory kinase I-κB. Activation of this pathway serves to shield nuclear localisation signals on NF-κB transcription factors which keeps them within the cytosol (Wertz and Dixit, 2010). Phosphorylation of I-κB results in proteasomal degradation and the localisation of NF-κB to the nucleus to begin target gene transcription. Non-canonical pathways help stabilise NF-κB inducing kinase. When NF-κB inducing kinase reaches substantial levels, it activates I-κK complexes to phosphorylate p100, a precursor NF-κB subunit, that when ubiquinised, couples with RelB to become transcriptionally active (Vallabhapurapu and Karin, 2009). These divergent pathways help NF-κB translocate to the nucleus to begin transcription of target pro-inflammatory genes (Latimer *et al.*, 1998).

Currently in the literature there are contrasting reports on whether TLR2 or TLR4 is the primary receptor for *B. pseudomallei* and the closely related species *B. thailandensis* (Wiersinga *et al.*, 2007c; Hii *et al.*, 2008; West *et al.*, 2008a; Novem *et al.*, 2009; West *et al.*, 2009). The aforementioned studies have used various methods including NF-κB reporter assays, qRT PCR, histological analysis and

survival studies in TLR knockout mice. Despite the growing breadth of experimental evidence no uniform answer has come to light, and the debate remains.

The disparity in these findings prompted us to examine two of our clinical *B. pseudomallei* isolates using similar *in vitro* assays of infection. In several studies we have shown significant differences in disease outcome in mouse models of infection as well as *in vitro* using a high virulence clinical isolate (NCTC 13178) and a low virulence clinical isolate (NCTC 13179) (Leakey *et al.*, 1998; Ulett *et al.*, 2000b; Ulett *et al.*, 2001b; Barnes, 2005). The work described in Chapters 5 and 6 of this thesis demonstrated differential transcriptional profiles for TLR2 and TLR4 in both murine and human macrophages infected with these isolates. We sought to determine if the observed differences in the inflammatory response to these isolates are due to a TLR2 or TLR4 signalling dominance and focused our study on the ability of these receptors to generate downstream signalling via activation of NF-κB.

The specific aim of the work described in this Chapter were to:

1) To determine the roles of TLR2 and TLR4 in innate immune responses to *B. pseudomallei* isolates from patients with different outcomes we assessed TLR2 and TLR4 individual recognition of clinical isolates of high and low virulence using stably transfected HEK293 cells

Our findings demonstrate that intracellular TLR mediated signalling during *B. pseudomallei* infection is influenced by isolate virulence, and that contrary to previous reports the degree of TLR2 and TLR4 mediated NF-κB activation may be dependant on the individual *B. pseudomallei* isolate encountered, rather than a clear cut TLR2 or TLR4 dominant response.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial Isolate Preparations

Details for all isolates and bacterial preparations are outlined in Chapter 3, section 3.1.

7.2.2 Cell Culture Conditions

HEK293 cells are generated from human embryonic kidney cells and were first described by Graham and colleagues in 1977 (Graham *et al.*, 1977). These cells are unique in that they are capable of incorporating foreign eukaryotic DNA and expressing that protein using a technique known as transfection. In the work described in this chapter, HEK293 cells were selected to express either TLR2 or TLR4 on the HEK293 cell surface to ascertain activation of those specific TLRs by *B. pseudomallei*. The HEK293 cells used in transfection experiments for this chapter were a gift from Ifor Beacham (Griffith University, Australia).

Cells were cultured in DMEM medium (Gibco Labs, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco Labs, Grand Island, NY, USA), with 20mM of L-glutamine (Sigma Aldrich, Sydney, NSW, Australia), and 12.5 mM HEPES buffer at 37°C with 5% CO_2.

7.2.3 Electrocompetent Cell Culture and Storage

Electrocompetent *E. coli* DH5α cells (Sigma Aldrich, Sydney, Australia) were commercially purchased and used to amplify TLR plasmids as these cells are generally have a higher transformation efficiency than chemically competent cells. *E. coli* DH5α cells were inoculated into 10 ml LB broth (Appendix I) and incubated at 37°C in a shaking incubator (150 rpm) for 24 h. Two, 250 ml culture flasks (500 ml total volume) of LB broth were then inoculated with 5 ml of the overnight *E. coli* DH5α cell culture. Inoculum was then cultured at 37°C with shaking (150 rpm) until the optical density (OD_{600}) of culture reached 0.5 to 0.7. Cells were chilled on ice for 20 min, without freezing, for all subsequent steps. Cells were transferred to a prechilled centrifuge tube and centrifuged at 4000 *g* for 15 min at 4°C. Following centrifugation, supernatants were discarded. Cells were then gently resuspended in 500 ml (2 x 250 ml) of ice cold 10% glycerol using a pre-chilled glass pipette (ensuring that the end of the pipette is undamaged to avoid lysis to cells). Cells were centrifuged at 4000 *g* for 15 min at 4°C, and the supernatant gently discarded. The pellet was then resuspended in 250 ml (2 × 125 ml) of ice cold 10% glycerol using a pre-chilled glass pipette, and centrifuged at 4000 *g* for 15 min at 4°C. Again, the supernatant was gently discarded. Each pellet was then resuspended in 15 ml ice cold

10% glycerol and transferred to a pre-chilled 50 ml Falcon tube and centrifuged at 4000 *g* for 15 min at 4°C. Each pellet was then resuspended in 2 ml ice cold 10% glycerol and aliquoted in 500 µl volumes into pre-chilled screw cap centrifuge tubes. Three 10 µl aliquots were taken to perform colony counts on LB agar (Appendix I) using three 10 fold dilutions. Cells were then snap frozen in liquid nitrogen and stored at -80°C until use.

7.2.4 Electrotransformation of Competent Cells with TLR Plasmids

Plasmids containing human TLR1, TLR2, TLR4, TLR6, CD14, MD-2, and β-actin dependant *Renilla* were a generous gift from Dr. Christopher Wilson at the University of Washington, Seattle, USA. Five separate electroporations were performed using 2 µl DNA with 50 µl of electrocompetent *E. coli* DH5α cells. Tubes containing 100 µl of *E. coli* DH5α cells were removed from -80°C storage and thawed on ice. In a pre-chilled 1.5 ml eppendorf tube, 2 µl of DNA (TLR1, TLR2, TLR4, TLR6, CD14, MD-2, NF-κB-dependant firefly ELAM luciferase, β-actin dependent *Renilla*, or pDisplayΔTMneo) was added to 50 µl *E. coli* DH5α cells and incubated for 1 min. DNA/cell mixture was transferred to a chilled 0.1 CM cuvette (Astral, Gimea, NSW, Australia), and gently tapped to ensure mixture settles to the bottom of the cuvette well. DNA/cell mixtures were placed into an electroporator (Astral, Gimea, NSW, Australia) at a time constant of 5 milliseconds and pulsed once at 1.80 kV. Immediately, 1 ml of SOC medium (Appendix I) was added to the cuvette and transferred to a 50 ml Falcon tube. Electroporations were repeated for a total of five reactions then pooled in a Falcon tube with an additional 3 ml of SOC medium. Cultures were then incubated at 37°C for 1 h with constant agitation (250 rpm). Following incubation, 1 µl, 10 µl, and 100 µl of 1 h culture were plated onto three separate LB/Amp/X-Gal/IPTG agar plates (Appendix I) and incubated overnight at 37°C. Three to four white colonies (white colonies are transformed) were selected and inoculated into 10 ml LB medium (Appendix I) and incubated for 24 h at 37°C.

7.2.5 Isolation of TLR Plasmids From Transformed DH5α Cells

Isolation of TLR plasmid inserts (TLR1, TLR2, TLR4, TLR6, CD14, MD-2, NF-κB-dependant firefly ELAM luciferase, β-actin dependant *Renilla*, and pDisplayΔTMneo) from transformed DH5α cells were carried out using the Fast Ion Plasmid Mini Kit (Real Biotech Corporation, Banqaio City, Taiwan) according to the manufacturer's instructions. Briefly, 50 ml of overnight culture of transformed DH5α cells (section 7.2.3) were centrifuged at 6000 × g for 15 min. A Plasmid-Midi column was placed into a 50 ml Falcon tube and the column was equilibrated with the addition of 5 ml PEQ Buffer. PEQ Buffer was allowed to empty through the column via gravity and the filtrate was discarded. The bacterial pellet was then resuspended in 4 ml PM1 Buffer and vortexed. PM1 Buffer (4 ml) was then added and tubes were gently mixed by inversion ten times (vortexing was avoided to prevent shearing of genomic DNA). The tubes were allowed to stand for 2 min at room temperature until lysate cleared. 4 ml of PM3 Buffer was then added and mixed immediately by inverting the tube ten times. Tubes were then centrifuged at 15, 000 × g for 20 min at room temperature. Supernatants were then added to the equilibrated Plasmid-Midi column and allowed to flow through the columns. The filtrate was then discarded. Columns were washed with 12 ml PW Buffer and allowed to empty. The filtrate was then discarded. The Plasmid-Midi column was placed into a clean centrifuge tube and 5 ml of PEL Buffer was used to elute DNA though the column. DNA was precipitated by addition of 3.75 ml of isopropanol to the eluted DNA. Tubes were gently mixed and centrifuged at 20,000 × g for 30 min at 4°C. Supernatants were carefully discarded and DNA pellets were gently washed with 5 ml of room temperature 75% ethanol. Tubes were then centrifuged at 20, 000 × g for 10 min at 4°C. Supernatants were gently discarded and pellets were air-dried for 10 min. DNA pellets were then dissolved in 100 µl TE Buffer. Purified plasmids were stored at -80°C until use.

7.2.6 LAL Assay

All reagents used for transfection experiments were assessed for endotoxin contamination using the E-Toxate Kit (Sigma, Castle Hill, NSW, Australia) according to the manufacturer's instructions.

7.2.7 General Transfection Scheme of HEK293 Cells with TLR Plasmids

All HEK293 transfected cells contained NF-κB-dependent firefly ELAM luciferase and control β-actin-dependant *Renilla* luciferase, human CD14, human MD-2 with either: TLR2, TLR2 and TLR1, TLR2 and TLR6 or TLR4. Transfection of HEK293 cells with human TLR plasmids were carried out according to methods previously described (West *et al.*, 2008a). Briefly, HEK293 cells were seeded into 96 well culture plates at a density of 4×10^4 cells/ml and incubated overnight at 37°C with 5% CO_2 to allow cells to stabilise. On the day of transfection stock solutions of $CaCl_2$ (2.5 M, Appendix I), were diluted 1:10 with sterile PBS to a final concentration of 0.25 M, filter sterilised and brought to room temperature. One 10 ml vial of frozen BES Buffered Saline (BBS, Appendix I) was removed from -20°C storage and brought to room temperature. Basic DNA preparations containing DNA common to all transfections (NF-κB-dependant firefly ELAM, β-actin-dependant *Renilla* luciferase, CD14, MD-2) were prepared in polystyrene tubes in 0.25 M $CaCl_2$. To a 500 µl volume of 0.25 M $CaCl_2$, 2 µg NF-κB-dependant firefly ELAM luciferase, 0.06 µg β-actin-dependent *Renilla* luciferase, 0.5 µg CD14, and 0.5 µg MD-2 were added and vortexed. 50 µl of basic DNA preparation was aliquoted into five labelled 5 ml polystyrene tubes. TLR plasmid DNA added to each of the tubes are listed in Table 7.1.

Table 7.1: TLR plasmid DNA concentrations added to basic master mix preparations described in section 7.2.7

Construct	TLR1(µg)*	TLR2(µg)*	TLR4(µg)*	TLR6 (µg)*	pDisplayΔTMneo (µg)*
TLR2	…	0.05	…	…	0.69
TLR2/TLR1	5	0.05	…	…	0.69
TLR2/TLR6	…	0.05	…	0.05	0.69
TLR4	0.4	…	…	…	0.69
empty vector	…	…	…	…	0.69

* all plasmid DNA concentrations are equivalent to µg DNA per 5 µl in 0.25M $CaCl_2$

Once unique TLR DNA was added to basic DNA preparation the mixture was vortexed. Slowly, 50 µl of 2 × BBS (Appendix I) was added to each tube while vortexing using medium agitation setting. The DNA mixture was incubated at room temperature for 15 min and vortexed. DNA mixtures were then incubated at room temperature for 4 h. Following 4h incubation, cells were washed with fresh DMEM

medium at room temperature (Appendix I). Fresh medium (150 µl) was then added to each well and plates were incubated overnight at 37°C with 5% CO_2.

The following day medium in 96 well plates was removed using a multi-channel pipette. Stimulation of transfected cells were carried out using 100 µl volumes of the following preparations: IL-1β (20 ng/ml), control TLR2/TLR1 ligand PamCSK4 (100 ng/ml), control TLR4 ligand *E .coli* 0111B4 LPS (10 ng/ml), NCTC 13178, and NCTC 13179 (MOI 10:1; 4×10^6 cfu/ml) for 4 h at 37°C with 5% CO_2. Optimisation for PamCSK4 stimulations were followed according to previously described methods (West *et al.*, 2008a). Stimulation medium was then removed from 96 well plates. Assessment of reporter plasmids were then carried out as detailed in section 7.2.8.

7.2.8 Background and Normalisation Protocols of MicoBeta Trilux for Anlaysis of NF-κB Reporter Assays

Background luminescence from a blank 96 well OptiPlate (Perkin Elmer, Red Hill, QLD, Australia) reading were subtracted from reported RLU values (Figure 7.1) via the MicroBeta Tri-Lux software. Prior to analysis of luminescence, normalisation protocols were carried out to correct for differing efficiencies between luminescence detectors of the MicroBeta Tri-Lux instrument from positive control samples (unstimulated transfected cells and media) and negative controls (media alone).

NF-κB activation was determined via analysis of Light Counts Per Second (LCPS) of two separate measurements using a MicroBeta Tri-Lux instrument (Perkin Elmer, Red Hill, QLD, Australia). All tests were carried out in triplicate. First, NF-κB-dependant firefly ELAM luciferase luminescense was determined from transfected cell stimulations (Table 7.1) following the addition of the Dual-Glo Luciferase Reagent (section 7.2.1). Secondly, measurements were carried out to determine β-actin-dependant *Renilla* luciferase luminescense from samples following addition of the Dual-Glo Stop & Glo Reagent (section 7.2.1). The ratio of experimental NF-κB-dependant firefly ELAM luciferase to control β-actin-dependant *Renilla* luciferase were calculated and reported as Relative Light Units (RLU).

7.2.9 Reporter Assay for NF-κB Activation in TLR2 and TLR4 Transfected HEK293 Cells

Following transfected cell stimulations (section 7.2.3), NF-κB activation was determined from 75 µl of transfected cell lysate (human CD14, human MD-2 with either: TLR2, TLR2 and TLR1, TLR2 and TLR6, TLR4, Δneo) using the Dual-Glo Luciferase Assay System (Promega, Sydney, Australia) according to the manufacturer's instructions.

7.2.10 Statistical Analysis

Comparisons between groups for transfection experiments were analysed using a two-way ANOVA with STATISTICA version 9.0 software (Statistica, Oklahoma, USA). Differences with a value of $p < 0.05$ were considered significant.

7.3 RESULTS

7.3.1 NF-κB Activation by *Burkholderia pseudomallei*

To determine the degree *B. pseudomallei* isolates of different virulence activate NF-κB via TLR2 and TLR4, we transfected HEK293 cells with either TLR2, TLR2/TLR1, TLR2/TLR6, and TLR4 with co-reporter plasmids. Both isolates activated NF-κB via TLR2, TLR2/TLR1, TLR1/TLR6 and TLR4 in comparison to transfected cells containing empty vector constructs ($p < 0.01$, Figure 7.1). However, in TLR4 transfected cells stimulated with NCTC 13178, NF-κB activation was greater than stimulations with NCTC 13179 and LPS controls ($p < 0.01$, Figure 7.1).

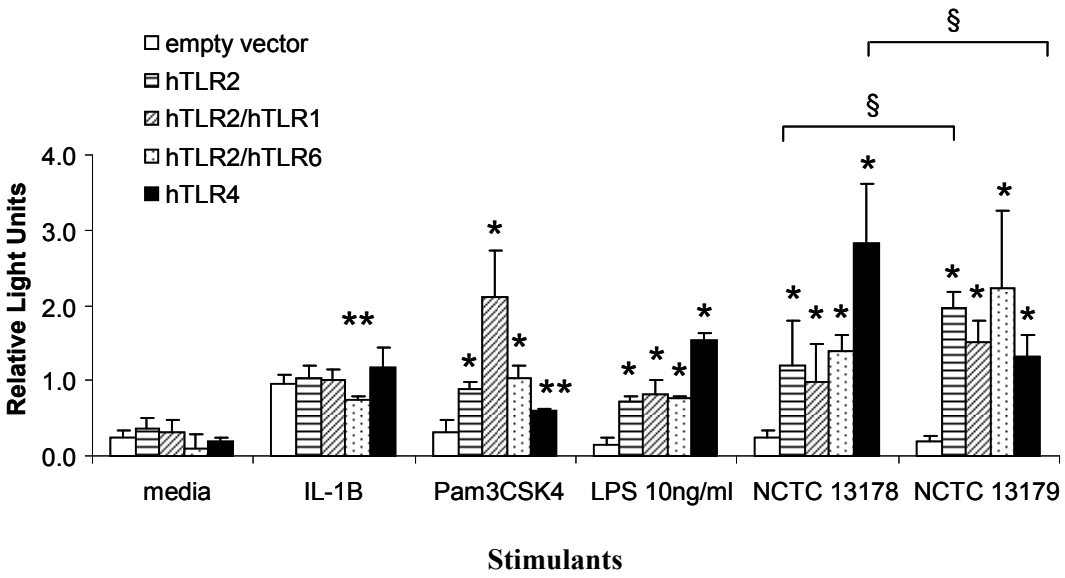

Figure 7.1 TLR2 and TLR4 mediated NF-κB activation in stably transfected HEK293 cells stimulated with *B. pseudomallei*.

HEK 293 cells were stably transfected with the following: human TLR2, TLR2/TLR1, TLR2/TLR6 or TLR4. All transfection preparations included co-reporters CD-14 and MD-2. Transfected cell stimulations were run in triplicate for 4 h and included: media alone, IL-1β (20ng/ml), Pam3CSK4 (100ng/ml), LPS (10ng/ml), *B .pseudomallei* NCTC 13178, *B. pseudomallei* NCTC 13179. NF-κB activation was measured by light emission and are expressed as the ratio of NF-κB-dependant firefly ELAM luciferase over control β-actin-dependant *Renilla* luciferase (Relative Light Units). Means ± SEM are reported. (*) represents significant differences to empty vector stimulated transfections and are equal to $p < 0.05$. (§) indicates differences between *B. pseudomallei* isolates and is equivalent to $p < 0.05$. *B. pseudomallei* isolates stimulated both TLR2 and TLR4 transfected cells. NCTC 13179 activation of NF-κB via TLR2 was significantly greater than NCTC 13178. NCTC 13178 NF-κB activation via TLR4 was significantly greater than NCTC 13179 and LPS controls.

7.4 DISCUSSION

The work described in this Chapter focused on the activation of TLR2 and TLR4 in stably transfected HEK293 cells challenged with clinical *B. pseudomallei* isolates of high and low virulence. The high virulence NCTC 13178 isolate was cultured from the cerebral spinal fluid of a six year old child who succumbed to melioidosis, while the low virulence NCTC 13179 isolate was cultured from a knee abcess of a non fatal case from a 56 year old male. Several studies within our laboratory have demonstrated a more severe inflammatory response to NCTC 13178 using both *in vitro* and *in vivo* models of infection. We sought to determine if the observed

difference in the inflammatory response to these isolates is due to a TLR2 or TLR4 signalling dominance and focused our study on the ability of these receptors to generate downstream activation of NF-κB. The findings described in this chapter will shed further light on the growing discrepancy in the literature regarding innate immune signalling in melioidosis and determine if TLR recognition of *B. pseudomallei* is ubiquitous, or isolate dependant.

The results of the current study demonstrate that TLR2 and TLR4 are capable of activating NF-κB when stimulated by two live *B. pseudomallei* isolates, however activation, whether TLR2 or TLR4 dominant, is dependent on the individual isolate. While both isolates were capable of activating both receptors, we found that the high virulence NCTC 13178 isolate displayed a significantly greater TLR4 dependent response in comparison to TLR2 constructs, as well as a greater TLR4 response in comparison to NCTC 13179 (Figure 7.1). In contrast, the low virulence NCTC 13179 isolate displayed a significantly greater TLR2 mediated response in comparison to TLR4, and a significantly higher TLR2 response in comparison to NCTC 13178. While TLR2/TLR1, TLR2/TLR6 transfected cell constructs stimulated with NCTC 13179 displayed a trend of increased NF-κB activation in comparison to NCTC 13178, these were not significant. This is the first time different live *B. pseudomallei* isolates have been compared using NF-κB reporter assays that demonstrate variable TLR responses and opens an avenue for further research.

Several studies have indicated a critical role for TLRs during innate immune signalling in melioidosis. While increased transcription of multiple TLRs including TLR2 and TLR4 have been reported in monocytes and granulocytes harvested from individuals presenting with acute melioidosis (Wiersinga *et al.*, 2007c), conflicting results have emerged regarding TLR2 or TLR4 as the primary receptor for *B. pseudomallei* LPS. In general, *B. pseudomallei* is capable of stimulating both TLR2 and TLR4 using *in vitro* analyses, however *in vivo* assays indicate that TLR2 alone impacts the host response. While TLR4 is considered the primary receptor for LPS, other intracellular Gram-negative bacterial pathogens including *L. pneumophila* and several *Yersinia spp* signal via TLR2 both *in vitro* and *in vivo* (Hirschfeld *et al.*, 2001; Sing *et al.*, 2002; Archer and Roy, 2006).

Using *in vivo* assays of *B. pseudomallei* infection Wiersinga and colleagues have demonstrated that TLR2 plays the preeminent role (Wiersinga *et al.*, 2007c). They found reduced injury, bacterial burden and pro-inflammatory cytokine levels in distant organs of TLR2 deficient mice (TLR2 -/-), while TLR4 -/- mice were similar to wild-type controls. NF-κB reporter assays were conducted in the same study using purified LPS stimulations from the *B. pseudomallei* 1026b isolate and their results demonstrated that signalling occurred through TLR2 rather than TLR4. However, these TLR4 constructs were not transfected with the co-receptor MD-2, which is essential for optimal TLR4 signalling (Shimazu *et al.*, 1999; Miyake, 2004; Kobayashi *et al.*, 2006; Hii *et al.*, 2008; West *et al.*, 2008a).

Several groups have used the closely related and avirulent species *B. thailandensis,* as a surrogate to study *B. pseudomallei* pathogenesis. In murine models of infection using aerosol challenge, the infectious dose of *B. thailandensis* is on the order of two to three logs greater than those reported for *B. pseudomallei* (West *et al.*, 2008b; Wiersinga *et al.*, 2008b). One such study investigating *B. thailandensis* (E264) aerosol infection in a panel of mouse strains, demonstrated no protective effect against infection in TLR4 competent C3H/HeN mice, which were asymptomatic following high dose challenge. In contrast, West and colleagues reported slight differences in pulmonary and systemic containment of *B. thailandensis* infection in TLR2-/- and TLR4-/- mice following low dose infection. However, in the same study they found in primary cells TLR4-/- and not TLR2-/- macrophages had impaired cytokine production. In bone marrow derived macrophages TNF-α production induced by *B. thailandensis* required TLR4 and not TLR2 (West *et al.*, 2009). Moreover, *B. thailandensis* mediated NF-κB activation via both TLR2 and TLR4 in a dose dependent manner (West *et al.*, 2009). Similar findings for *B. pseudomallei* were also reported, where both TLR2 and TLR4 mediated NF-κB activation in co-reporter assays (Hii *et al.*, 2008; West *et al.*, 2008a). Preparations of heat killed *B. pseudomallei* stimulated TLR2 and TLR4, however purified lipid A and LPS signalled in a TLR4 dependent manner (West *et al.*, 2008a). Interestingly, LPS preparations from separate isolates (BP-1 and K96243) demonstrated differential NF-κB activation in TLR4 transfected constructs. Our findings support this observation as *B. pseudomallei* NCTC 13178 activated NF-κB to a greater extent than NCTC 13179.

In a study comparing the structural and antigenic diversity of *B. pseudomallei* (KHW) and *B. thailandensis* LPS (ATCC 700388) , it was found that *B. pseudomallei* LPS stimulated less TNF-α, IL-6 and IL-10 production in both human THP-1 and murine RAW 264.7 macrophage cell lines in comparison to *B. thailandensis* LPS (Novem *et al.*, 2009). In addition, Novem and colleagues evaluated TLR receptor specificity from *B. pseudomallei* and *B. thailandensis* LPS preparations by assessing NF-κB reporter assays for TLR2 and TLR4 in transfected HEK293 cells. In contrast to results reported by Wiersinga e*t al.* 2007, they found *B. pseudomallei* and *B. thailandensis* LPS activated NF-κB exclusively via TLR4. The observed variations in immunological activity between the two species were thought to be attributed to their unique lipid A moieties which were characterised using mass spectrometry. The authors contend that *B. pseudomallei* weakly activates TLR4, and reduced immunological activity induced by *B. pseudomallei* LPS serves as a mechanism for host invasion (Novem *et al.*, 2009). Another study using the same *B. pseudomallei* KHW isolate found that live bacterial stimulations were capable of activating NF-κB in cells transfected with TLR2, TLR4 and TLR5, albeit at low levels (Hii *et al.*, 2008). In addition, transfected HEK293 cells stimulated with their respective TLR ligands in combination with *B. pseudomallei* suppressed NF-κB activation through secretion of TssM, which interferes with signalling intermediates TRAF-6, TRAF-3 and IκBα (Tan *et al.*, 2010). In the same study, live *B. pseudomallei* suppressed NF-κB activation in RAW 264.7 macrophages 2.5 fold less than heat killed *B. pseudomallei* (Tan *et al.*, 2010). These findings support results for live bacterial stimulations observed in the work described in this Chapter in regards to the low virulent NCTC 13179 isolate (Figure 7.1). However, we observed greater activation of NF-κB by the high virulent NCTC 13178 isolate in comparison to NCTC 13179 and LPS controls. These discrepancies may be a result of the different isolate used as well as variations in reporter assay methodology.

While the presence of exogenous contaminants, either lipoproteins or LPS can effect reporter assays, the breadth of studies conducted in several independent laboratories would indicate that *B. pseudomallei* is capable of activating TLR2 and TLR4 (Lee *et al.*, 2002). Caution should be taken when considering a dominant TLR response during infection as most pathogens are capable of engaging multiple TLRs. The concerted synergy between multiple recognition mechanisms is crucial for effective

responses and the results reported here are a small component of the overall design. In a pneumonia model of *P. aeruginosa* it was found that LPS and flagellin stimulation of TLR4 and TLR5 respectively, resulted in a protective immune response whereas TLR2 activation was deleterious (Skerrett *et al.*, 2007). Our findings suggest that TLR2 or TLR4 signalling dominance observed *in vitro* may be attributed to the specific isolate. To our knowledge, no *in vivo* survival analysis in TLR knockout mice has been conducted using the *B. pseudomallei* isolates that demonstrate TLR4 signalling specificity *in vitro* and therefore further studies assessing the LPS structure of NCTC 13178 and NCTC 13179, as well as the use of TLR2 and TLR4 knockout mice are necessary to confirm these hypotheses.

CHAPTER 8

GENERAL DISCUSSION

The innate immune system is comprised of chemical, cellular and physical components that act as barriers to infection. These components include the epidermis, ciliated respiratory epithelium, vascular endothelium and mucosal surfaces (Mogensen, 2009). The cellular component includes antigen-presenting dendritic cells, macrophages and lymphocytes in the case of infection. Initiation of innate immunity occurs by the recognition of microbial structures by the Toll-like receptor family (Takeda *et al.*, 2003). Over the past twelve years our understanding of Toll-like receptors and their relation to the innate immune response have increased exponentially (Takeda *et al.*, 2003). The innate immune response is mediated by phagocytic cells which work in concert to orchestrate the development of protective immunity. Because of their distribution on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) and within endosomes (TLR3, TLR7, TLR8, and TLR9), TLRs are quite diverse in their ability to patrol both extra- and intracellular environments for the presence of pathogens. Once detection occurs a signalling cascade is initiated by the dimerisation of TLRs on the plasma membrane coupled with the aggregation of specific adaptor molecules (MyD88, TIRAP/Mal, TRIF/TICAM-1, TIRP/TRAM/TICAM-2) at the C terminus (O'Neill *et al.*, 2003). This process culminates in the release of DNA binding proteins that initiate transcription of pro-inflammatory mediators that ultimately enable the clearance of the pathogen (Vogel *et al.*, 2003). The innate immune systems role as the first line of defence against bacterial infection and how TLRs initiate this response is the subject of intense study across a multitude of pathogens. Given that pathogens express different structural and biochemical compositions with various niche requirements in the host, it is surprising the degree of specificity to which TLRs are capable. Therefore, understanding TLR activation in the presence of different pathogens, and different isolates of the same pathogen will enable us to determine how these receptors help shape the immune response and to develop protective strategies.

Prior to the commencement of the work described in this thesis no literature had been published regarding *B. pseudomallei* and TLRs, and the first paper appeared in press in 2007 (Wiersinga *et al.*, 2007a). The studies in this thesis served as a preliminary investigation analysing which TLRs were involved in *B. pseudomallei* recognition (Chapter 4). As such, a panel of isolates cultured from twenty-six patients from Townsville, Australia were surveyed for TLR2, TLR4, TLR5, and TLR9 activation in a murine cell line and primary cultures. The results of this study emphasize important PAMPs on *B. pseudomallei*, by assessing multiple isolates. Transcriptional increase of TLR mRNA in host macrophages infected with *B. pseudomallei* was uniform and not dependant on isolate virulence at the early stages of infection. In murine models of melioidosis, the kinetics and concentration of various pro-inflammatory cytokines are critical in generating resistance and or contributing to pathology, and the delayed onset of excessive cytokine production may serve as an evasion strategy of the bacteria (Ulett *et al.*, 2000b; Ulett *et al.*, 2000c). Several studies have demonstrated that *B. pseudomallei* LPS does not induce a strong response and this may serve as a mechanism to avoid TLR4 detection and establish an intracellular niche (Wiersinga *et al.*, 2007c; Novem *et al.*, 2009). In this study, the nominal production of NO and the failure to efficiently clear bacteria in macrophages supports this hypothesis at least during the first 6 h of infection.

The kinetics of TLR2 and TLR4 mRNA expression were assessed for clinical *B. pseudomallei* isolates where greater expression of TLR2 was evident for a low virulent isolate NCTC 13179 (Chapter 5). The low virulent isolate demonstrated a strong TLR2 dominant response, suggesting that this particular isolate possesses lipoprotein and or LPS structural characteristics that activate TLR2. Indeed, this is supported by TLR2 mediated NF-κB activation by NCTC 13179 in transfected HEK293 cells (Chapter 7). Furthermore, in inactive monocytes, TLR2 is expressed on the plasma membrane, in early and late endosomes and in rab11+ compartments (Nilsen *et al.*, 2008). Upon stimulation, TLR2 is accumulated in lipid rafts then rapidly traffics to maturing phagosomes (Underhill *et al.*, 1999; Ozinsky *et al.*, 2000b). TLR2, TLR1 and TLR6 are recruited to all maturing phagosomes, not only those containing TLR2 ligands, thus allowing them to survey the contents of phagocytic vesicles. This may explain the elevated levels of TLR2 noted in this study for both high (NCTC 13178) and low (NCTC 13179) virulence isolates. However,

the increase in transcription of TLR2 for the low virulence isolate suggests a potentially larger accumulation of TLR2 ligands in macrophages infected with this isolate. This result, coupled with a decreased survival of the low virulence isolate indicates more efficient clearance of the bacteria. Further studies assessing TLR2 and TLR4 trafficking to early and late endosomes in macrophages infected with *B. pseudomallei* would assist our understanding of phagocytic mechanisms during melioidosis. The kinetics of cytokine production are considered an important indicator of disease progression and outcome in murine melioidosis (Ulett *et al.*, 2000c; Ulett *et al.*, 2000b). In the current study we observed different cytokine profiles between our clinical *B. pseudomallei* isolates in activated THP-1 macrophages. The secretion of pro-inflammatory cytokines was less in macrophages stimulated with the low virulence isolate with the exception of IL-12 suggesting decreased capacity to induce hyperproduction of cytokines thus enabling macrophages to mount an adequate response for pathogen clearance. This may correlate with the delayed onset of sepsis observed clinically. Conversely, the high virulence NCTC 13178 isolate induced significantly greater amounts of selected pro-inflammatory cytokines which emphasizes the importance of determining mRNA expression in addition to total protein when characterising inflammatory responses either *in vitro* or *in vivo*.

Gene expression profiles of several pro-inflammatory cytokines have been described in both murine and human melioidosis (Ulett *et al.*, 2000c; Ulett *et al.*, 2000b; Wiersinga *et al.*, 2007a; Barnes *et al.*, 2008a; Barnes *et al.*, 2008b; Wiersinga *et al.*, 2008b). The pro-inflammatory cytokines IL-1, IL-12 and TNF-α all play vital roles during *B. pseudomallei* infection. The results in this thesis support previous findings on the importance of genetic factors in regards to host recognition of *B. pseudomallei,* and the differences in recognition and response between hosts may be attributed to variations in TLR function and transcription of unique inflammatory profiles (Chapter 6). A comprehensive assessment of cellular mediated responses in healthy and immunocompromised hosts is crucial to understanding how *B. pseudomallei* establishes its intracellular niche and causes resultant pathology. Improvements in treatment for melioidosis rely on a detailed understanding of the cellular mediated responses to the bacteria, and assessment of inflammatory cytokines at the initial stages of infection will help determine the link to protective immunity. Future

studies analysing innate immune responses in diabetic mice should be assessed to determine how *B. pseudomallei* effects host cell responses in at risk individuals. These studies are essential to identify potentially novel treatment targets in melioidosis.

In the present studies, a comparison of TLR2 and TLR4 activation of NF-κB demonstrated that downstream signalling elicited by these receptors differs amongst individual isolates (Chapter 7), which futher supports variant transcriptional pro-flammatory cytokine profiles elcited by the same isolates in human macrophages (Chapter 5). However, several independent laboratories contend either TLR2 or TLR4 as the specific receptor involved in *B. pseudomallei* recognition. Certainly the data presented here requires further studies using survival analyses and histological data gathered from TLR2 and TLR4 knockout mice infected with our clinical isolates to support these hypotheses. Currently, preliminary work is underway to purify LPS preparations from NCTC 13178 and NCTC 13179 to determine structural characteristics using Matrix Laser Desorption Ionisation Mass Spectrometry. Identification of acylation patterns of the lipid A species from these isolates would clarify the activation of TLR2 and TLR4 observed in this thesis. The endotoxic activity of lipid A species is dependant on structural variance in fatty acid, polar head group, and carbohydrate composition. The most biologically active lipid A species, isolated from *E. coli*, *Neisseria meningitides* and *V. cholerae*, are hexa-acylated with side chains of twelve to fourteen carbons in length (Zughaier *et al.*, 2005). Deviations in the acylation pattern of lipid A species effect the strength of the inflammatory signal (Raetz and Whitfield, 2002; Bäckhed *et al.*, 2003). Certain studies point to the weakly immunogenic activity of *B. pseudomallei* LPS as a potential mechanism for host evasion and a means to establish an intracellular niche (Utaisincharoen *et al.*, 2000; Novem *et al.*, 2009). During cases of acute infection this point may be moot, as exponential numbers of bacteria and LPS overwhelm the immune system as evident by clinical outcomes. However, determining the structures of our clinical isolates may provide further insight into the pathogenesis of *B. pseudomallei*.

Hyper-production of pro-inflammatory cytokines plays a vital role in the pathogenesis of sepsis and targeting innate immune activation and signalling holds a potentially promising therapeutic role. The potential clinical benefit of TLR antagonism is to

modulate excessive inflammatory responses in order to dampen signalling and circumvent immunopathology. In the case of Gram-negative bacterial sepsis, TLR4 antagonists act to prevent LPS induced shock. Initially, studies focusing on the pathogenesis of sepsis by inhibiting LPS and TNF-α were largely unsuccessful (Grau and Maennel, 1997). However, the development and use of two lipid A analogues, TAK-22 and eritoran tetrasodium (E5564) have recently been approved for clinical trials (Mullarkey *et al.*, 2003). Phase 2 testing of Eritoran in patients with severe sepsis has shown a trend toward reduced mortality with various dosing regimens, however further studies are warranted (Tidswell *et al.*). The application of these two compounds in infection models of melioidosis would be of interest given the high incidence of sepsis generated during *B. pseudomallei* infection, as well as the involvement of TLR4. The TLR4 co-receptor CD14 has been shown to have a negative impact on the host response against *B. pseudomallei* and may also be a target for treatment in addition to E5564 and TAK-22 (Wiersinga *et al.*, 2008c).

Another avenue of therapeutic potential in melioidosis is the use of NF-κB inhibitors. Specific inhibitors of IKK kinase activity have been conducted which disrupt phosphorylation of NF-κB by IκBα and prevent nuclear translocation (Yamamoto and Gaynor, 2001). Controlling excessive cytokine secretion during sepsis induced by *B. pseudomallei* via this treatment maybe plausible, however, most treatments have focused on more chronic inflammatory diseases such as Alzheimers, inflammatory bowel disease, and rheumatoid arthritis. Complete inhibition of NF-κB pathways pose some safety concerns including regulation of inflammation and apoptosis, but treatment with these compounds could provide a novel approach to controlling chronic *B. pseudomallei* infection.

Collectively, the results contained in this thesis provide basic data supporting the role of TLR recognition and signalling during *B. pseudomallei* infection. In addition to different inflammatory mRNA expression and secretion profiles in susceptible and partially resistant mice, it appears that host genetic factors may also effect TLR expression in macrophages. Furthermore, the recognition of *B. pseudomallei* by TLRs may be dependant on the structural characteristics of individual isolates. Future studies characterising the LPS structure of the two prominent clinical isolates (NCTC 13178 and NCTC 13179) used in this study are warranted. Infection studies

examining TLR function and activation in other important cell types during acute *B. pseudomallei* infection, namely dendritic cells, neutrophils and natural killer cells, would further our understanding of disease progression and the development of effective immune responses in melioidosis.

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APPENDIX I

SOLUTIONS AND REAGENTS

A1.1 GENERAL SOLUTIONS

A1.1.1 2M Glucose

Glucose	36 g
ddH_2O	to 100 ml

A1.1.2 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG)

IPTG	2.383 g
ddH_2O	to 10 ml

Dissolve IPTG in water. Filter sterilise (22 μm) and store in 1 ml aliquots at -20°C until use.

A1.1.3 2M Magnesium stock (Mg^{2+})

$MgCl^2 \cdot 6H_2O$	20.33 g
$MgSO_4 \cdot 7H_2O$	24.65 g
ddH_2O	to 100 ml

Combine ingredients until completely dissolved. Filter sterilise through a disposable 0.22 micron filter.

A1.1.4

1× Phosphate buffered saline (PBS), pH 7.2

NaCl	8 g
(S-9888, Sigma Chemicals, USA)	
Na_2HPO_4	0.64 g
(S-0876, Sigma Chemicals, USA)	
KCl	0.2 g
(AnalaR, BDG Chemicals, USA)	
KH_2PO_4	0.16 g

Make up to 950 ml with single distilled water and adjust pH to 7.2, before bringing volume to 1000 ml. Autoclave at 121°C for 15 min.

A1.2 RT PCR SOLUTIONS AND REAGENTS

A1.2.1 Agarose gel (1%)

TAE Buffer	100 ml
DNA grade agarose	1 g
(200-0011, Progen Industries)	

Mix ingredients in a glass beaker and microwave for 1 min. Mix agarose TAE Buffer mixture gently and microwave for an additional 2 min or until agarose is dissolved. Cool at room temperature for 2 min. Add 2.5 ml of ethidium bromide (10 mg/ml) and swirl to mix. Pour into electrophoresis tank and leave to set at room temperature for 15 to 20 min.

A1.2.2 Ethidium Bromide

Ethidium bromide	100 mg
(200271, Boehringer Manheim)	
Distilled Water	10 ml

A1.2.3 6X Loading buffer

Glycerol	30 %
Bromophenol blue	0.025 %

Make in TE Buffer and store at 4°C till use.

A1.2.24TE Buffer

Tris-HCl pH 8.0	10 mM
EDTA	1 mM

Combine ingredients and store at 4°C until use.

A1.2.5 Tris-acetate (TAE) buffer (50X)

Tris-HCl	242 g
(T 6791, Sigma Chemicals, USA)	
Glacial acetic acid	57.1 ml
(100015N, BDH Laboratory Supplies)	
0.5M EDTA pH 8.0	100 ml
Double distilled water	up to 1000 ml

Combine ingredients and autoclave at 121°C for 15 min. Dilute 1/50 with distilled water for use.

A1.3 CELL CULTURE MEDIA AND REAGENTS

A1.3.1 Transport medium

RPMI 1640 (21870-076, Gibco)	1 L
Penicillin (15070-063, Life Technologies)	100,000 Units
Streptomycin (15070-063, Life Technologies)	100 mg

A1.3.2 Culture medium (RPMI)

RPMI 1640 (21870-076, Gibco)	1 L
Penicillin (15070-063, Life Technologies)	100,000 Units
Streptomycin (15070-063, Life Technologies)	100 mg
HEPES Buffer (15630-080, Life Technologies)	20 mM
L-Glutamine	2 mM
Heat-inactivated serum	100 ml

For culture of human cells, 10% heat-inactivated pooled human serum is added. For culture of murine cells, 10% heat-inactivated foetal bovine serum (HIFBS) is added.

A1.3.3 Culture medium (DMEM)

DMEM (21870-076, Gibco)	1 L
Penicillin (15070-063, Life Technologies)	100,000 Units
Streptomycin (15070-063, Life Technologies)	100 mg
HEPES Buffer (15630-080, Life Technologies)	20 mM
L-Glutamine	2 mM
Heat-inactivated serum	100 ml

A1.3.4 L-glutamine stock solution

FCC grade L-glutamine (G-5763, Sigma Chemicals, USA)	15 g
RPMI 1640 (21870-076, Gibco)	1000 ml

Combine ingredients and filter sterilise (0.22 μm). Dispense into 2 ml aliquots and store at -70°C till use.

A1.3.5 Heat-inactivated foetal bovine serum (HIFBS)

Heat 500 ml of FBS (Life Technologies) at 56°C for 25 min. Cool to room temperature. Aliquot into 10 ml tubes and store at -20°C until use.

A1.3.6 Trypan blue solution

Trypan blue powder (Difco Laboratories, England)	0.02 g
PBS pH 7.4	10 ml

Combine ingredients and store at room temperature.

A1.4 BACTERIAL BROTHS AND AGARS

A1.4.1 Luria-Bertani (LB) Medium

Tryptone (LP0042, Oxoid)	10 g
Yeast extract (LP0021, Oxoid)	5 g
NaCl	10 g
dH_2O	to 1000 ml

Combine ingredients, stirring until dissolved. Autoclave at 121°C for 15 min. Store at 4°C until use.

A1.4.2 Luria-Bertani Agar

LB Medium	500 ml
Agar Technical No.3 (LP0013, Oxoid)	15 g
Antibiotics as required	

Dissolve agar base in LB medium by boiling for 15 min. Autoclave at 121°C for 15 min, cool to 50°C and aseptically add antibiotics before pouring into petri plates

A1.4.3 5% Sheep Blood Agar

Blood Agar Base No.2 (CM0271, Oxoid)	40 g
Sheep blood, defibrinated	50 ml
dH_2O	to 1000 ml

Dissolve base in water and boil for 15 min until agar has completely dissolved. Autoclave at 121°C for 15 min, cool to 50°C and aseptically add blood. Mix by gently swirling and pour into petri plates.

A1.4.4 SOC Medium

tryptone (LP0042, Oxoid)	2 g
yeast extract (LP0021, Oxoid)	0.5 g
NaCl (1M)	1 ml
KCl (1M)	0.25 ml
Mg^{2+} (2M) stock	1 ml
glucose (2M)	1 ml
dH_2O	to 100 ml

Dissolve tryptone, yeast extract, NaCl and KCl in 97 ml distilled water. Autoclave at 121°C for 15 min and cool to room temperature. Add Mg^{2+} stock and glucose, each to a final concentration of 20 mM. Add sterile, distilled water to 100 ml. Final pH 7

A1.5 TRANSFECTION ASSAY REAGENTS

A1.5.1 2X BBS (BES Buffered Saline)

BES (391334, Calbiochem)	50 mM
NaCl (S-9888, Sigma Chemicals, USA)	280 mM
Na_2HPO_4 (S-0876, Sigma Chemicals, USA)	1.5 mM

The pH must be adjusted to 6.95 at room temperature. Prepare 100 ml per experiment and filter (0.22 μm) into 10 ml Falcon tubes for storage at -20°C.

A1.6 ANTIBIOTIC SOLUTIONS

A1.6.1 Ampicillin (100 mg/ml)

Ampicillin	10 g
ddH_2O	100 ml

Dissolve ampicillin in water. Filter sterilise (0.22 μm) and store at -20°C in 1 ml aliquots.

A1.6.2 Kanamycin (25 mg/ml)

Kanamycin sulphate	2.5 g
ddH_2O	100 ml

Dissolve kanamycin in water. Filter sterilise (0.22 μm) and store at -20°C in 1 ml aliquots.

APPENDIX II

CALCULATION OF COPY NUMBER FOR qRT PCR ANALYSIS

DNA Quantitation: ng conversion to fmols

For a given quantity of DNA in nanograms the equation to convert femtomoles is as follows (assuming 50% GC)

$$\frac{\text{X ng plasmid DNA } \mu l^{-1}}{0.66 \text{ ng fmol}^{-1} \text{ kbp}^{-1} \times \text{plasmid and insert size kbp}}$$

Where X is the quantity of DNA in nanograms as measured on a spectrophotometer or other method. The plasmid and insert size is in Kbp so a plasmid and insert of 3500 base pairs is 3.5 kbp.

Conversion from fmols to copies

$$\frac{\text{x fmol } \mu\text{l-1} \times 1 \text{ mol} \times 6.02 \times 10^{23} \text{ copies mol}^{-1}}{1 \times 10^{15} \text{ fmol}}$$

where x fmol µl-1 is calculated in the first equation.

APPENDIX III

OPTIMISATION FIGURES

Figure A3.1 Temperature gradient for optimising binding of TLR4 primers. Standard PCR conditions using 100 ng RAW 264.7 RNA templates were carried out as described in section 4.2.5, with the exception of varied annealing temperatures as indicated. DNA size markers are shown on the far right column and negative template control in the far left lane.

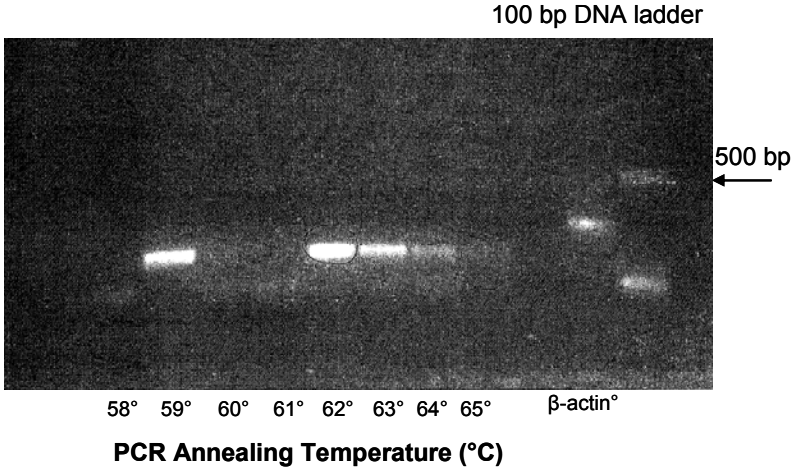


Figure A3.2 Temperature gradient for optimising binding of TLR5 primers. Standard PCR conditions using 100 ng RAW 264.7 RNA templates were carried out as described in section 4.2.5, with the exception of varied annealing temperatures as indicated. DNA size markers are shown on the far left column and negative template control in the far right lane.

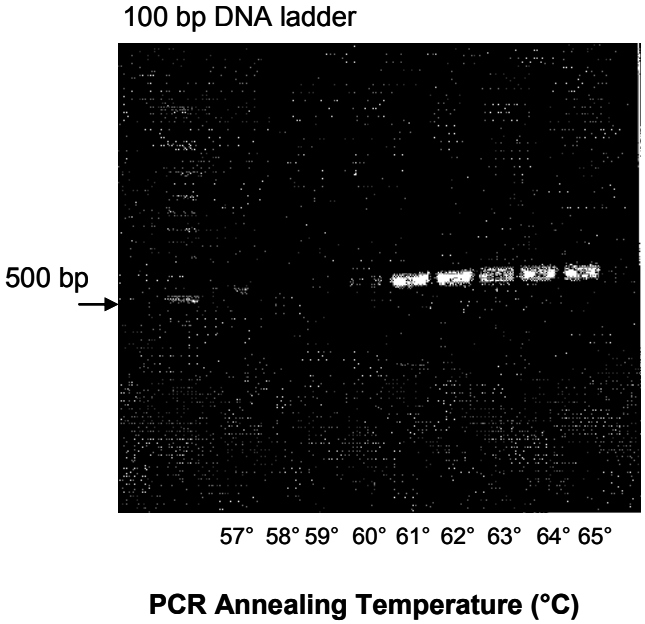


Figure A3.3 TLR insert verification into pGEM®-T vector plasmids using M13 primers

pGEM-T vector plasmids were successfully transformed with TLR2, TLR4, TLR5, TLR9 and β-actin PCR products. DNA size markers are shown on the far left column and positive template control is indicated in the far right lane (300bp).

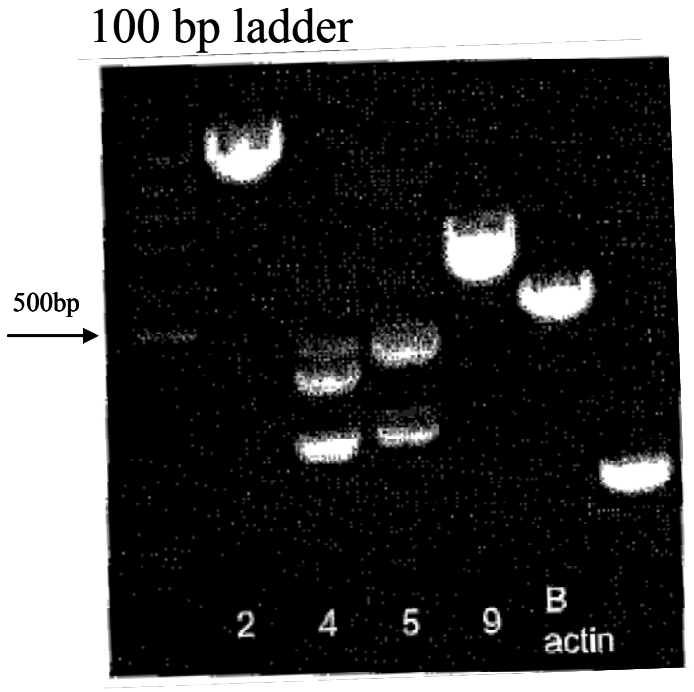


Figure A3.4 Standard curve quantitation report of TLR2 transcription in *B. pseudomallei* infected THP-1 cells

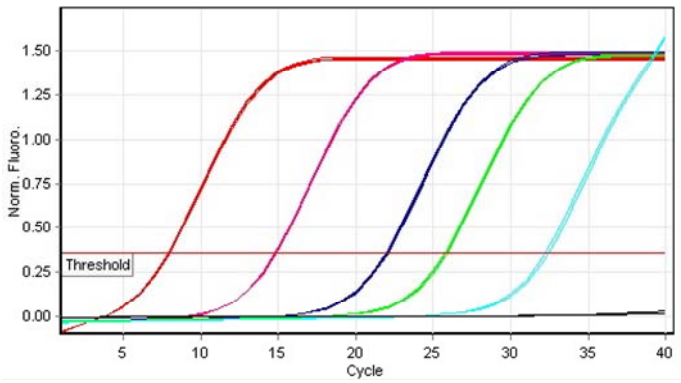
Quantitative real-time polymerase chain reaction was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for TLR2 using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Standard curve values are reported as copy number of TLR2 transcripts. Quantitation details, cycling parameters, standard curve and raw data for TLR2 standard curve are listed below.

Quantitation Information

Threshold	0.3573
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.283*CT + 10.240)
Standard Curve (2)	CT = -3.530*log(conc) + 36.143
Reaction efficiency (*)	0.92009 (* = 10^(-1/m) - 1)
M	-3.52957
B	36.1429
R Value	0.99974
R^2 Value	0.99947
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Profile

Cycle	Cycle Point
Hold @ 95°c, 10 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 10 secs
	Step 2 @ 60°c, hold 15 secs
	Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	



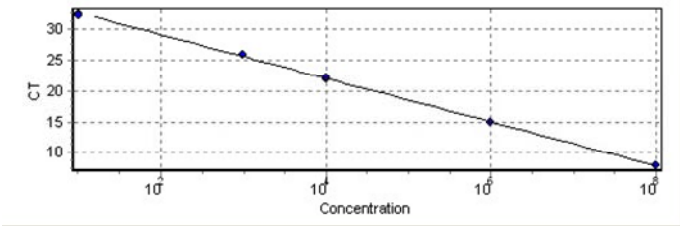


Table A3.1 qRT PCR raw data for TLR2 standard curve

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
1		Std 1 TLR2	7.95	1.00E+08	9.70E+07	3.0%	7.93
2		Std 1 TLR2	7.99	1.00E+08	9.47E+07	5.3%	
3		Std 1 TLR2	7.88	1.00E+08	1.02E+08	1.7%	
4		Std 1 TLR2	7.89	1.00E+08	1.01E+08	0.9%	
5		Std 2 TLR2	14.85	1.00E+06	1.08E+06	8.0%	14.83
6		Std 2 TLR2	14.89	1.00E+06	1.05E+06	5.1%	
7		Std 2 TLR2	14.80	1.00E+06	1.12E+06	11.8%	
8		Std 2 TLR2	14.81	1.00E+06	1.11E+06	11.0%	
9		Std 3 TLR2	21.92	1.00E+04	1.07E+04	6.7%	22.01
10		Std 3 TLR2	21.98	1.00E+04	1.03E+04	2.9%	
11		Std 3 TLR2	22.09	1.00E+04	9.60E+03	4.0%	
12		Std 3 TLR2	22.05	1.00E+04	9.86E+03	1.4%	
13		Std 4 TLR2	25.77	1.00E+03	8.66E+02	13.4%	25.82
14		Std 4 TLR2	25.82	1.00E+03	8.39E+02	16.1%	
15		Std 4 TLR2	25.79	1.00E+03	8.57E+02	14.3%	
16		Std 4 TLR2	25.91	1.00E+03	7.93E+02	20.7%	
17		Std 5 TLR2	32.42	1.00E+01	1.13E+01	13.3%	32.32
18		Std 5 TLR2	32.22	1.00E+01	1.30E+01	29.6%	
19		Std 5 TLR2	32.18	1.00E+01	1.33E+01	31.2%	

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
20		Std 5 TLR2	32.20	1.00E+01	1.28E+01	28.7%	

Figure A3.5 Standard curve quantitation report of TLR4 transcription in *B. pseudomallei* infected THP-1 cells

Quantitative real-time polymerase chain reaction was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for TLR4 using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Standard curve values are reported as copy number of TLR4 transcripts (Chapter 5, section 5.3). Quantitation details, cycling parameters for TLR4 standard curve are listed below.

Quantitation Information

Threshold	0.3573
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.276*CT + 9.905)
Standard Curve (2)	CT = -3.630*log(conc) + 35.951
Reaction efficiency (*)	0.88584 (* = 10^(-1/m) - 1)
M	-3.62971
B	35.95069
R Value	0.99974
R^2 Value	0.99947
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Profile

Cycle	Cycle Point
Hold @ 95°c, 10 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 10 secs
	Step 2 @ 60°c, hold 15 secs
	Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

αα

Table A3.2 qRT PCR raw data for TLR4 standard curve

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
1		Std 1 TLR4	7.19	1.00E+08	8.41E+07	15.9%	7.06
2		Std 1 TLR4	6.99	1.00E+08	9.51E+07	4.9%	
3		Std 1 TLR4	7.00	1.00E+08	9.45E+07	5.5%	
4		Std 1 TLR4	7.03	1.00E+08	9.40E+07	6.2%	
5		Std 2 TLR4	14.12	1.00E+06	1.03E+06	3.2%	14.12
6		Std 2 TLR4	14.10	1.00E+06	1.05E+06	4.9%	
7		Std 2 TLR4	14.14	1.00E+06	1.02E+06	2.3%	
8		Std 2 TLR4	14.13	1.00E+06	1.03E+06	3.0%	
9		Std 3 TLR4	21.27	1.00E+04	1.10E+04	10.5%	21.31
10		Std 3 TLR4	21.41	1.00E+04	1.01E+04	1.5%	
11		Std 3 TLR4	21.23	1.00E+04	1.14E+04	13.5%	
12		Std 3 TLR4	21.25	1.00E+04	1.10 E+04	12.8%	
13		Std 4 TLR4	25.01	1.00E+03	1.03E+03	3.3%	24.95
14		Std 4 TLR4	24.84	1.00E+03	1.15E+03	15.3%	
15		Std 4 TLR4	24.99	1.00E+03	1.05E+03	4.7%	
16		Std 4 TLR4	24.96	1.00E+03	1.07E+03	6.7%	
17		Std 5 TLR4	32.63	1.00E+01	8.20E+00	18.0%	32.47
18		Std 5 TLR4	32.35	1.00E+01	9.85E+00	1.5%	
19		Std 5 TLR4	32.03	1.00E+01	1.20E+01	20.1%	
20		Std 5 TLR4	32.87	1.00E+01	7.06E+00	29.4%	

Figure A3.6 Standard curve quantitation report of IL-1β transcription in *B. pseudomallei* infected THP-1 cells

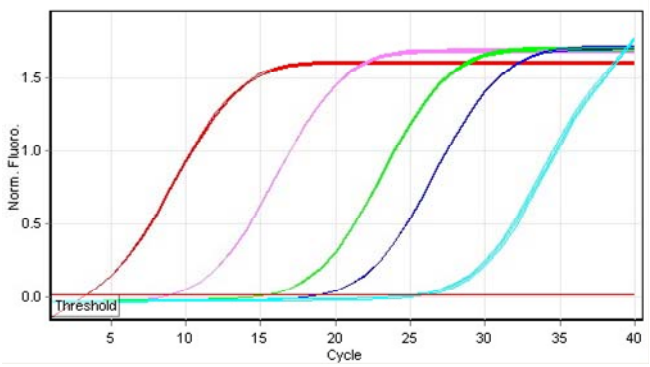
Quantitative real-time polymerase chain reaction was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for IL-1β using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Standard curve values are reported as copy number of IL-1β transcripts. Quantitation details, cycling parameters, standard curve for IL-1β standard curve are listed below.

Quantitation Information

Threshold	0.0184
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.311*CT + 8.951)
Standard Curve (2)	CT = -3.216*log(conc) + 28.788
Reaction efficiency (*)	1.04603 (* = 10^(-1/m) - 1)
M	-3.21633
B	28.78815
R Value	0.99913
R^2 Value	0.99827
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Profile

Cycle	Cycle Point
Hold @ 95°c, 10 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 10 secs
	Step 2 @ 60°c, hold 15 secs
	Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	



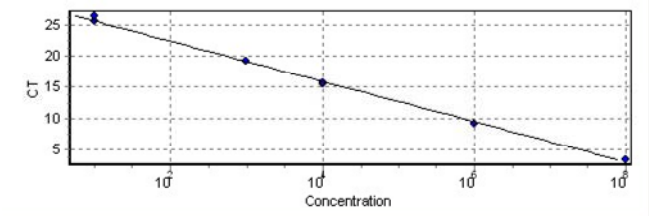


Table A3.3 qRT PCR raw data for IL-1β standard curve

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
1		STD1 IL-1B	3.39	1.00E+08	7.87E+07	21.3%	3.39
2		STD1 IL-1B	3.39	1.00E+08	7.89E+07	21.1%	
3		STD1 IL-1B	3.40	1.00E+08	7.85E+07	21.5%	
4		STD1 IL-1B	3.39	1.00E+08	7.90E+07	21.0%	
5		STD2 IL-1B	9.18	1.00E+06	1.25E+06	24.9%	9.19
6		STD2 IL-1B	9.19	1.00E+06	1.24E+06	24.4%	
7		STD2 IL-1B	9.19	1.00E+06	1.24E+06	24.2%	
8		STD2 IL-1B	9.19	1.00E+06	1.24E+06	24.1%	
9		STD3 IL-1B	15.60	1.00E+04	1.26E+04	25.9%	15.62
10		STD3 IL-1B	15.64	1.00E+04	1.23E+04	22.7%	
11		STD3 IL-1B	15.63	1.00E+04	1.23E+04	23.3%	
12		STD3 IL-1B	15.62	1.00E+04	1.24E+04	24.1%	
13		STD4 IL-1B	19.16	1.00E+03	9.84E+02	1.6%	19.18
14		STD4 IL-1B	19.14	1.00E+03	1.00E+03	0.2%	
15		STD4 IL-1B	19.16	1.00E+03	9.82E+02	1.8%	
16		STD4 IL-1B	19.28	1.00E+03	9.07E+02	9.3%	
17		STD5 IL-1B	25.61	1.00E+01	9.71E+00	2.9%	25.87
18		STD5 IL-1B	25.61	1.00E+01	9.71E+00	2.9%	
19		STD5 IL-1B	26.40	1.00E+01	5.54E+00	44.6%	
20		STD5 IL-1B	25.38	1.00E+01	9.86E+00	2.1%	

Figure A3.7 Standard curve quantitation report of IL-12p40 transcription in *B. pseudomallei* infected THP-1 cells

Quantitative real-time polymerase chain reaction was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for IL-12p40 using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Standard curve values are reported as copy number of IL-12p40 transcripts. Quantitation details, cycling parameters, standard curve for IL-12p40 standard curve are listed below.

Quantitation Information

Threshold	0.0184
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.324*CT + 10.376)
Standard Curve (2)	CT = -3.086*log(conc) + 32.025
Reaction efficiency (*)	1.10864 (* = 10^(-1/m) - 1)
M	-3.08639
B	32.02527
R Value	0.99876
R^2 Value	0.99752
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Profile

Cycle	Cycle Point
Hold @ 95°c, 10 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 10 secs
	Step 2 @ 60°c, hold 15 secs
	Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

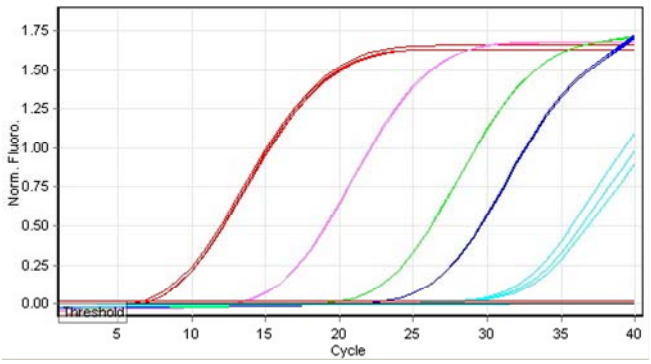


Table A3.4 qRT PCR raw data forIL-12p40 standard curve

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
1		STD1 IL-12	6.63	1.00E+08	1.69E+08	69.3%	7.05
2		STD1 IL-12	7.27	1.00E+08	1.05E+08	5.1%	
3		STD1 IL-12	7.27	1.00E+08	1.05E+08	5.0%	
4		STD2 IL-12	7.27	1.00E+08	1.05E+08	5.3%	
5		STD2 IL-12	13.54	1.00E+06	9.73E+05	2.7%	11.99
6		STD2 IL-12	13.56	1.00E+06	9.62E+05	3.8%	
7		STD2 IL-12	13.58	1.00E+06	9.48E+05	5.2%	
8		STD2 IL-12	13.62	1.00E+06	9.44E+05	5.1%	
9		STD3 IL-12	20.12	1.00E+04	7.19E+03	28.1%	20.14
10		STD3 IL-12	20.16	1.00E+04	6.99E+03	30.1%	
11		STD2 IL-12	20.18	1.00E+04	6.93 E+03	32.3%	
12		STD2 IL-12	20.14	1.00E+04	7.28E+03	28.7%	
13		STD4 IL-12	23.09	1.00E+03	7.84E+02	21.6%	22.13
14		STD4 IL-12	23.10	1.00E+03	7.78E+02	22.2%	
15		STD4 IL-12	23.09	1.00E+03	7.85E+02	21.5%	
16		STD2 IL-12	23.09	1.00E+03	7.85E+02	21.5%	
17		STD5 IL-12	28.71	1.00E+01	1.19E+01	18.5%	28.42
18		STD5 IL-12	28.40	1.00E+01	1.49E+01	49.3%	
19		STD5 IL-12	28.14	1.00E+01	1.82E+01	81.8%	
20		STD5 IL-12	28.22	1.00E+01	1.76 E+01	77.3%	

Figure A3.8 Standard curve quantitation report of TNF-α transcription in *B. pseudomallei* infected THP-1 cells

Quantitative real-time polymerase chain reaction was carried out on template cDNA using SYTO9 (Invitrogen, New South Wales, Australia), ImmoMix (Bioline, New South Wales, Australia) and gene-specific primers for TNF-α using the Rotor-Gene 6000 instrument (Qiagen, Victoria, Australia). Standard curve values are reported as copy number of TNF-α transcripts. Quantitation details, cycling parameters, standard curve for TNF-α standard curve are listed below.

Quantitation Information

Threshold	0.0184
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.314*CT + 9.453)
Standard Curve (2)	CT = -3.184*log(conc) + 30.096
Reaction efficiency (*)	1.06101 (* = 10^(-1/m) - 1)
M	-3.1839
B	30.09644
R Value	0.99935
R^2 Value	0.99871
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
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Cycle	Cycle Point
Hold @ 95°c, 10 min 0 secs	
Cycling (40 repeats)	Step 1 @ 95°c, hold 10 secs
	Step 2 @ 60°c, hold 15 secs
	Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

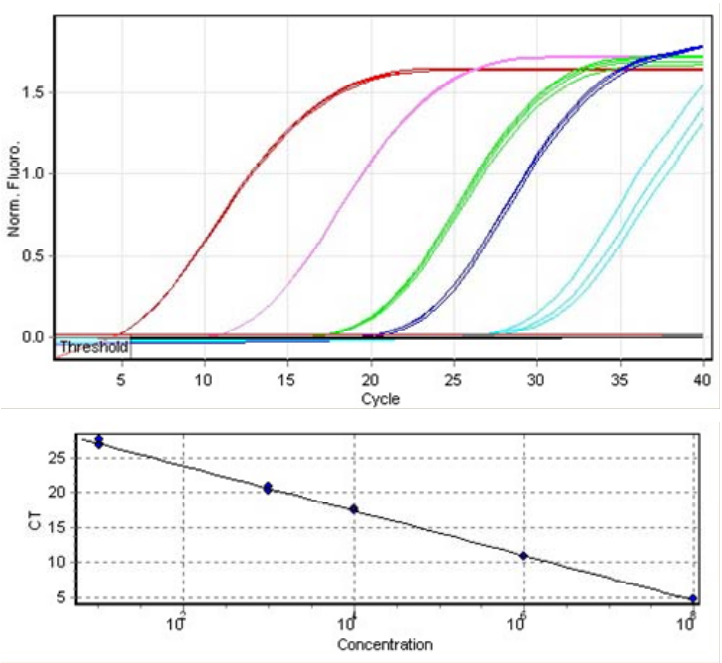

Table A3.5 qRT PCR raw data for TNF-α standard curve

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
1		STD1 TNFa	4.77	1.00E+08	8.99E+07	10.1%	4.78
2		STD1 TNFa	4.78	1.00E+08	8.96E+07	10.4%	
3		STD1 TNFa	4.78	1.00E+08	8.96E+07	10.4%	
4		STD1 TNFa	4.81	1.00E+08	8.90E+07	10.8%	
5		STD2 TNFa	10.81	1.00E+06	1.14E+06	14.2%	10.82
6		STD2 TNFa	10.81	1.00E+06	1.14E+06	14.5%	
7		STD2 TNFa	10.84	1.00E+06	1.11E+06	11.3%	
8		STD2 TNFa	10.82	1.00E+06	1.09E+06	10.6%	
9		STD3 TNFa	17.33	1.00E+04	1.02E+04	2.3%	17.43
10		STD3 TNFa	17.52	1.00E+04	8.92E+03	10.8%	
11		STD3 TNFa	17.48	1.00E+04	9.15E+03	8.5%	
12		STD3 TNFa	17.37	1.00E+04	9.93E+03	0.7%	
13		STD4 TNFa	20.22	1.00E+03	1.26E+03	26.5%	20.38
14		STD4 TNFa	20.93	1.00E+03	7.59E+02	24.1%	
15		STD4 TNFa	20.19	1.00E+03	1.30E+03	29.6%	
16		STD4 TNFa	20.18	1.00E+03	1.31E+03	30.6%	
17		STD5 TNFa	26.99	1.00E+01	9.46E+00	5.4%	27.07
18		STD5 TNFa	26.74	1.00E+01	8.77E+00	11.5%	
19		STD5 TNFa	27.54	1.00E+01	6.33E+00	36.7%	

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var	Rep. Ct
20		STD5 TNFa	26.68	1.00E+01	1.18E+01	18.0%	