THE STUDY OF

BURKHOLDERIA PSEUDOMALLEI

LIPOPOLYSACCHARIDE

VIDHYA D/O NOVEM

(B.Sc, NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2009

Acknowledgements

I wish to express my profound gratitude and indebtedness to my supervisor Associate Professor Ooi Eng Eong for his constant encouragement and guidance in my research endeavours.

In addition, I wish to acknowledge Dr Gladys Tan, Dr Yichun Liu, Dr Sivalingam SP, Wang Dongling and Sim Siew Hoon from BD laboratory at Defence Medical and Environmental Research Institute, DSO National Laboratories for their support during the course of my research.

I gratefully acknowledge Associate Professor Markus Wenk and Dr Shui Guanghou from the Department of Biochemistry and Department of Biological Sciences for their guidance in structural analysis.

I would also like to thank my research colleagues in BD laboratory at Defence Medical and Environmental Research Institute, DSO National Laboratories for their help, stimulating discussions and maintaining a cheerful working atmosphere.

Last but not the least, I would like to extend my profound sense of gratitude to my beloved parents, sister and husband for their sacrifices, perpetual support, patience and understanding.

CONTENTS

CHAPTER 2: Extraction of Lipopolysaccharides

Chapter 3: Determination of the effect of magnesium on the virulence of *Burkholderia pseudomallei*

CHAPTER 4: Structural and biological characterization of lipid A from *B. pseudomallei* and *B. thailandensis*

CHAPTER 5: Determination of the Toll-like receptor (TLR) involved in the recognition of LPS from *B. pseudomallei* and *B. thailandensis*

Summary

Burkholderia pseudomallei, the etiological agent of melioidosis, is a Gram-negative bacterium. In this thesis, for the first time, the lipopolysaccharide (LPS) of *B. pseudomallei* was characterised and compared to that of *B. thailandensis,* a close relative of *B. pseudomallei* that rarely causes disease, to uncover important virulence mechanisms that may contribute to *B. pseudomallei*'s pathogenesis.

Initial fatty acid (FA) compositional analysis revealed that the LPS of *B. pseudomallei* differed from that of *B. thailandensis,* by the presence of 2-hydroxy-tetradecanoic acid [C14:0(2-OH)]. Similarly, *in-vitro* experiments indicated that *B. thailandensis* LPS induced two times higher levels of cytokine production in murine macrophages than *B. pseudomallei* LPS. The LPS of *B. pseudomallei* and *B. thailandensis* were also found to activate immune cells through Toll-like Receptor 4 (TLR4). The presence of contaminating proteins in extracted LPS is likely to be responsible for the observation by other groups that TLR2 is the pattern recognition receptor of *B. pseudomallei* LPS.

As the structure of the lipid A moiety has been described to influence its biological activity, the lipid A from *B. pseudomallei* was subjected to further structural characterization by mass spectrometry, and compared to that of *B. thailandensis* to identify unique features that contribute to the differences in biological activity observed *in-vitro*. The major lipid A species identified in *B. pseudomallei* consisted of a bi-phosphorylated disaccharide backbone modified with 4-amino-4-deoxy-arabinose

(Ara4N) at both phosphate groups and penta-acylated with FA 3-hydroxy-tetradecanoic acid [C14:0(3-OH)], 3-hydroxy-hexadecanoic acid [C16:0(3-OH)] and either tetradecanoic acid (C14:0) or C14:0(2-OH). The major lipid A species identified in *B. thailandensis* had a similar architecture with some essential differences. These include lipid A species without C14:0(2-OH) and major lipid A species with only one or none of the phosphate groups modified with Ara4N. These unique features may be responsible for the differences in the ability of the two pathogens to activate murine macrophages *in-vitro* and may play an important role in the pathogenesis of the pathogen by allowing *B. pseudomallei* to evade the innate immune system and survive intracellularly. Further studies are required to confirm these suggestions.

Some Gram-negative bacteria, such as *Salmonella enterica* serovar Typhimurium modify their lipid A structure when cultured in low magnesium medium, an environment that mimics the macrophage phagosomal environment. Here, the effect of low magnesium culture medium on the LPS of *B. pseudomallei* was investigated. However, no changes were observed in both FA composition and cytokine induction in *in-vitro* stimulation assays with LPS extracted from *B. pseudomallei* cultured in magnesium supplemented or magnesium minimal medium. The LPS of *Salmonella* serovar Typhimurium was similarly extracted from magnesium supplemented or magnesium minimal medium and as shown in literature, changes were observed in both FA composition and cytokine induction. Taken together, the data in this thesis suggest that *B. pseudomallei* LPS is able to evade innate immune activation and does not require external stimulus to alter its structure.

List of Tables

List of Figures

List of Publications

Manuscripts

1. **Novem, V.**, Shui, G., Wang, D., Bendt, A. K., Sim, S. H., Liu, Y., Thong, T. W., Sivalingam, S. P., Ooi, E. E., Wenk, M. R., and Tan, G. Structural and biological diversity of *Burkholderia pseudomallei* and *Burkholderia thailandensis* LPS. Clin Vaccine Immuno **16:** 1420.

Conference presentations

1. **Novem, V.**, Wang, D., Sim, S. H., Lau, Y. F., Tang, L. H., Tan, G., and Ooi, E. E. Lipid A analysis of *Burkholderia pseudomallei.* The 5th World Melioidosis Congress, November 2007. Khon Kaen, Thailand.
2. **Novem, V.**, Wang, D., Sim, S. H., Liu, Y., Thong, T. W., Tan, G., and Ooi, E. E. Purification of *Burkholderia pseudomallei* LPS eliminates recognition through TLR2. Keystone Symposia : Pattern Recognition Molecules and Immune Sensors of Pathogens, March 2009. Alberta, Canada.

List of Abbreviations

Ara4N	4-amino-4deoxy-arabinose
B. cepacia	*Burkholderia cepacia*
B. mallei	*Burkholderia mallei*
B. pseudomallei	*Burkholderia pseudomallei*
bsa	*Burkholderia* secretion apparatus
BSL2	Biosafety level 2
BSL3	Biosafety level 3
B. thailandensis	*Burkholderia thailandensis*
C12:0	Dodecanoic acid
C14:0	Tetradecanoic acid
C14:0 (2-OH)	2-hydroxy-tetradecanoic acid
C14:0 (3-OH)	3-hydroxy-tetradecanoic acid
C16:0	Hexadecanoic acid or palmitate
C16:0 (3-OH)	3-hydroxy-hexadecanoic acid
CAMPS	Cationic antimicrobial peptides
CD14	Cluster of differentiation 14
C. elegans	*Caenorhabditis elegans*
CF	Cystic fibrosis
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray Ionisation
FBS	Fetal bovine serum
g	Gram
GC-MS	Gas chromatography mass spectrometry
HEK 293	Human embryonic kidney cell-line
IFN-β	Interferon-beta
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
iNOS	Inducible nitric oxide synthase
Kdo	3-Deoxy-D-*manno*-octulosonate
K. pneumoniae	*Klebsiella pneumoniae*
LAL	Limulus amoebocyte lysate
LBP	LPS binding protein
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
Mg^{2+}	Magnesium
mg	Milli gram
ml	Milli liter
mM	Milli molar
MNGC	Multinucleated giant cell
MS	Mass spectrometry
MyD88	Myeloid differentiation primary response protein
m/z	Mass to charge ratio
n	Number
NF-κβ	Nuclear factor kappa beta
ng	Nano gram
nm	Nano meter

p	Probability
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
P. gingivalis	*Porphyromonas gingivalis*
PMA	Phorbol 12-myristate 13-acetate
pNifty	NF-κβ inducible reporter plasmid
pRen	Constitutive renilla reporter plasmid
QTOF	Quadrupole time of flight
RAW 264.7	Mouse macrophage cell-line
RG	Risk group
R-LPS	Rough LPS
RPM	Revolutions per minute
Salmonella serovar Typhimurium	*Salmonella enterica* serovar Typhimurium
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S-LPS	Smooth LPS
THP-1	Human monocyte cell-line
TLR	Toll-like receptor
TMP-SMX	trimethoprim and sulfamethoxazole
TNF-α	Tumor necrosis factor-alpha
TTSS	Type III secretion system
T-test	Student's *t*-test
µg	Micro gram
µl	Micro liter
µM	Micro molar
Y. pestis	*Yersinia pestis*

Chapter 1

Literature Review

1.1 INTRODUCTION

Burkholderia pseudomallei, formerly known as *Pseudomonas pseudomallei* (Yabuuchi *et al.,* 1992), is a Gram-negative bacterium that was first identified by Whitmore in 1911 (Miller *et al.,* 1948). It is the etiological agent of melioidosis, an infectious disease of major public health importance in southeast Asia and northern Australia (Cheng and Currie, 2005).

Over the past 5 years, basic research on this pathogen has progressed rapidly. This is partly due to the increased recognition of the organism, with the discovery of sporadic cases outside endemic areas, the fear that the bacterium may be used as a biological weapon and the availability of genomic sequence data that facilitated the identification of virulence factors (Vietri and DeShazer, 2007).

The fatality rate of melioidosis remains high in endemic countries despite the availability of intensive treatment with appropriate antibiotics (Woods *et al.,* 1999). The time between acquisition and the onset of melioidosis is reported to vary from 2 days in acute disease to 62 years in sub-clinical, chronic or relapse cases (Leelarasamee and Bovornkitti, 1989; Mays and Ricketts, 1975; Ngauy *et al.,* 2005). As *B. pseudomallei* is a facultative intracellular pathogen, many groups have postulated that after the initial phase of infection, the bacterium may exist in a dormant stage in macrophages for months and years (Vietri and DeShazer, 2007). This, and the ability of *B. pseudomallei* to escape the innate immune response and appear inconspicuous to the host immune system may be responsible for the long

latency period (Brett *et al.,* 2000; Stevens *et al.,* 2004; Woods *et al.,* 1999) in melioidosis.

One mechanism used by *B. pseudomallei* to evade host detection is the weak activation of the innate immune system (Utaisinchroen *et al.,* 2001). This may be in part due to the synthesis of lipopolysaccharide (LPS) molecules of weak activity *in-vitro* (Matsuura *et al*., 1996; Utaisinchroen *et al.,* 2000). Since unique structural features in the LPS of some Gram-negative bacteria have been shown to be responsible for intracellular survival (Ernst *et al.,* 2003; Guo *et al.,* 1997), LPS may similarly play an important role in the intracellular survival of *B. pseudomallei.* This literature review will look into the background of melioidosis and the role of LPS in the pathogenesis of this bacterium.

1.2 MELIOIDOSIS

1.2.1 Distribution

Melioidosis is predominantly a disease of tropical climates, especially in southeast Asia and northern Australia where it is endemic (Cheng and Currie, 2005; Dance, 1991, 2000; Dharakul and Songsivilai, 1999). In northeast Thailand, melioidosis accounts for 20% of community acquired septicaemia (Chaowagul *et al.*, 1989), and is responsible for 40% of sepsis related deaths (White *et al*., 2003). The annual incidence in Ubon Ratchathani province in northeast Thailand was 4.4 cases per 100,000 between 1987 and 1991 (Suputtamongkol *et al*., 1994). Sporadic cases have been reported throughout the world, including Africa (Dance, 1991), the Middle East (Pourtaghva *et al*., 1977), the Caribbean and Central and South America (Inglis *et al*., 2006; Rolim *et al*., 2005). The number of cases around the world has significantly increased with melioidosis becoming endemic in New Caledonia (Le Hello *et al.*, 2005) and following the Asian Tsunami (Cierakul *et al*., 2005; Nieminen and Vaara, 2005).

In Singapore, the first case of melioidosis was reported in 1920, but it was not recognized as an important health threat until three fatal septicaemic cases among healthy young adults were reported in 1989. Since this incident in 1989, melioidosis was made administratively notifiable to the Ministry of Health (Ministry of Health, 2004b), and in December 2008, this was upgraded to legally notifiable for disease surveillance. Between 1990 and 2006, an average of 63 cases was reported each year with an annual case fatality rate of 25.9%. The incidence rate was 1.3 per 100,000 population in 2006 (Ministry of Health, 2006). In 2004, between January and July, a

higher number of cases, 58 compared to 13 and 14 cases during the same period in 2002 and 2003, respectively were notified to the Ministry of Health, with a case fatality rate of 40% (Ministry of Health, 2004b). This peak of cases coincided with the heavy rainfall (Ministry of Health, 2004c) experienced during that period and no common source was found between the different infections (Liu *et al.,* 2006).

1.2.2 Acquistion of disease

Melioidosis is a disease often reported in individuals who have been exposed to environments containing *B. pseudomallei* (Wiersinga *et al.*, 2006). However, one case of perinatal transmission of melioidosis from mother to baby has been described after a vacation in Thailand (Abbink *et al.*, 2001). The precise mode of acquisition of melioidosis is unknown but majority of melioidosis infections are assumed to result from percutaneous inoculation through open cuts. In Thailand, where the highest rates of melioidosis are described, exposure occurs very frequently during rice farming, which is the main occupation in the region (White, 2003). The farmers rarely wear protective footwear and their feet often show signs of repeated trauma and injuries (Wiersinga *et al*., 2006). Serological studies have shown that in Thailand, exposure arises as soon as the infant makes contact with soil and water and 25% of children seroconvert every year for the first 4 years of life (Kanaphun *et al*., 1993). Of the total melioidosis cases notified between March to April 2004 in Singapore, 61% had significant exposure to soil (construction workers and outdoor sports), suggesting inoculation as an important mode of infection (Ministry of Health, 2004a).

Inhalation has also been postulated to be an important mode of disease acquisition. This is partly due to the strong association of melioidosis cases with the monsoonal

rainy season in endemic countries (Chaowagul *et al*., 1989; White, 2003). Aerosols created during heavy rain results in inhalation of the organism, shifting the primary mode of acquisition from cutaneous inoculation to inhalation (Currie *et al*., 2003). Further, higher rainfall is significantly associated with pneumonia. The higher case fatality observed in Singapore during the first seven months in 2004 was strongly correlated to the higher rainfall experienced during that period. A higher proportion of patients presented with pneumonic form of disease; 73.1% compared to 36.4% in the same period, the year before, was also observed (Ministry of Health, 2004c). During the Vietnam war, a fairly high incidence of melioidosis was reported among helicopter crew (Howe *et al*., 1971). It has been postulated that the rotor blades of the helicopters probably produced aerosols from the wet paddy fields, hence spreading the disease to the soldiers via inhalation.

More recently, involvement in the 2004 tsunami has been described as a major risk factor for melioidosis (Chierakul *et al*., 2005). Ingestion may also pose as a mode of infection (Cheng and Currie, 2005) as contamination of potable water had been implicated in a cluster of cases in Northern Australia (Currie *et al*., 2001).

Presence of underlying disease is a well-recognised risk factor for melioidosis. These include diabetes mellitus, renal failure, alcohol excess, chronic lung disease and low immune status (Cheng and Currie, 2005; White, 2003). Incidence of melioidosis cases peaks between the fourth and sixth decade of life in Thailand, with more than 80% of the adults having one or more underlying diseases (Wiersinga *et al*., 2006). A population-based study in Australia found higher risk of melioidosis among those aged more than 45 years, diabetics or those with excess alcohol consumption, chronic

lung disease, and chronic renal disease (Cheng and Currie, 2005). In Singapore, the highest incidence rate was observed among adults aged 65 and above (5.4 per 100,000) (Ministry of Health, 2006). Higher mortality rates were also observed among those with co-morbidities (Ministry of Health, 2006). Among the 62 cases reported in 2006, 86.4% had co-morbidities (Ministry of Health, 2006). The most common was pneumonia (55.9%), followed by diabetes mellitus (52.5%) and hypertension (30.5%).

1.2.3 Clinical presentation and treatment

Infection with melioidosis occurs primarily in three clinical forms: Asymptomatic, localised infection, or septicaemia (Wiersinga *et al*., 2006). The clinical symptoms of melioidosis mimic those of many other diseases and thus differentiating melioidosis from other acute and chronic bacterial infections is difficult without laboratory tests (Peacock, 2006). Confirmation of melioidosis requires laboratory culture and isolation of *B. pseudomallei,* which is not readily available in many hospitals in endemic regions. Infection with *B. pseudomallei* may be clinically silent for many years after exposure, and the development of disease usually occur as a result of a change in immune status (Woods *et al*., 1999).

The most severe clinical manifestation in melioidosis is septic shock, which is often associated with pneumonia and bacterial dissemination to distinct sites (Wiersinga *et al*., 2006) Other clinical presentations include genitourinary infection, encephalomyelitis, liver and spleen abscesses, neurological or brain abscesses, and skin or soft tissue infection (Cheng and Currie, 2005).

Currently, no effective method of prevention of melioidosis exists (Leelarasamee *et al*., 2004). There is no vaccine against *B. pseudomallei* and the bacterium is intrinsically resistant to penicillin and gentamicin, the usual treatment for suspected septicaemia in many parts of the developing world where melioidosis is endemic (Brett *et al*., 2000). Even under the best circumstances, prolonged treatment with expensive antibiotics is required for the eradication of this organism, although relapse is common. The current treatment of choice are parenteral ceftazidime for ten to fourteen days or longer as clinically indicated, followed by oral TMP-SMX with doxycycline for at least twelve to twenty weeks (Wuthiekanun *et al*., 2006).

Recurrent melioidosis is an important cause of morbidity and mortality in patients who survive a primary infection. The rate of relapse is approximately 6% in the first year and 13% during the next 10 years of follow up (Peacock, 2006). Among relapse patients, 75% were caused by re-infection with the same strain. This could be a result of failure in antibiotic treatment or that the primary infection was caused by more than one strain of the bacterium (Peacock, 2006). One study suggested that the failure to complete at least 12 weeks of therapy was an important determinant of relapse, reinforcing the necessity for adherence to therapy (Cheng and Currie, 2005).

1.3 BURKHOLDERIA PSEUDOMALLEI

1.3.1 Environmental niche

Burkholderia pseudomallei is a soil saprophyte, and can be recovered readily from water and wet soils in endemic areas (White *et al.*, 2003). *B. pseudomallei* is able to survive hostile environmental conditions such as nutrient deficiency, acidic environments and dehydration (Cheng and Currie 2005; Inglis and Sagripanti 2006). Interestingly, *B. pseudomallei* has been shown to survive in sterile triple distilled water for more than 17 years in a non-replicative state (Wuthiekanun *et al*., 1995; Moore *et al*., 2008). Based on these observations, it has been suggested that the increase in *B. pseudomallei* infection observed during the rainy season may partly be due to the switching of the bacteria from a dormant and non-culturable state to a more replicative state (Currie, 2008).

1.3.2 Military relevance

B. pseudomallei is currently classified as a category B bioterrorism agent by the US Centers for Disease Control and Prevention, because of the ease of dissemination, and the ability to result in moderate morbidity and low mortality rates due to the lack of appropriate treatment. This pathogen has been studied by the United States, the former Soviet Union and possibly Egypt as a potential biological warfare weapon, although they have never been used in this capacity (Vietri and DeShazer, 2007). The use of *B. pseudomallei* as a possible biological warfare agent is a serious concern due to the ease of acquiring strains from the environment and the lack of a vaccine.

1.3.3 Intracellular survival

An important aspect of interaction with host cells is the ability of *B. pseudomallei* to survive intracellularly in both phagocytic and non-phagocytic cells (Egan *et al*., 1996; Jones *et al*., 1996; Wiersinga *et al*., 2006). Upon internalization, *B. pseudomallei* may either reside within the acidic environment of phagolysosomes, or escape into the cytoplasm (Stevens *et al*., 2002). Within the cytoplasm it induces the formation of actin associated membrane protrusions, which facilitates cell-to-cell spread and the formation of multinucleated giant cells (MNGC) (Kespichayawattana *et al*., 2000). MNGC has been identified in tissues of melioidosis patients and it is has been suggested that the formation of such fused cells sequesters the pathogen and shields it from host surveillance (Wong *et al*., 1995).

Intracellular survival of *B. pseudomallei* may play an important role in evading the host system (Brett *et al*., 2000; Stevens *et al*., 2004; Woods *et al*., 1999). *In-vitro* models indicate that *B. pseudomallei* uses multiple mechanisms to survive intracellularly. These include its type III secretion system (TTSS) and capsular polysaccharide. Cluster three of TTSS (TTSS3) was required for full virulence of *B. pseudomallei* in a hamster model of infection (Warawa *et al.,* 2005). TTSS3 in *B. pseudomallei* (termed *bsa*, *Burkholderia* secretion apparatus) shares homology with the *inv/spa/prg* TTSS of *Salmonella enterica* serovar Typhimurium, which is required for the invasion and survival of this pathogen within host cells. The *bsa* cluster in *B. pseudomallei* has similarly been demonstrated to be required for its intracellular survival. Mutants lacking components of *bsa* show reduced invasion, replication in murine macrophages and inability to escape from endocytic vacuoles (Stevens *et al.,* 2002). For example, BopE and BipD secreted via the Bsa type III

apparatus were required for bacterial entry into HeLa cells. Furthermore, *B. pseudomallei* BipD mutants were attenuated following infection of mice, and showed impaired bacterial replication in liver and spleen (Stevens *et al.*, 2002).

B. pseudomallei produces an extracellular capsular polysaccharide. The presence of capsule may play an important role in its intracellular survival as phagocytosis was shown to be greater in capsule deficient mutant than wild type (Reckseidler-Zenteno *et al.*, 2005). It has been described that the capsule may act as a barrier, shielding the organism from the host. This is based on the observation that deposition of complement factor 3b by host cells was lower in the presence of capsule.

The ability of *B. pseudomallei* to survive intracellularly may partly be due to its resistance to antimicrobial activity and ability to evade macrophage killing. *In-vitro*, *B. pseudomallei* is resistant to the cationic antimicrobial peptide, human defensin and protamine sulfate (Jones *et al*., 1996). It has also been observed that *B. pseudomallei* inhibits DNA and protein synthesis in host cells (Mohamed *et al*., 1989). The exact mechanisms by which *B. pseudomallei* evades macrophage killing are unknown. However, upon invasion of macrophages, *B. pseudomallei* failed to substantially activate inducible nitric oxide synthase (iNOS), which plays an important role in the killing of intracellular bacteria (Utaisincharoen *et al*., 2001). The failure to activate iNOS may result from the inability to activate IFN-β, which is an upstream activator of iNOS. Addition of exogenous IFN-β was demonstrated to restore the macrophage ability to activate iNOS expression and consequently resulted in enhanced killing of the pathogen (Utaisincharoen *et al*., 2004).

1.4 LIPOPOLYSACCHARIDE (LPS)

1.4.1 General architecture

LPS is the outer membrane molecule found in Gram-negative bacteria. It consists of a hydrophobic domain termed lipid A, a non-repeating core oligosaccharide region, and a distal polysaccharide termed O-specific antigen that is the outermost region of LPS (Raetz *et al*., 2002). Generally, two forms of LPS exist. The S-form (smooth) LPS consists of all the 3 segments of LPS and is so called due to the appearance of glossy and shiny colonies. The R-form (rough) of LPS consists of only lipid A and the core region, and gives rise to dull colonies. The presence of Lipid A and Kdo (anchor to core) is the minimal requirement for the growth of Gram-negative bacterium (Caroff *et al*., 2002). The additional core and O-specific antigen are not required for growth, but may help the bacteria resist the effects of antibiotics, the complement system and other environmental stress.

1.4.2 Lipid A

Lipid A is the most conserved region of LPS. It is the bio-active centre (Dixon *et al*., 2005) and endotoxic component of the LPS, and the only region of the LPS that is recognised by the innate immune system (Miller *et al*., 2005). The innate immune system is the first line of defence that recognises evolutionarily conserved patterns associated with pathogens such as LPS. It is able to detect pico-molar concentrations of LPS and trigger cellular response (Jerala, 2007). LPS is responsible for several symptoms and signs of sepsis such as fever, diarrhoea, hypotension and shock. The classical lipid A structure is represented by the LPS from *Escherichia coli* and

consists of a biphosphorylated disaccharide backbone acylated with hydroxy and non-hydroxy fatty acids at specific positions (Caroff *et al*., 2003).

1.4.3 Activation of host cells by LPS (lipid A)

The LPS receptor complex in host cells is made up of Toll-like receptor 4 (TLR4), MD2 and CD14. Lipid A is first recognised by the LPS binding protein (LBP), which transfers monomers of LPS to CD14. CD14 is a high affinity LPS receptor but does not have a cytoplasmic domain, and hence cannot transduce signal upon binding to LPS (Chow *et al*., 1999). Instead, CD14 concentrates LPS and transfers it to TLR4-MD2, which then initiates a complex signal transduction across the plasma membrane, ultimately leading to the activation of nuclear factor kappa beta (NF-κβ), with subsequent transcription and release of pro-inflammatory mediators including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor alpha (TNF-α). LPS can bind directly to the TLR4/MD2 complex without LBP and CD14, although with lower affinity (Miyake *et al*., 2006). There are 2 proposed signalling pathways after TLR4 activation, the MyD88 dependent pathway and independent pathways (Zughaier *et al*., 2005). In the MyD88 dependent pathway, there is rapid NF-κβ activation and release of pro-inflammatory cytokines and chemokines. On the other hand, LPS activation of the MyD88 independent pathway leads to IFN-β release and delayed NF-κβ activation. This pathway appears to be important for dendritic cell maturation and is a link between the innate and adaptive immune response (Hoebe *et al*., 2003).

Although lipid A was once thought to be a conserved molecule, it is diverse in both number and length of fatty acid side chains and other associated modifications. These

variations results in altered recognition of the molecule by TLR4. The hexa-acylated lipid A of *E. coli* with side chains of 12 to 14 carbons, has been shown to be maximally stimulating (Caroff *et al*., 2003). Any deviation from this structure such as the number or length of fatty acyl chains would reduce the magnitude of signal (Caroff *et al*., 2003; Raetz *et al*., 2002). An *E. coli* strain that was genetically modified to express penta-acylated lipid A showed reduced immunogenicity and acted antagonistically by also inhibiting pro-inflammatory signalling by wild-type *E. coli* and LPS from other bacteria (Backhed *et al*., 2003). Ernst *et al*., (2003) also demonstrated that LPS preparations from *Pseudomonas aeruginosa* clinical isolates expressing penta and hexa-acylated species stimulated more robust pro-inflammatory responses than LPS purified from clinical isolates expressing only penta-acylated species.

The capacity of lipid A to stimulate host cells and induce cytokine production is dependent not only on the number and nature of fatty acids but also on the species of the host cells. Tanamoto and coworkers showed that chemically synthesized *E. coli* type hexa-acylated lipid A (compound 506) and *Salmonella* type hepta-acylated lipid A (compound 516) were able to strongly induce TNF-α release in mouse peritoneal macrophages and mouse macrophage like cell-line J774-1 (Tanamoto and Azumi, 2000). However, in the human cell-lines THP-1 and U937, compound 516 was completely inactive while compound 506 displayed strong activity. These results suggests that the mechanism of activation by LPS or the recognition of lipid A structure by human cells may differ from that in mouse cells.

Several groups have shown that the shape of lipid A may influence its biological activity and consequently the receptors that are activated (Netea *et al*., 2002; Seydel *et al*., 2000). The hexa-acylated shape of lipid A from *E. coli* assumes a conical shape and when intercalated into the membrane, induces a mechanical stress onto putative signalling proteins and signals through TLR4. *Porphyromonas gingivalis,* which assumes a conical shape to a lesser degree stimulates through TLR2 (Netea *et al*., 2002). On the other hand, lipid A from *Chromobacterium violaceum* that assumes a cylindrical shape would instead act antagonistically by occupying the binding site but not exerting the necessary stress for activation (Seydel *et al*., 2000) (Figure 1.1).

1.4.4 Modification of lipid A structures

For many pathogens, early innate responses slow the progress of infection, allowing the adaptive immune response to develop. Some Gram-negative bacteria have evolved mechanisms to modify their lipid A structures in different environments. This manipulates the pattern recognition motif used by the innate immune system to delay, evade or eliminate host recognition, hence subverting host immune response. Changes in acylation pattern and the modification of phosphate groups with 4-amino-4deoxy-arabinose (Ara4N) are two common mechanisms used to modify the lipid A structures.

In *Salmonella enterica* serovar Typhimurium, modification of lipid A occurs via the activation of a two component regulatory system PhoP-PhoQ (García Véscovi *et al*., 1996). PhoQ is a sensor kinase that senses the different environmental conditions such as that inside a macrophage phagosome, or those that destabilise the bacterial membrane, such as magnesium limited growth medium and exposure to antimicrobial

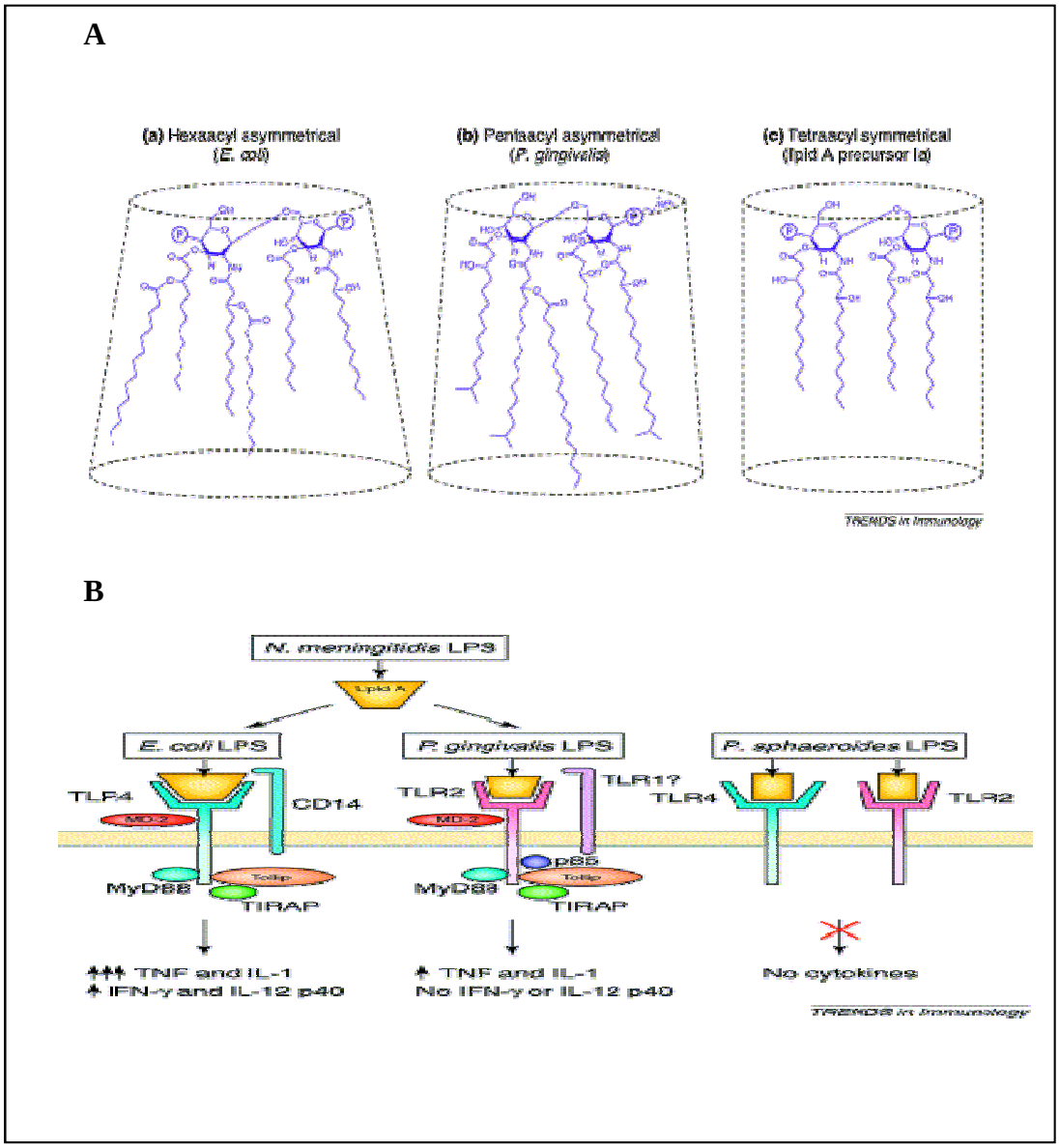


Figure 1.1: Shape of lipid A and its influence on biological activity. The three dimensional structures of various types of lipid A (A), and the hypothesis linking the shape of lipid A to the type of receptor it binds (B) is illustrated above. Figure adapted from Netea *et al*., 2002 (Trends in Immunology).

peptides (Kawasaki *et al*., 2005). Upon recognition of the different environmental conditions, PhoQ activates PhoP by phosphorylation, leading to the activation of more than forty genes. The roles of the PhoP-PhoQ system and other activated enzymes were ascertained by the establishment of mutants for the specific genes (Guo *et al*., 1998). PmrA/PmrB is another two component system activated by PhoP-PhoQ that catalyses the substitution of Ara4N at the terminal phosphates (Zhou *et al*., 2001) of lipid A. This modification allows the bacterium to lower their affinity for cationic antimicrobial peptides (CAMPS) and cationic antibiotics by decreasing the overall negative charge of their cell surfaces (Gunn, 2001; Trent, 2004). In addition, PhoP-PhoQ activates lipid A palmitoyltransferase and 3-O-deacylase that catalyses the modification of acylation patterns of its lipid A moieties (Gibbons *et al*., 2005). These modifications have been shown to increase the virulence of the bacteria by enhancing intracellular survival.

Changes to the lipid A structure of *Pseudomonas aeruginosa* has also been shown to occur in magnesium limited conditions (Ernst *et al*., 1999). These changes are similar to those identified between clinical isolates from patients with cystic fibrosis (CF) and environmental isolates (Ernst *et al*., 2007). LPS from environmental isolates and laboratory adapted strains of *P. aeruginosa* has a penta-acylated structure. In contrast, the isolates from airway of CF patients synthesize hexa-acylated LPS with added palmitate (C16:0) and Ara4N in the lipid A moiety (Ernst *et al*., 1999). Consequently, CF LPS has been demonstrated to stimulate robust production of inflammatory cytokines but not the penta-acylated LPS from environmental isolates. In addition, only human cells are able to discriminate between the different lipid A structures of *P. aeruginosa* but not murine cells (Hajjar *et al*., 2002). This differential recognition

is due to the hypervariable 82 amino acid region in the extracellular domain of human TLR4 that is not present in any region of the murine TLR4. The modifications in the lipid A structure of *P. aeruginosa* are thought to lead to the chronic colonisation of the airways of patients with CF, by promoting resistance to CAMPs and other active components of the immune system (Ernst *et al*., 2007).

Yersinia pestis has similarly been shown to modulate their lipid A structure but in response to temperature. *Y. pestis* is the causative agent of plague (Aussel *et al*., 2000). When *Y. pestis* is grown between 21^{o}C to 27^{o}C, the body temperature of fleas, the lipid A structure is mainly a hexa-acylated form. However, when the growth temperature is increased to 37^{o}C, the body temperature of humans, the lipid A changes to a tetra-acylated form (Knirel *et al*., 2005). The hexa-acylated lipid A is a strong activator of TLR4 in humans, whereas the tetra-acylated form has very weak stimulatory activity (Kawahara *et al*., 2002). The changes in acylation patterns significantly contribute to the virulence of this bacterium by reducing the recognition by human TLR4 and hence prevent containment by local inflammation. This allows *Y. pestis* to achieve a high bacterial load in mammalian blood required for efficient flea infection (Montminy *et al*., 2006).

1.5 LPS OF *B. PSEUDOMALLEI*

Perry and group reported the presence of 2 types of LPS in *B. pseudomallei* (Perry *et al*., 1995), although later studies demonstrated that one of the LPS corresponded to the capsular polysaccharide that co-purified with the LPS (Isshiki *et al*., 2001). To determine the heterogeneity in LPS from different isolates, LPS from 12 strains of *B. pseudomallei* were compared using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from different geographical locations and the profile from the different strains were found to be homogenous (Pitt *et al*., 1992). This study concluded that LPS was not an important virulence factor, although the number of samples studied was limited. A bigger study compared LPS profiles from 214 strains of *B. pseudomallei*. With the exception of 4 isolates, the rest showed identical ladder patterns (Anuntagool *et al*., 1998). These 4 isolates were obtained from patients with a relapse of melioidosis. Furthermore, this atypical LPS could not react with the monoclonal antibody (Anuntagool *et al*., 2000) that was reactive against the other strains of LPS. In 2006, the same group compared LPS from 1,327 *B. pseudomallei* isolates (Anuntagool *et al*., 2006) and found a third type of LPS, which was of a rough form. Although no association was found between LPS type and disease severity, the atypical LPS and R-LPS were found more frequently in clinical than environmental isolates, and more in Australian than Thai isolates. These isolates were also more often associated with relapse than with primary infection. The 3 LPS types showed similar endotoxic activity in the Limulus amoebocyte lysate (LAL) assay but there was a lack of immunological cross-reactivity on western blot, indicating a difference in host immune response (Anuntagool *et al*., 2000).

1.5.1 The role of LPS in the pathogenesis of *B. pseudomallei*

LPS is the most common bacterial component that is implicated in initiating septic syndrome (Bosshart *et al*., 2007). *B. pseudomallei* LPS has been suggested to play a possible role in the pathogenesis of melioidosis because patients with septicaemic melioidosis (Friedland *et al*., 1992; Suputtamongkol *et al*., 1992) show significantly elevated levels of pro-inflammatory cytokines, the release of which is known to be activated by LPS in other bacterial infections. Furthermore, these patients displayed high levels of anti-LPS antibodies (Petkanjanapong *et al*., 1992). In another study, the level of antibody to *B. pseudomallei* LPS was found to be significantly higher in melioidosis patients who survived than in those who died. These antibodies were also significantly higher in patients with nonsepticaemic than septicaemic melioidosis (Charuchaimontri *et al*., 1999). In an *in-vitro* model using polymorphonuclear leukocytes, antibodies to the LPS O-antigen of *B. pseudomallei* were protective against the pathogen by promoting phagocytic killing (Ho *et al*., 1997). These antibodies were also demonstrated to develop during human infection in a clinical study and this may facilitate clearance of the organisms *in-vivo*.

Relatively few functional studies have focused on the LPS of *B. pseudomallei*. Recently, the O-antigen of *B. pseudomallei* LPS was demonstrated to modulate host response, as a lipopolysaccharide (LPS) mutant that lacks the O-antigenic polysaccharide moiety, was more susceptible to macrophage killing during the early phase of infection than the parental wild-type strain (Arjcharoen *et al*., 2007). Kinetic studies have shown that *B. pseudomallei* LPS was less stimulatory in activating RAW 264.7 (mouse macrophage cell-line) than LPS from *E. coli* (Utaisincharoen *et al*., 2000). Similarly, Matsuura and co-workers have demonstrated that LPS from

B. pseudomallei had weaker pyrogenic and lower macrophage activating activity than LPS from *E. coli*, despite inducing stronger mitogenic activity in murine splenocytes (Matsuura *et al*., 1996). Both studies seemed to indicate that the LPS of *B. pseudomallei* may be unique and is less capable in activating immune cells. Hence, synthesis of weakly stimulatory LPS may be one strategy used by *B. pseudomallei* to evade the immune system.

Interestingly, *B. pseudomallei* LPS was able to stimulate splenocytes from C3H/HeJ mice that carry a loss of function mutation in TLR4 (Matsuura *et al.,* 1996). This finding suggests that *B. pseudomallei* LPS was unique and does not stimulate pro-inflammatory responses through TLR4. Supporting this conclusion is a recently published data that indicated that TLR2, and not TLR4 was involved in the signal transduction *of B. pseudomallei* LPS (Wiersinga *et al*., 2007).

1.5.2 LPS as subunit vaccines

As high levels of anti-LPS antibodies appear to be protective in melioidosis patients, various groups have explored using *B. pseudomallei* LPS as subunit vaccines. A study evaluating LPS and capsular polysaccharide as possible subunit vaccines against experimental melioidosis (Nelson *et al*., 2004) suggested that LPS was a potential vaccine candidate against melioidosis. Mice immunised with LPS developed a strong IgG3 and IgM response and an increased mean time to death was observed compared with unvaccinated controls when the mice were further challenged via the intra-peritoneal or aerosol routes. The passive transfer of antibodies from immunized mice into naive animals also provided protection against a subsequent challenge with *B. pseudomallei* (Nelson *et al*., 2004). Another study investigated the varying

protection conferred to mice by various polysaccharides subunits through immunisation with *B. pseudomallei* strains with mutations in O-antigen, capsular polysaccharide, type III O-PS or type IV O-PS gene clusters (Sarkar-Tyson *et al*., 2007). Immunization with all the polysaccharide mutant strains resulted in delayed time to death compared to the naive controls, when challenged with wild-type *B. pseudomallei* strain. However, contrary to previous results, immunisation with *B. pseudomallei* strains with mutations in O-antigen and capsular polysaccharide conferred greater protection (Sarkar-Tyson *et al*., 2007). This was attributed to the exposure of other protective surface antigens upon removal of the highly abundant polysaccharide.

1.6 PRELIMINARY DATA AND OBJECTIVES OF CURRENT PROJECT

Preliminary unpublished results from our laboratory's collaborative study with Dr. Patrick Tan's laboratory, Genome Institute of Singapore (GIS), indicated the presence of an environmental transcriptional regulatory system (similar to PhoP-PhoQ in *Salmonella* serovar Typhimurium) in *B. pseudomallei* based on microarray data and sequence homology. As described above, in *Salmonella* serovar Typhimurium, the two component regulatory system (PhoP-PhoQ) has been demonstrated to detect changes in the environment, such as the low magnesium ion concentration in culture medium or macrophage phagosome (García Véscovi *et al*., 1996). Based on this finding, it was hypothesized that environmental magnesium may also play a significant role in modulating the virulence in *B. pseudomallei*. This was supported by the increase in melioidosis cases during rainy seasons, where heavy rains could result in dilution of soil ions resulting in the increased virulence of *B. pseudomallei*.

To confirm this phenomenon, the effect of magnesium on the virulence of *B. pseudomallei* was investigated using a *Caenorhabditis elegans* model. Wild-type *B. pseudomallei* was grown in either magnesium-supplemented or magnesium-minimal culture medium. Results indicated that a lower percentage of the worms survived when infected with *B. pseudomallei* cultured in magnesium minimal medium, signifying that *B. pseudomallei* became more virulent under such conditions (Figure 1.2). Similar phenomenon was observed with both the young adult stage worms and the L4 stage worms. To ascertain that the increase in virulence was not due to the difference in the bacterial load, the growth kinetics of *B. pseudomallei* in magnesium supplemented and magnesium minimal culture medium was investigated. Results

showed that the growth of *B. pseudomallei* was significantly enhanced when extracellular magnesium was present and that growth was at least 30% slower when the media contained minimal amount of magnesium (Figure 1.3).

The *C. elegans* model described above suggests a role of magnesium in the virulence of *B. pseudomallei*. Magnesium has been shown to play an important role in maintaining the outer membrane intergrity of Gram-negative bacteria and bacteria such as *Salmonella* serovar Typhimurium and *P. aeruginosa* are known to modulate host response in response to divalent cations such as magnesium with the aid of the PhoP-PhoQ environment regulatory system. Host response is modulated primarily through the modification of the lipid A moiety of LPS in these pathogens. Since our preliminary studies indicate the presence of a similar regulatory system in *B. pseudomallei,* and that magnesium may modulate the virulence of *B. pseudomallei*, we hypothesize that magnesium plays an important role in the virulence of *B. pseudomallei* and that virulence is determined by surface remodeling of LPS in response to magnesium limitation.

Other groups have shown that the LPS of *B. pseudomallei* is weakly immunogenic and this characteristic may contribute to the ability of the bacterium to survive intracellularly for prolonged periods by evading host response (Utaisincharoen *et al*., 2000). However, no studies have compared the functional activities of *Burkholderia thailandensis* LPS to *B. pseudomallei* LPS. *B. thailandensis* is a close relative of *B. pseudomallei* that rarely causes diseases. Hence, differences in functional activities between the two LPS, if any, would reveal an important possible

pathogenesis mechanism in *B. pseudomallei*. We further hypothesize that differences in *B. pseudomallei* LPS and *B. thailandensis* LPS may modulate host response.

In order to evaluate the above hypotheses, the following studies were undertaken:

1. Characterisation of LPS extracted from *B. pseudomallei* grown under magnesium rich and deficient conditions. The LPS profile was also compared to that of *B. thailandensis.*

2. Investigation of the effect of magnesium on the LPS of *B. pseudomallei* in a cell-based *in-vitro* model.

3. Detection of differences in virulence between LPS of *B. pseudomallei* and *B. thailandensis.*

4. Determination of structural differences between LPS of *B. pseudomallei* and *B. thailandensis.*

5. Determination of the TLR involved in the recognition of *B. pseudomallei* and *B. thailandensis LPS*

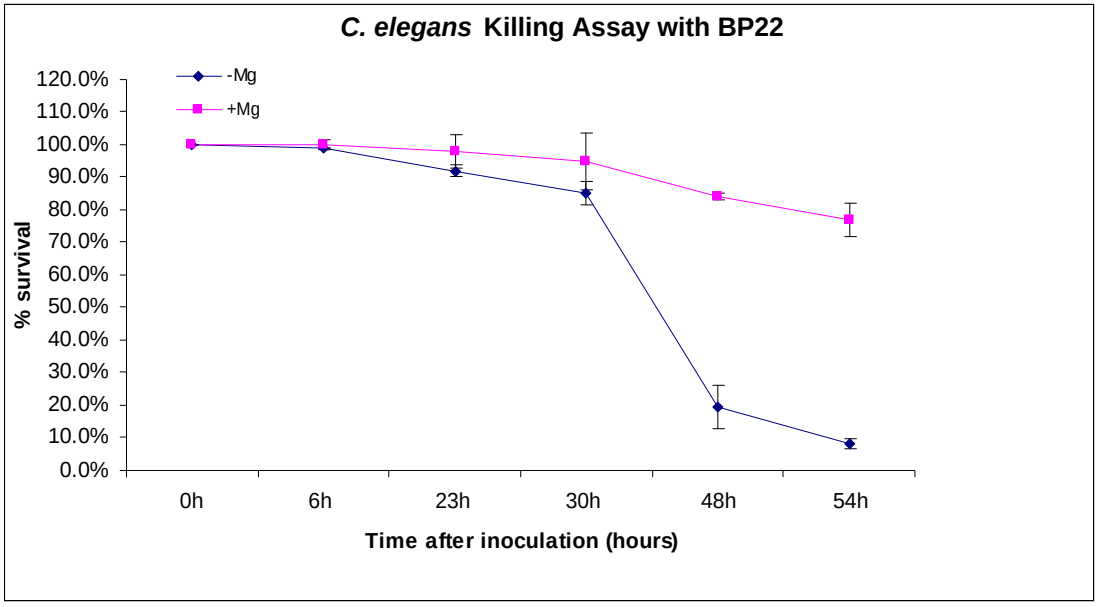


Figure 1.2: *C. elegans* killing assay with *B. pseudomallei* grown in magnesium-supplemented and magnesium-minimal medium. +Mg and –Mg indicates growth in magnesium-supplemented or minimal medium respectively (data courtesy of Sim Siew Hoon, DSO National Laboratories).

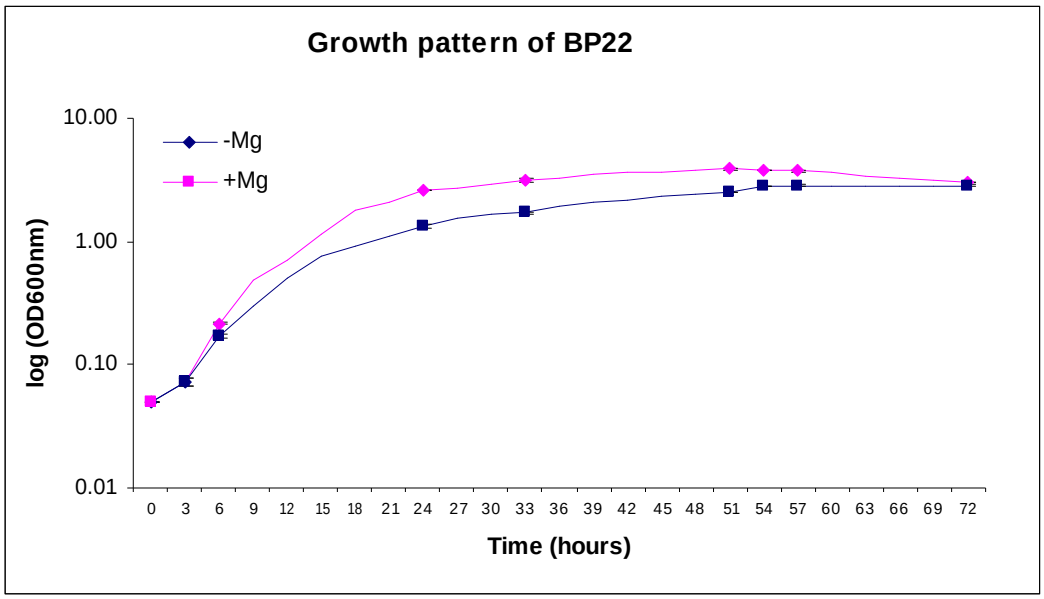


Figure 1.3: Growth of *B. pseudomallei* in magnesium-supplemented and magnesium-minimal medium. +Mg and –Mg indicates growth in magnesium-supplemented or minimal medium respectively. Results shown are average of 3 experiments (data courtesy of Sim Siew Hoon, DSO National Laboratories).

Chapter 2

Extraction of Lipopolysaccharides

2.1 INTRODUCTION

Lipopolysaccharides (LPS) are outer membrane molecules found in Gram-negative bacteria (Raetz *et al.*, 2002) and play an important role in disease caused by this group of bacteria (Bryn and Jantzen, 1982). Structural investigations of LPS have found that these molecules consist of three distinct regions (Weinbaum *et al.*, 1971): the outermost regions consist of a polysaccharide chain with repeating oligosaccharide units of variable length, known as the O-antigen region; this is in turn connected to the core polysaccharide chain, which is linked to lipid A via one or more units of a unique 8 carbon sugar, 3-deoxy-d-manno-octulosonate (KDO). Lipid A is the bio-active component of the LPS that is responsible for the endotoxicity of the bacteria (Rosner *et al.*, 1979).

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a simple and convenient method that provides information on the fundamental structure of LPS. In an electric field, LPS migrates towards the anode, due to the highly negatively charged lipid A. The LPS chains (O-antigen) with the same number of repeating sugar units will co-migrate to give the characteristic ladder pattern common to LPS. The distance the LPS chain migrates depend on the length of polysaccharide chains, thus long-chain LPS move a short distance, whereas those with short chains or none move very close to the dye front.

The most commonly used method for the extraction of LPS from Gram-negative bacteria, is the hot phenol-water procedure introduced by Westphal and Jann (Westphal and Jann, 1965). However, a review of the experimental protocol by our Institutional Biosafety Committee had indicated that our biosafety level-3 (BSL-3) laboratory does not have the necessary chemical containment to minimize the risk posed by the hazardous chemical to our laboratory staff. We thus took this opportunity to explore an alternative method for LPS extraction and characterize the resultant extract.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and culture medium

Burkholderia thailandensis (ATCC 700388), *Salmonella enterica* serovar Typhimurium (BAA 189), *Pseudomonas aeruginosa and Klebsiella pneumoniae are* risk group 2 (RG2) agents and were cultured in the biosafety level 2 laboratory. *Burkholderia pseudomallei* strains, BP22 and K96243 are risk group 3 (RG3) agents and were obtained from the *B. pseudomallei* collection at Defence Medical and Environmental Research Institute (DMERI), DSO (Kent Ridge). For growth under magnesium (Mg^{2+}) poor and Mg^{2+} rich conditions, the basal medium used was N-minimal medium (5mM KCl, 0.5mM K_2SO_4, 1mM KH_2PO_4, 1.8µM $FeSO_4.7H_2O$, 2µg/ml thiamine-HCl, 0.1M tris-HCl, pH7.4 and 22mM glucose) and the Mg^{2+} concentration was 10mM $MgSO_4$ for Mg^{2+} rich medium and 8µM $MgSO_4$ for Mg^{2+} poor medium.

2.2.2 Culture of RG2 bacteria for LPS extraction

The various bacterial strains were streaked out from frozen stock and cultured for 2 days on TSA plates at 37^{o}C (recovery plate). Colonies from the recovery plate were then streaked onto TSA plate and cultured overnight at 37^{o}C. The next day, one colony was picked and inoculated into 5ml of either Mg^{2+} rich (+) or Mg^{2+} poor (–) N-minimal broth, and shaken at 200rpm for 24 hours at 37^{o}C. The bacterial culture was passaged in the respective culture media for four times; each time a 1:100 dilution of overnight culture was inoculated into fresh broth and incubated for 24 hours at 37^{o}C. For the fourth passage, 2ml of culture was inoculated into 200ml of fresh broth to prepare enough

bacterial culture for LPS extraction. At the end of passage four, 200ml of bacterial broth was centrifuged at 2000rpm for 10 minutes and the supernatant was discarded. The bacterial pellet was reconstituted in 10ml of respective broth. 1ml of the reconstituted bacterial culture was spread onto the respective "+" or "-" N-minimal agar plates and incubated overnight at 37^{o}C.

To harvest the bacteria, 6ml of the respective "+" or "-" N-minimal medium was added to the plates and scraped. The bacterial suspension was transferred to a pre-weighed 50ml falcon tube and the plates were washed with an additional 6ml of broth. The bacteria suspension from 3 plates was collected in one 50ml falcon tube. The tubes were centrifuged at 3750rpm for 20 min at 4^{o}C. The supernatant was aspirated and discarded. Each tube was re-weighed to determine the cell pellet mass. 1ml of lysis buffer (iNtRON LPS Extraction Kit, Intron Biotechnology) was added to every 50mg wet weight of bacteria. The tubes were vortexed vigorously until all cell pellets were dispersed completely. Tubes were stored at 4^{o}C until use.

2.2.3 Culture of RG3 bacteria for LPS extraction

B. pseudomallei was cultured similarly as above with some changes. All work was carried out in the BSL-3 laboratory located at DMERI, DSO (Kent Ridge). For safety considerations, broth cultures were shaken at 70rpm in an orbital shaker instead of 200rpm. After harvesting the cells in lysis buffer, sterility test was conducted by inoculating 5% of the total volume onto TSA plates. If no bacterial growth was observed

after 48 hours of incubation, the tubes were removed from the BSL3 laboratory and stored at 4^{o}C in the BSL2 laboratory until ready for further processing.

2.2.4 LPS extraction

As a first step, the lysis buffer and bacterial lysate mixture, which had been stored at 4^{o}C, was vortexed vigorously to resuspend any lysate that could have sedimented during the storage. 0.2% volume of chloroform was then added to each tube, followed by a short vortex for 10 to 20 seconds to mix the chloroform well. The tubes were then left at room temperature for a further five minutes to allow the phases to separate before centrifuging at 3000g for 45 minutes at 4^{o}C using the minimum breaking force. The supernatant (top phase) was then harvested into clean centrifuge tubes. Two volumes of purification buffer (iNtRON LPS extraction kit) was added to one volume of supernatant and mixed well. The centrifuge tubes were placed in a -20^{o}C freezer for 2 hours to allow the LPS to precipitate, following which, the tubes were centrifuged at 13000rpm for 15 minutes at 4^{o}C in a high-speed centrifuge. The supernatant was discarded and the pellet was washed twice with 70% ethanol. Finally, the pellet was air-dried, lyophilized and stored at 4^{o}C until LPS analysis.

2.2.5 Whole cell LPS profile

One full loop of bacterial lawn was harvested from the agar plates (passage 5) and transferred into a pre-weighed 1.5ml micro-centrifuge tube. The micro-centrifuge tube was reweighed and 50µl of solubilisation buffer was added to every milligram of bacteria. The bacterial cells were then resuspended by vortexing to break up cell clumps.

This suspension was heated at 99^{o}C for 10 minutes to kill and homogenize bacteria with occasional vortexing during the heating. After heating, an equal volume of solubilisation buffer containing proteinase K (100μg for every 30μl of solubilisation buffer) was added and the bacteria were heated at 60^{o}C for 2 hours. Finally the proteinase K was inactivated by boiling for 10 minutes. The sample was stored at -20^{o}C until use.

For work with bacterial strains requiring BSL-3 containment, after heating at 99^{o}C for 10 minutes to kill and homogenize bacteria, a sterility test was conducted. 25μl of the homogenate was inoculated onto TSA plate and incubated for 48 hours at 37^{o}C in an incubator. If no growth was observed, the samples were considered sterile and brought out of the BSL3 laboratory. The protocol was continued in the biosafety level 2 (BSL2) laboratory with the addition of solubilisation buffer containing proteinase K.

2.2.6 SDS-PAGE analysis

SDS-PAGE was carried out using 16% resolving gel. LPS or whole cell lysate was loaded per well. After electrophoresis, the separated components were detected using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen, Singapore). Stained gels were visualized using a 300 nm UV trans-illuminator.

2.3 RESULTS

The LPS from *Salmonella* serovar Typhimurium, *P. aeruginosa, K. pneumoniae, B. thailandensis* and *B. pseudomallei* were extracted using an alternative extraction method (iNtRON LPS Extraction Kit). To compare if the LPS obtained using this kit was representative of the whole LPS from the bacterium, crude LPS was obtained from whole cells using proteinase K digestion.

Figure 2.1 shows the profile of LPS from *Salmonella* serovar Typhimurium obtained from the kit. The ladder pattern obtained was similar to the whole cell LPS profile. No missing bands were observed. Although the ladder pattern was similar, a slight difference was observed between the LPS obtained from *Salmonella* serovar Typhimurium grown under Mg^{2+} rich and Mg^{2+} poor N-minimal medium. Similarly no difference was seen in the LPS profile of *P. aeruginosa* (Figure 2.2)

Figure 2.3 shows the LPS profile of *B. thailandensis*. The LPS profile of *B. thailandensis* did not have the characteristic ladder pattern seen in the LPS profile of *Salmonella* serovar Typhimurium. The LPS profile was similar to the whole cell LPS profile with the exception of an additional band of intermediate molecular weight that was present when the kit extraction method was used. Similar profile was observed between bacteria grown under Mg^{2+} rich and Mg^{2+} poor conditions.

Similar to *B. thailandensis, B. pseudomallei* (Figure 2.4) did not show any difference in the ladder pattern between the Mg^{2+} rich and Mg^{2+} poor conditions. An additional band of intermediate molecular weight was also observed when the extraction kit was used. Furthermore, LPS profile from *B. pseudomallei* was similar to that of *B. thailandensis*.

K. pneumoniae is an excessively capsulated bacterium. To check if the kit extraction method was suitable for the extraction of LPS from such bacteria, we compared the LPS profile to that of the whole cell LPS (Figure 2.5). Unlike the other bacteria studied here, we were unable to obtain a proper LPS profile even from the whole cell lysate. A smear pattern, characteristic of capsular polysaccharide was observed.

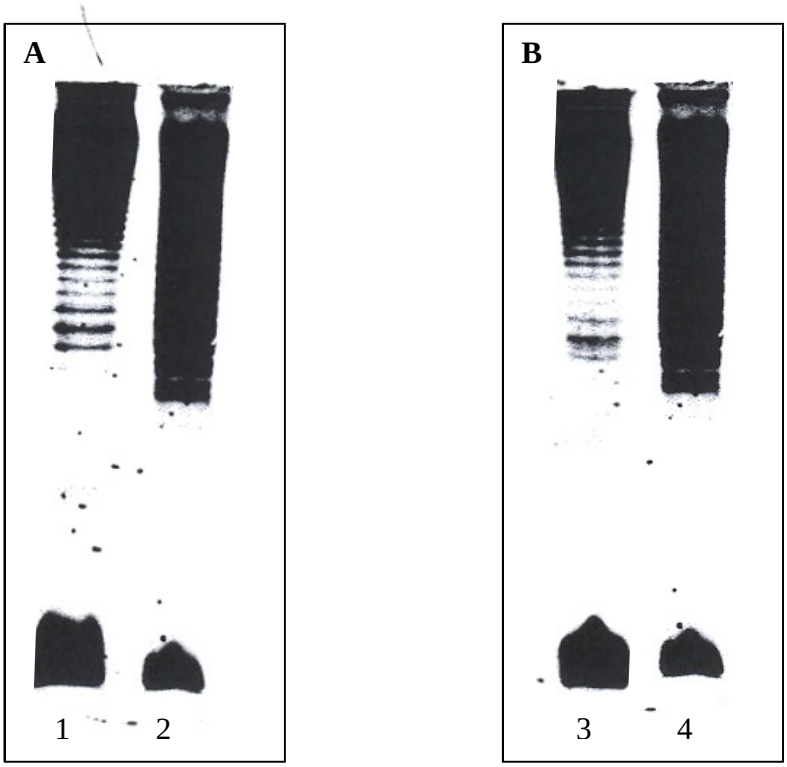


Figure 2.1: SDS-PAGE profile of *Salmonella* serovar Typhimurium LPS. LPS was extracted using an LPS extraction kit (A) and compared to the whole cell LPS profile (B). Lanes 1 and 3 represent LPS extracted from bacteria grown under Mg^{2+} rich medium and lanes 2 and 4 represent LPS extracted from bacteria grown under Mg^{2+} poor medium.

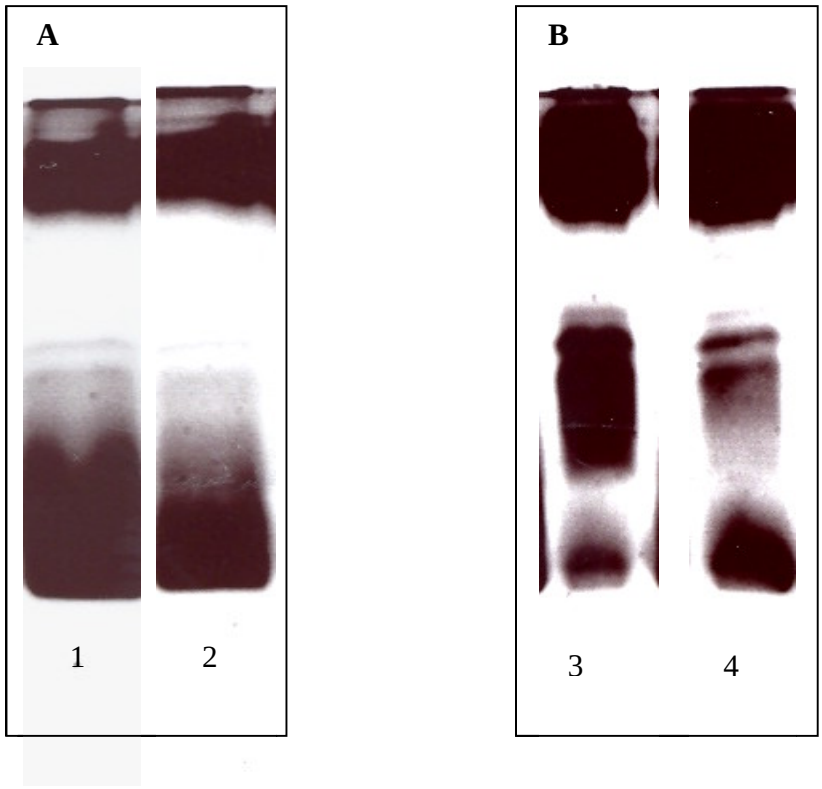


Figure 2.2: SDS-PAGE profile of *P. aeruginosa* LPS. LPS was extracted using an LPS extraction kit (A) and compared to the whole cell LPS profile (B). Lanes 1 and 3 represent LPS extracted from bacteria grown under Mg^{2+} rich medium and lanes 2 and 4 represent LPS extracted from bacteria grown under Mg^{2+} poor medium.

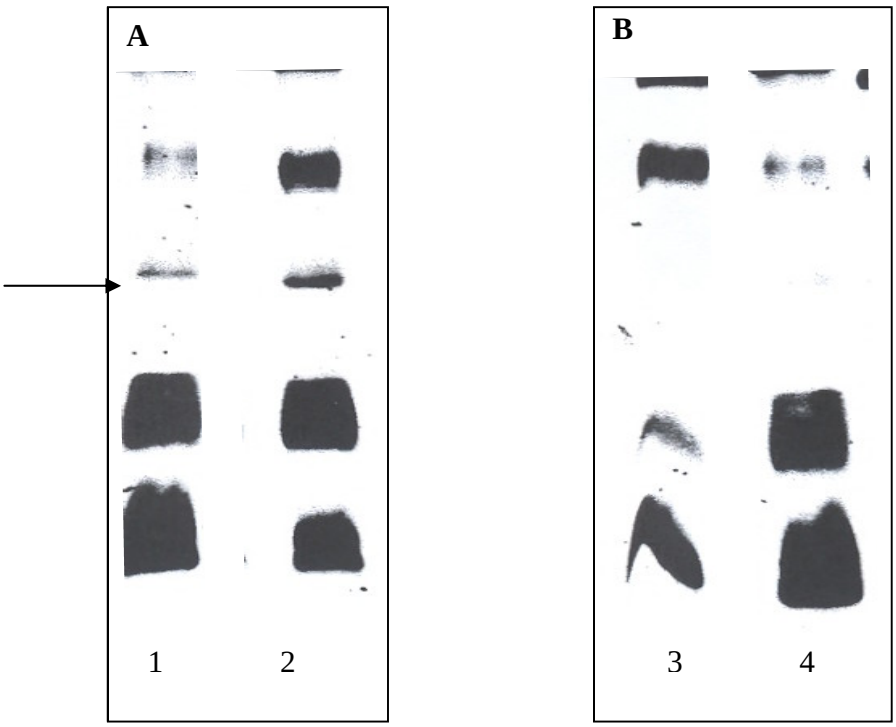


Figure 2.3: SDS-PAGE profile of *B. thailandensis* LPS. LPS was extracted using an LPS extraction kit (A) and compared to the whole cell LPS profile (B). Lanes 1 and 3 represent LPS extracted from bacteria grown under Mg^{2+} rich medium and lanes 2 and 4 represent LPS extracted from bacteria grown under Mg^{2+} poor medium. Arrow indicates band present in kit extracted LPS but absent in whole cell LPS.

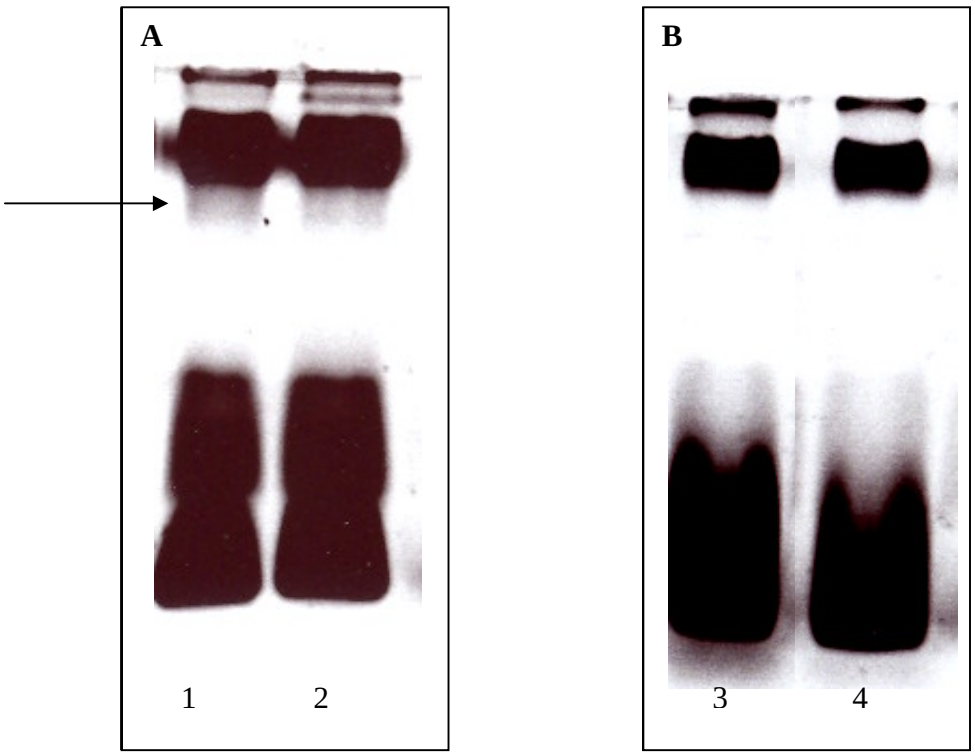


Figure 2.4: SDS-PAGE profile of *B. pseudomallei* LPS. LPS was extracted using an LPS extraction kit (A) and compared to the whole cell LPS profile (B). Lanes 1 and 3 represent LPS extracted from bacteria grown under Mg^{2+} rich medium and lanes 2 and 4 represent LPS extracted from bacteria grown under Mg^{2+} poor medium. Arrow indicates band present in kit extracted LPS but absent in whole cell LPS.

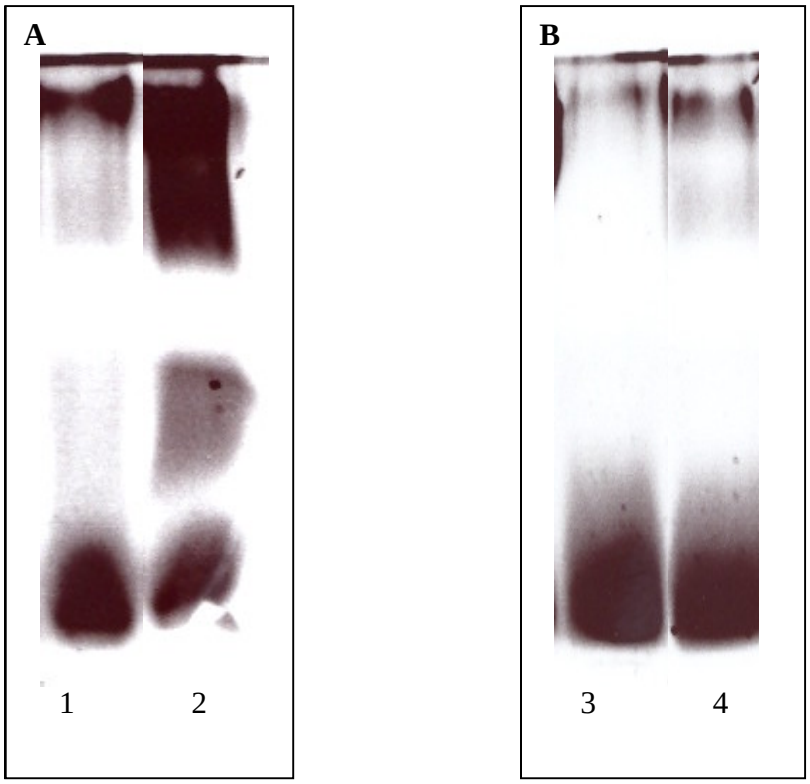


Figure 2.5: SDS-PAGE profile of *K. pneumoniae* LPS. LPS was extracted using an LPS extraction kit (A) and compared to the whole cell LPS profile (B). Lanes 1 and 3 represent LPS extracted from bacteria grown under Mg^{2+} rich medium and lanes 2 and 4 represent LPS extracted from bacteria grown Mg^{2+} poor medium.

2.4 DISCUSSION

Several methods exist for the extraction of LPS from Gram-negative bacteria. The most efficient and commonly used method is the hot phenol-water procedure introduced by Westphal and Jann (Westphal and Jann, 1965). This procedure is based on the theory that proteins are soluble in the phenol phase and LPS in the water phase, enabling the extraction of LPS from the aqueous phase. The hot phenol-water method has gone through several modifications and specific methods have been introduced for rough and smooth types of LPS. For rough type bacteria, an extraction method (PCP) involving a solvent mixture of aqueous phenol, chloroform and petroleum ether was developed (Galanos *et al.,* 1969). However, all of the above methods require the use of phenol, which is toxic and consequently dialysis is needed to remove the phenol. This also makes the procedure complex and time-consuming. Also, in some instances, LPS may be recovered from the phenol phase instead. Darveau and Hancock (1983) proposed a method that was suitable for the isolation of both smooth and rough LPS without the requirement of phenol, although the need for a multi-step procedure has probably resulted in many investigators shunning this method of extraction (Darveau and Hancock, 1983). Other new methods have been proposed for the extraction of LPS. These include the extraction of LPS from small quantities of cells (Li *et al.,* 1998) or the use of an RNA-isolating agent (Yi *et al.,* 2000). However, these methods still require 2 to 3 days for the extraction or the use of phenol. El Hamidi and colleagues (2005) introduced a new lipid A micro-isolation method that allows the direct isolation of lipid A from whole bacterial cells (El Hamidi *et al.,* 2005). Although this method takes just a day and is broadly

applicable to different Gram-negative bacteria, it allows only the extraction of rough-type LPS.

The LPS from *B. pseudomallei* is usually obtained using the hot phenol-water based procedure. However, it was shown upon separating the phases, only 0.7% of LPS enters the aqueous phase while 3.5% enters the phenol phase (Perry *et al.,* 1995). Chemical analyses also showed that the aqueous product was essentially a rough type of LPS and the smooth type of LPS was essentially in the phenol phase. Hence, all groups working on this LPS have since combined the 2 phases to obtain the maximal amount of LPS. However the high molecular weight capsular polysaccharide expressed by the virulent isolates of *B. pseudomallei* also partitions together with the LPS into the phenol phase (Brett *et al.,* 2007). Removal of the capsular polysaccharide is difficult and would require the use of size exclusion chromatography.

Due to the above reasons, and that we were unable to use the hot phenol-water method in our BSL3 facilities for safety reasons, we searched for an alternative method for the extraction of LPS. The LPS extraction kit from Intron Biotechnology has been used by several groups for the extraction of LPS from bacteria such as *Salmonella* species (Baldauf *et al.,* 2007), and *P. aeruginosa* (Bucki *et al.,* 2007). The kit extraction method is based on the hot phenol-water method, but without the need of hot phenol. In the kit extraction method, the bacterial cell membranes are disrupted with the provided aqueous lysis buffer, which consists of a mixture of chemicals, including a low concentration of phenol. Compared to the hot phenol-water method, the need for mechanical cell

disruption and heating are eliminated, and hence is safe for use in the BSL3 facilities. To isolate the aqueous phase LPS, chloroform is used to separate the phenol and aqueous phase. After centrifugation, the aqueous layer is isolated and the LPS is precipitated with the provided purification buffer, waiving the need for extensive dialysis as required in the hot phenol-water method. By avoiding the phenol phase, the possible contamination with capsular polysaccharide is also reduced significantly (Brett *et al.*, 2007). Since for some bacteria, LPS may be associated strongly with outer-membrane proteins causing them to enter the phenol phase, the LPS extracted using the kit was compared to whole cell LPS profile to enable the detection of such LPS.

This study is focused on LPS from *B. pseudomallei* and *B. thailandensis*. However, to determine the suitability of the extraction method, we also extracted LPS from *Salmonella* serovar Typhimurium, *P. aeruginosa* and *K. pneumoniae.* With the exception of *K. pneumonia,* the LPS extraction kit was suitable for the extraction of LPS from the other bacteria tested here. Although LPS from *B. pseudomallei* and *B. thailandensis* showed an extra band of intermolecular weight compared to whole cell lysate, these bands probably coincide with those from smooth LPS, which have been shown in *B. pseudomallei* to migrate into the phenol phase (Perry *et al.*, 1995). The bands are not likely to be due to protein contaminants as the staining used for the visualization of the gel is specific for LPS. Conventionally, LPS patterns are detected using silver staining. However, silver staining cannot differentiate LPS from proteins in the sample. The staining method used here stains only the periodate-oxidized carbohydrate groups, and hence is specific for the staining of LPS.

A difference was noted in the LPS ladder appearance of *Salmonella* serovar Typhimurium grown under Mg^{2+} rich and Mg^{2+} poor conditions. Although it is known that the lipid A of *Salmonella* serovar Typhimurium is modulated under varying magnesium concentrations (García Véscovi *et al.,* 1996), changes in the ladder pattern was not expected. This phenomenon seen here may be due to the different loading of LPS. Changes in LPS profile between Mg^{2+} rich and poor conditions were not seen for *P. aeruginosa, B. pseudomallei and B. thailandensis*. This is expected as SDS-PAGE LPS profile is not sensitive enough to detect changes in lipid A, which is the lowest molecular weight band in the gel. Changes in fatty acid acylation of lipid A will require further structural analysis.

Chapter 3

Determination of the effect of magnesium on the virulence of *Burkholderia pseudomallei.*

3.1 INTRODUCTION

Some Gram-negative bacteria have evolved mechanisms to modify their structures in different environments. This alteration in the pattern recognition motif delays or evades recognition by the innate immune system, hence subverting host immune response. For example, *Salmonella enterica* serovar Typhimurium is able to modify its lipid A molecules via the activation of a two component regulatory system PhoP-PhoQ (García Véscovi *et al.,* 1996). PhoQ is a kinase that senses the different environmental conditions, such as the reduced pH inside the macrophage phagosome, or the exposure to antimicrobial peptides, which destabilises the bacterial membrane (Fields *et al.*, 1989; Kawasaki *et al.*, 2005). This results in the activation of genes that enable the bacterium to respond to such environmental stimuli appropriately, enhancing its chances of survival.

In both prokaryotes and eukaryotes, magnesium (Mg^{2+}) is a membrane and ribosome stabilizer and is essential in reactions requiring ATP (Reinhart, 1988). In some bacteria, Mg^{2+} is the signal that regulates the PhoP/PhoQ system, which in turn controls several pathogenic properties of the facultative intracellular parasite including intra-macrophage survival (Fields *et al.,* 1986). In the case of *Salmonella* serovar Typhimurium, low Mg^{2+} environment mimics the macrophage phagosomal environment; sensing of the host environment (phagosome) enables the bacterium to resist host cationic antimicrobial peptides (CAMPS) and cationic antibiotics by decreasing the overall negative charge of their cell surfaces (Zhou *et al.*, 2001). This provides a competitive edge to the survival of the pathogen.

To date, much of the intricacies of *Burkholderia pseudomallei* pathogenesis remain to be elucidated and dissecting the molecular basis underlying bacterial virulence may provide new insights into the disease process. Preliminary unpublished results from our collaboration with Dr Patrick Tan's laboratory at the Genome Institute of Singapore indicated the presence of an environmental transcriptional regulatory system similar to that of *Salmonella* serovar Typhimurium, based on microarray and sequence homology data. Upon further investigation, we found that *B. pseudomallei* exhibited increased virulence when cultured in N-minimal medium containing low concentration of Mg^{2+} in a *Caenorhabditis elegans* model. Hence, the focus of the study in this chapter is to investigate the effects of low magnesium concentration on the LPS of *B. pseudomallei*.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

RAW 264.7 (mouse macrophage cell-line) and THP-1 (human monocyte cell-line) were purchased from American Type Culture Collection (ATCC). RAW 264.7 was cultured at 37°C in a humidified incubator (95 % air with 5% CO_2) in Dulbecco's Modified Eagle's Medium **(**DMEM) containing 10% fetal bovine serum (FBS) and sub-cultured every 3 to 4 days at a split ratio of 1:6. THP-1 cells were grown in suspension in RPMI 1640 with 10% FBS and 2mM L-glutamine and sub-cultured once a week at a ratio of 1:10.

3.2.2 Optimisation of the priming of THP-1 cells

THP-1 cells were primed for 8, 14 or 24 hours with 10 or 100ng/ml of phorbol 12-myristate 13-acetate (PMA). After priming, cells were washed with sterile phosphate buffered saline (PBS) twice, and complete medium added. Cells were allowed to rest for 24 hours to ensure that they revert to a resting phenotype before stimulation with 100ng/ml of commercial *Salmonella* serovar Typhimurium LPS (Sigma, Singapore) for 24 hours. The priming and stimulation of cells were monitored by light microscopy, and production of TNF-α to choose the optimal parameters for priming. The optimal concentration of PMA and length of priming was chosen for subsequent studies.

3.2.3 LPS stimulation assay

For RAW 264.7, 5 x 10^5 cells were seeded per well in a 6-well plate and incubated overnight prior to LPS stimulation assay. For THP-1 cells, 1 x 10^6 cells, stimulated with

10ng/ml PMA were seeded into each well of a 6-well plate. After 24 hours incubation, culture medium was removed and the cells were washed twice with PBS before fresh medium was added. THP-1 cells were allowed to rest for further 24 hours before being used for the LPS stimulation assay.

Both RAW 264.7 and THP-1 cells were stimulated with increasing concentration of LPS extracted from *B. pseudomallei* and *B. thailandensis* grown under Mg^{2+} rich or Mg^{2+} poor N-minimal medium. LPS extracted from *Salmonella* serovar Typhimurium was used as a control. Assay was carried out for 12 and 24 hours, respectively and the cell culture supernatant was quantitated for various cytokines (TNF-α, IL-10 and MCP-1) using Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

3.2.4 Fatty Acid extraction and analysis

4ml of methanolic HCl was added to 8mg of extracted LPS, and was heated for 18 hours at 90^{o}C in a Teflon-lined screw capped glass tube. Pentadecanoic acid was used as an internal standard. After overnight incubation, an equal volume of 50% saturated NaCl solution was added, and the fatty acid methyl esters were extracted with chloroform. Nitrogen gas was used to concentrate the extracted methyl esters to almost dryness but not completely and analysed by gas chromatography mass-spectrometry (GC-MS).

3.2.5 Statistical analysis

Cytokine production was analysed by unpaired Student's *t* test (2-tailed distribution). Values of $p<0.05$ were considered statistically significant.

3.3 RESULTS

3.3.1 Analysis of fatty acids in lipid A

Six different LPS extracts were analysed by GC-MS and the results are shown in Table 3.1. Modifications in the lipid A structure, when exposed to Mg^{2+} limiting environment are well characterized in *Salmonella* serovar Typhimurium (Guo *et al.*, 1997). Hence, LPS was extracted from this bacterium cultured in either Mg^{2+} rich or Mg^{2+} poor N-minimal medium and used as a positive control to detect changes in lipid A. When cultured in Mg^{2+} rich N-minimal medium, dodecanoic acid (C12:0), tetradecanoic acid (C14:0) and 3-hydroxy-tetradecanoic acid [C14:0(3-OH)] were the fatty acids detected in the lipid A of *Salmonella* serovar Typhimurium. When Mg^{2+} poor N-minimal medium was used to culture the bacteria, 2-hydroxy-tetradecanoic acid [C14:0(2-OH)] and hexadecanoic acid (C16:0) were present in addition to the above fatty acids.

Our analysis also showed that *B. pseudomallei* and *B. thailandensis* (Table 3.1) had different fatty acid composition compared to *Salmonella* serovar Typhimurium. C12:0 was absent in the LPS of both *B. pseudomallei* and *B. thailandensis* but present in *Salmonella* serovar Typhimurium. Another major difference was the presence of 3-hydroxy hexadecanoic acid [C16:0 (3-OH)] in the lipid A of *B. pseudomallei* and *B. thailandensis* but was not found in *Salmonella* serovar Typhimurium.

The lipid A fatty acid composition in *B. pseudomallei* was also different from that of *B. thailandensis* (Table 3.1). The LPS extract from *B. thailandensis* lacked C14:0(2-

Table 3.1: Fatty acid composition of LPS from *B. pseudomallei*[1], *B. thailandensis*[1] and *Salmonella* serovar Typhimurium[1].

LPS	*B. pseudomallei*		*B. thailandensis*		*Salmonella* serovar Typhimurium	
	+Mg	-Mg	+Mg	-Mg	+Mg	-Mg
C12:0	x[2]	x	x	x	✓	✓
C14:0	✓	✓	✓	✓	✓	✓
C14:0(2-OH)	✓	✓	x	x	x	✓
C14:0(3-OH)	✓	✓	✓	✓	✓	✓
C16:0	✓	✓	✓	✓	x	✓
C16:0(3-OH)	✓	✓	✓	✓	x	x

[1] Bacteria were cultured in either Mg^{2+} rich (+Mg) or Mg^{2+} poor (-Mg) N-minimal medium.

[2] ✓ and x indicates the presence and absence of the corresponding fatty acid, respectively.

OH) compared to *B. pseudomallei*, although the profile of the other fatty acids in the LPS molecule was qualitatively similar. No difference in the fatty acid composition of their lipid A was observed, when either of these two species of bacteria was cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium.

3.3.2 Effect of magnesium on the virulence of *B. pseudomallei* LPS in human monocytes *in-vitro*

To determine the optimal conditions required to prime THP-1 cells, phorbol 12-myristate 13-acetate (PMA) was included in the culture medium for 8, 14 or 24 hours at a concentration of 10 or 100ng/ml. The priming and stimulation of cells were monitored by light microscopy, and TNF-α protein measurement. Priming with 10ng/ml of PMA for 24 hours was found to be optimal. Higher concentrations of PMA were toxic to the cells and priming for less than 24 hours was not sufficient to stably activate cells. Figure 3.1 shows the morphology of the cells after priming with 10ng of PMA. THP-1 cells are suspension cells (Figure 3.1A), which upon priming with PMA for 24 hours (Figure 3.1B), form spindle-like adherent cell monolayer in the flask. The cells remained adherent during the next 24 hours of recovery (Figure 3.1C) and upon stimulation with 100ng/ml of commercial *Salmonella* serovar Typhimurium LPS (Figure 3.1D) assumed a macrophage-like morphology.

To ensure that basal levels of TNF-α was not affected by PMA priming and that the cells would be able to respond to LPS stimulation, increasing concentrations of commercial *Salmonella* serovar Typhimurium LPS was added to 1×10^6 of THP-1 cells (Figure 3.2).

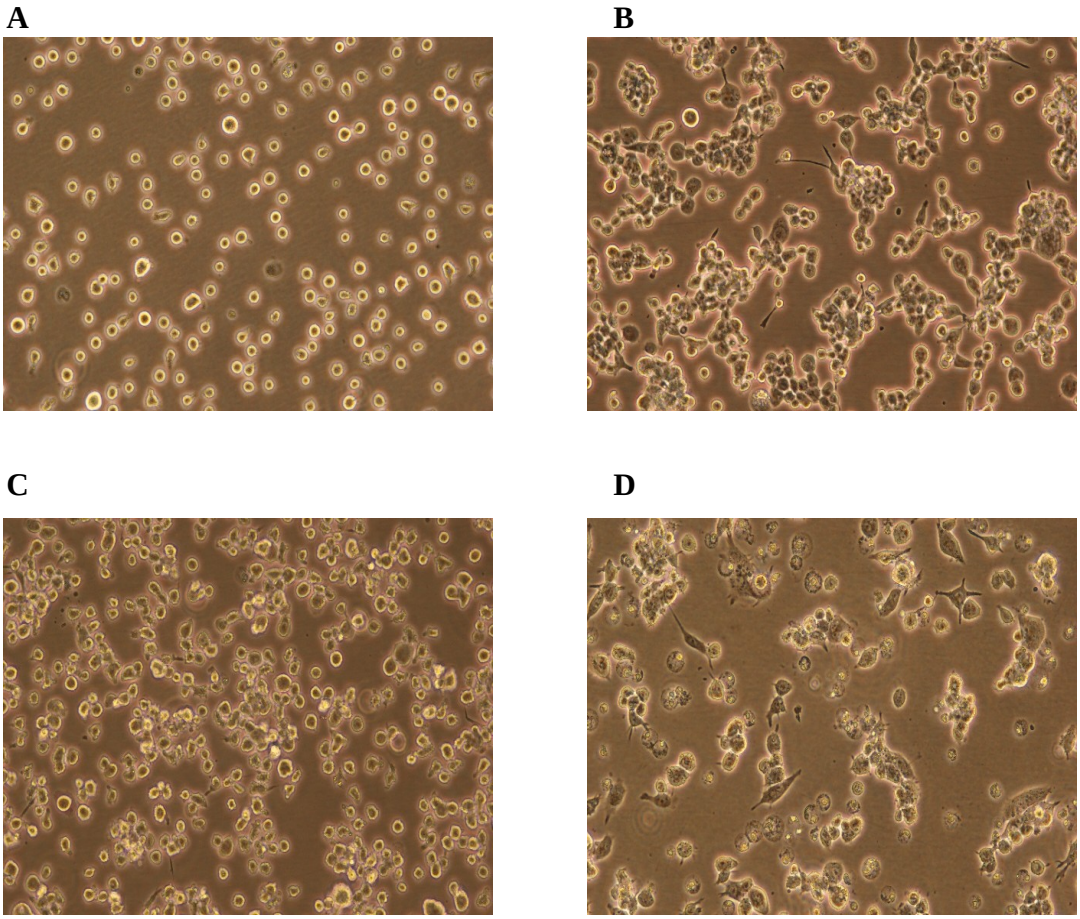


Figure 3.1: Morphology of THP-1 cells primed with PMA. (A) Normal THP-1 (x 20), (B) THP-1 stimulated with 10ng/ml PMA for 24 hours (x 20), (C) Primed THP-1 after 24 hours of recovery (x 20), and (D) primed THP-1 stimulated with 100ng/ml of commercial *Salmonella* serovar Typhimurium LPS for 24 hours after recovery (x 20).

At basal levels, without LPS stimulation, no differences in TNF-α concentration between primed and unprimed cells was observed. Upon PMA stimulation, the cells were able to respond to LPS in a dose-dependent manner. Hence, the parameters for THP-1 priming were fixed as mentioned above.

Primed THP-1 cells were stimulated with increasing concentrations of LPS from *Salmonella* serovar Typhimurium, *B. thailandensis* and *B. pseudomallei* cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium (Figure 3.3). THP-1 cells were able to respond to LPS from all three species in a dose dependent manner. LPS extracted from *Salmonella* serovar Typhimurium cultured in Mg^{2+} poor medium, was significantly more potent in activating THP-1 cells than LPS from bacteria cultured in Mg^{2+} supplemented medium ($p<0.05$), when 0.1ng/ml to 100ng/ml of LPS was used. Regardless of the culture medium used to grow the bacteria, LPS from both *B. pseud*omallei and *B. thailandensis* showed no significant difference ($p>0.05$) in stimulating TNF-α production from THP-1 cells.

3.3.3 Effect of magnesium on the virulence of *B. pseudomallei* LPS in mouse macrophages *in-vitro*

To check if mouse macrophages would respond to LPS differently from human cells, RAW 264.7 mouse macrophage cell-line was stimulated with LPS extracted from *Salmonella* serovar Typhimurium, *B. thailandensis and B. pseudomallei* cultured in either Mg^{2+} rich or Mg^{2+} poor medium. Figures 3.4, 3.5 and 3.6 show the cytokine profile of increasing concentrations of LPS on TNF-α, MCP-1 and IL-10 production,

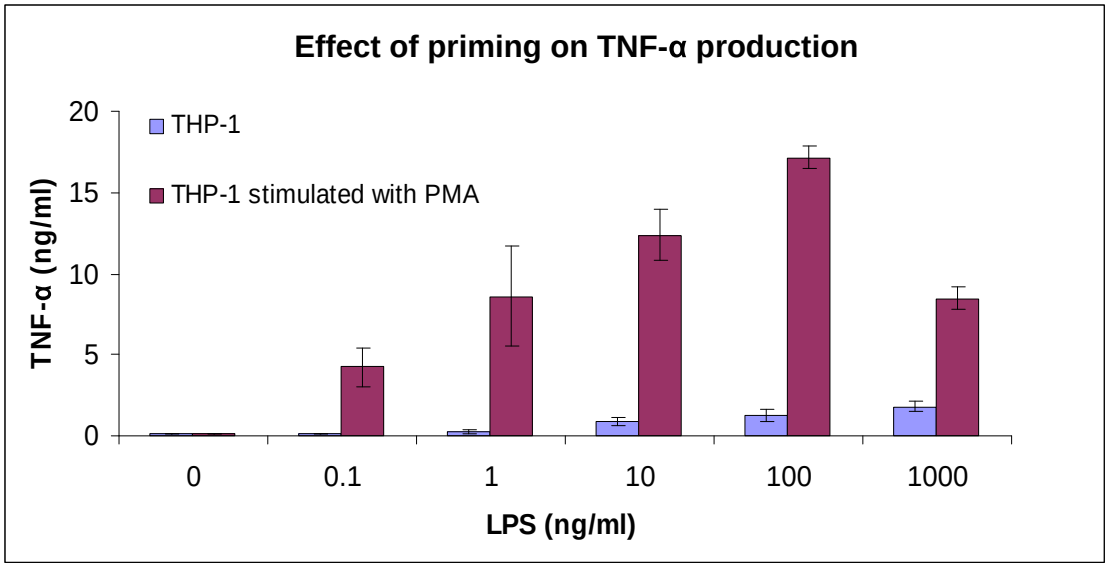


Figure 3.2: Effect of priming on TNF-α production. THP-1 cells were primed with 10ng/ml of PMA for 24 hours. After resting for 24 hours, primed and unprimed cells were stimulated with commercial *Salmonella* serovar Typhimurium LPS for 24 hours. Data-points represent the mean of 3 independent experiments and error bars represent the standard deviation.

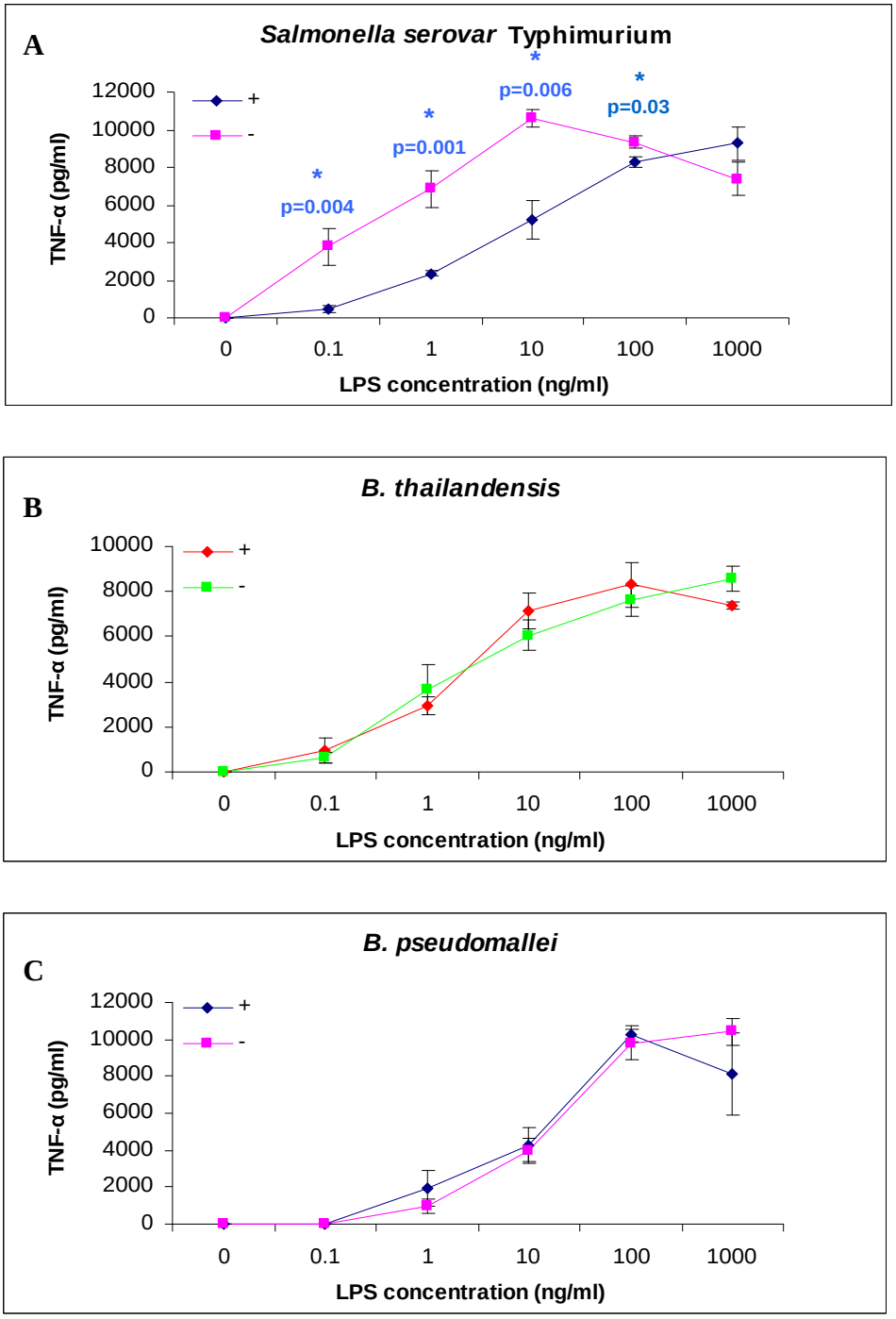


Figure 3.3: TNF-α production in primed THP-1 cells. Primed THP-1 cells were stimulated with LPS from *Salmonella* serovar Typhimurium (A), *B. pseudomallei* (B) and *B. thailandensis* (C), and were assayed for levels of TNF-α. Data-points represent the mean of 3 independent experiments and error bars represent standard deviation. '+' and '-' represent LPS extracted from bacteria cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium, respectively. * represents significant differences ($p<0.05$) in TNF-α production.

respectively. RAW 264.7 produced significantly more TNF-α and MCP-1 when stimulated with 10 to 1000ng/ml of LPS extracted from *Salmonella* serovar Typhimurium cultured in Mg^{2+} poor medium than Mg^{2+} rich medium. There was no difference in TNF-α and MCP-1 production when RAW 264.7 was stimulated with LPS extracted from *B. pseudomallei* and *B. thailandensis* grown in either Mg^{2+} rich or Mg^{2+} poor N-minimal medium. Generally, the level of IL-10 produced by RAW 264.7 was low when stimulated with LPS from the three species. A significant level of IL-10 production could be detected only when the cells were stimulated with 1000ng/ml of LPS. RAW 264.7 produced more IL-10 in response to 1000ng/ml of LPS extracted from only *Salmonella* serovar Typhimurium cultured in Mg^{2+} poor than Mg^{2+} rich N-minimal medium. No difference in IL-10 expression was detected when stimulated with LPS from *B. pseudomallei* and *B. thailandensis* cultured in either Mg^{2+} rich or Mg^{2+} poor N-minimal medium.

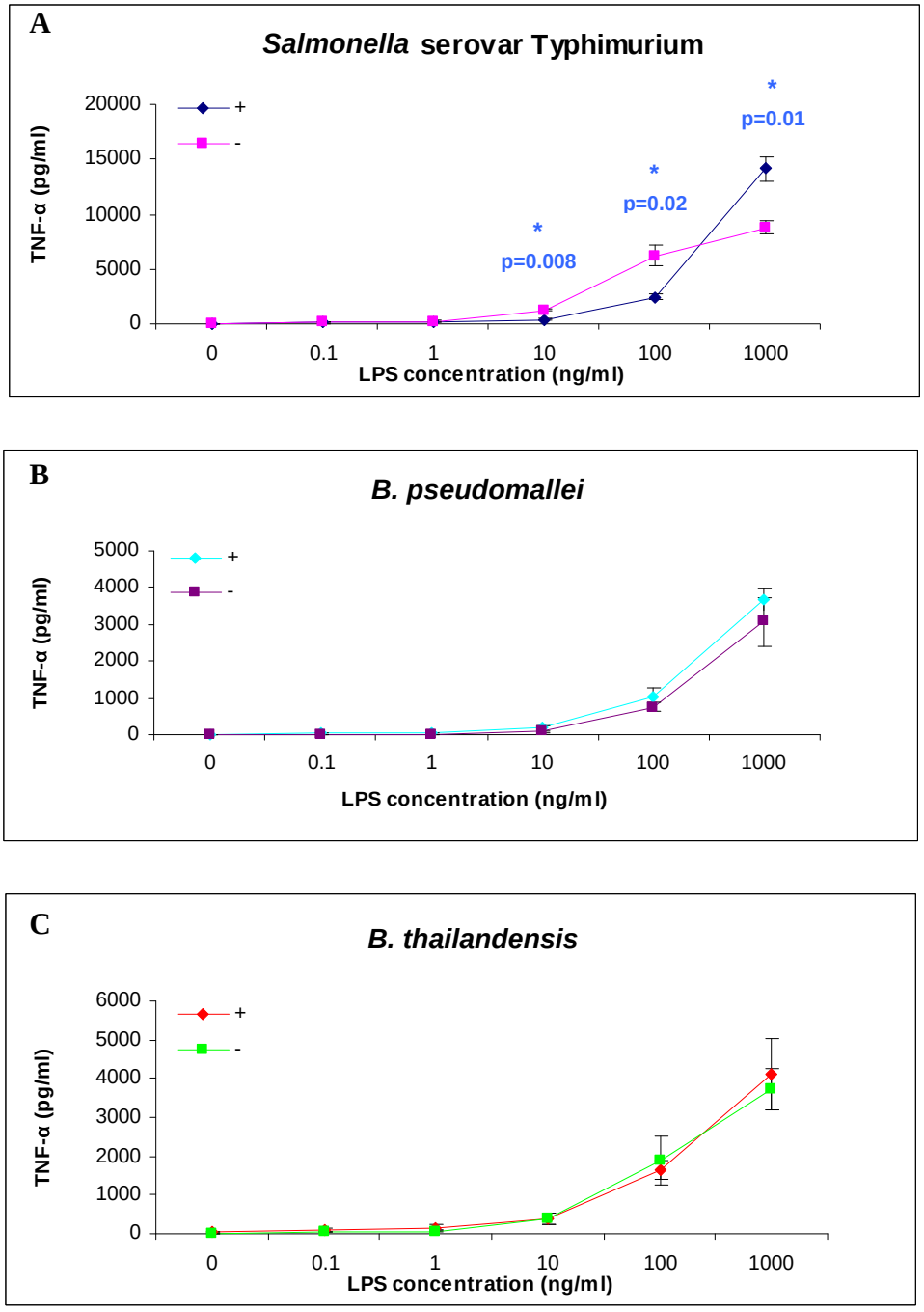


Figure 3.4: TNF- α production in RAW 264.7. RAW 264.7 were stimulated with LPS from *Salmonella* serovar Typhimurium (A), *B. pseudomallei* (B) and *B. thailandensis* (C), and were assayed for levels of TNF-α. Data-points represent the mean of 3 independent experiments and error bars represent standard deviation. '+' and '-' represent LPS extracted from bacteria cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium, respectively. * represents significant differences ($p<0.05$) in TNF-α production.

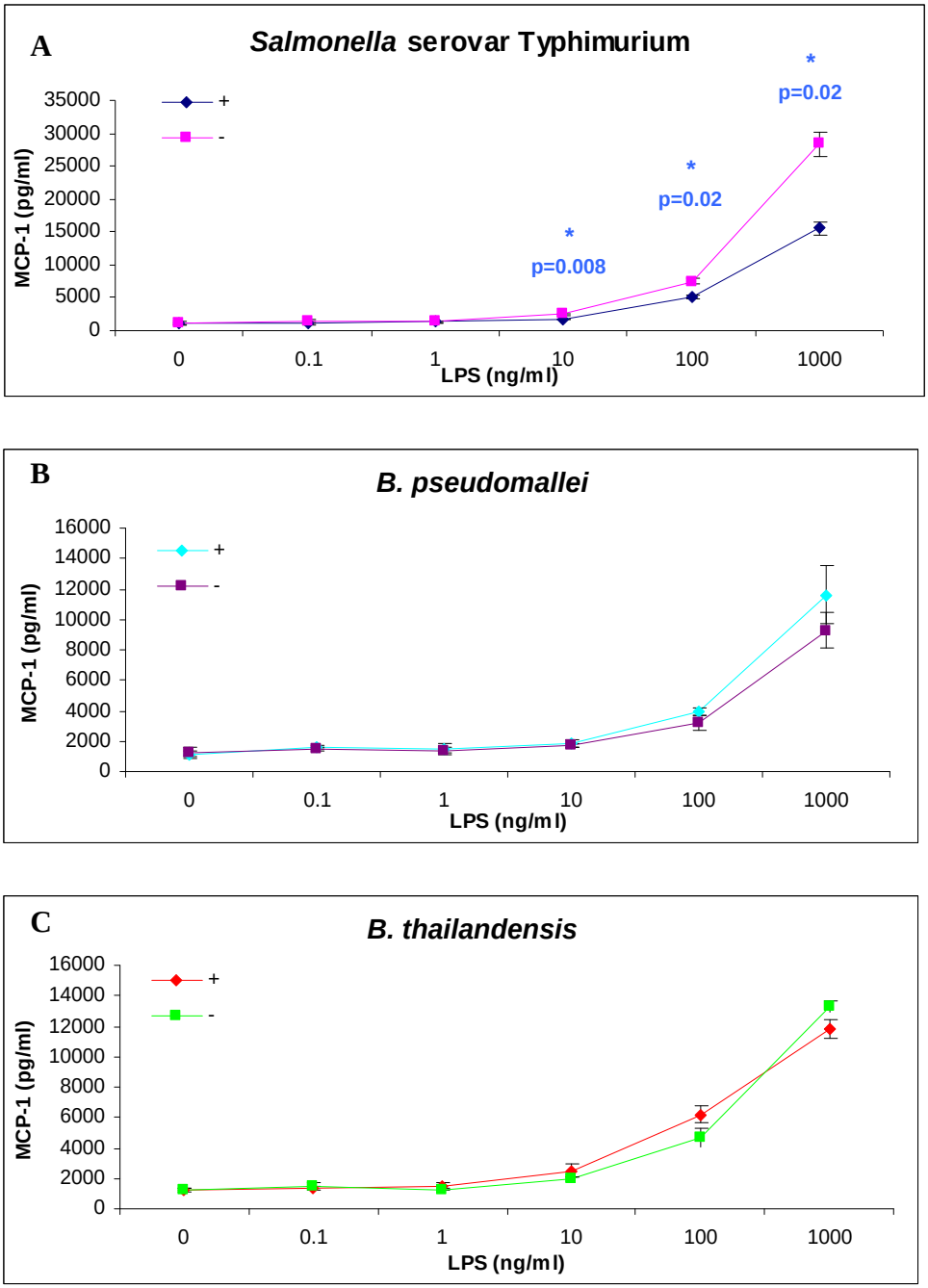


Figure 3.5: MCP-1 production in RAW 264.7. RAW 264.7 were stimulated with LPS from *Salmonella* serovar Typhimurium (A), *B. pseudomallei* (B) and *B. thailandensis* (C), and were assayed for levels of MCP-1. Data-points represent the mean of 3 independent experiments and error bars represent standard deviation. '+' and '-' represent LPS extracted from bacteria cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium, respectively. * represents significant differences ($p<0.05$) in MCP-1 production.

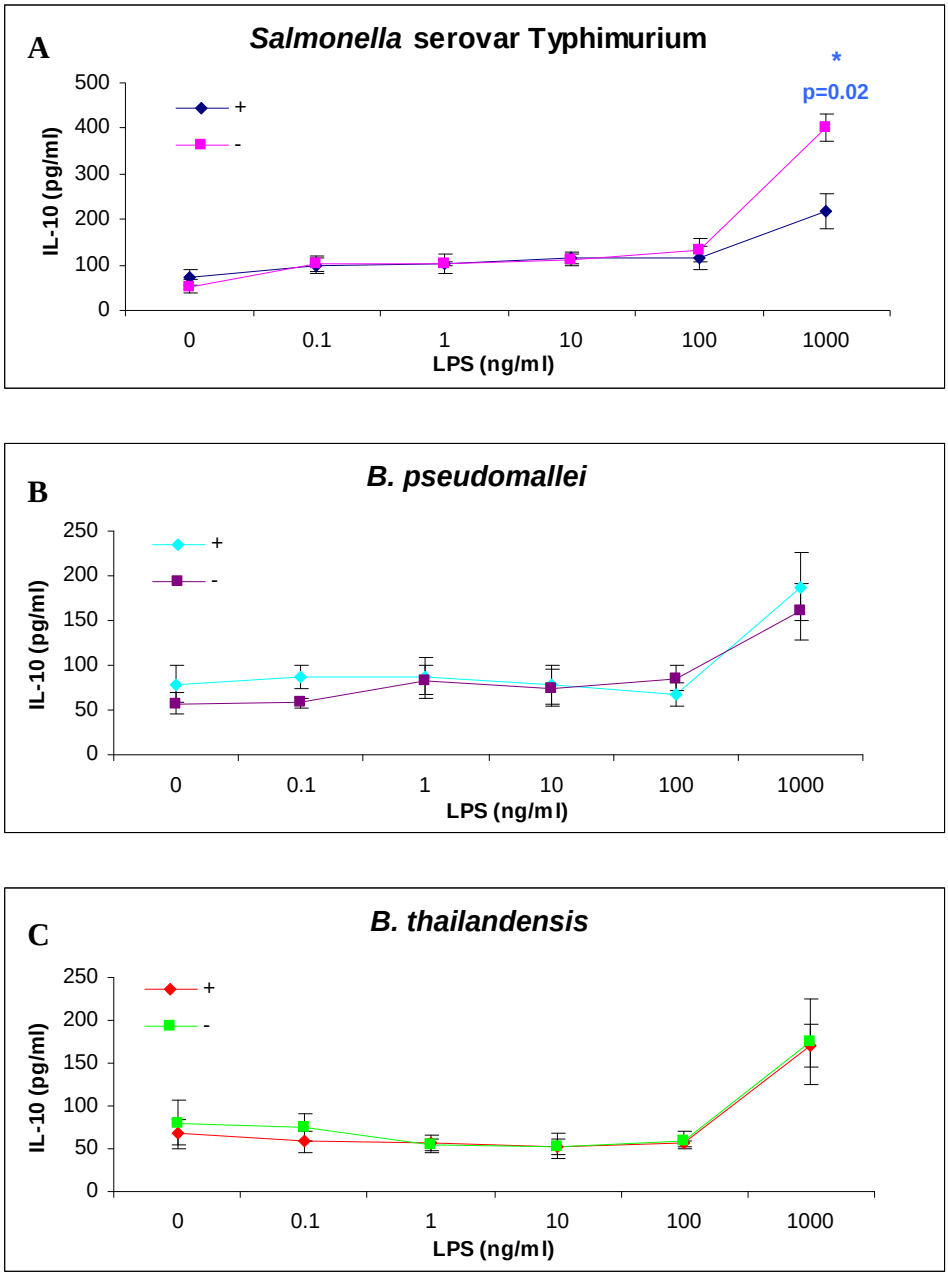


Figure 3.6: IL-10 production in RAW 264.7. RAW 264.7 were stimulated with LPS from *Salmonella* serovar Typhimurium (A), *B. pseudomallei* (B) and *B. thailandensis* (C), and were assayed for levels of IL-10. Data-points represent the mean of 3 independent experiments and error bars represent standard deviation. '+' and '-' represent LPS extracted from bacteria cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium, respectively. * represents significant differences ($p<0.05$) in IL-10 production.

3.4 DISCUSSION

Preliminary results based on a *C. elegans* infection model suggested that Mg^{2+} deficiency in the bacterial environment alters the virulence phenotype of *B. pseudomallei*. Increased death was observed in nematodes infected with *B. pseudomallei* cultured in magnesium minimal medium, suggesting that *B. pseudomallei* became more virulent under such conditions. As intact LPS is required for *Salmonella* mediated induction of programmed cell death in *C. elegans* (Aballay *et al.,* 2003) and others have demonstrated that bacteria such as *Salmonella* serovar Typhimurium and *Pseudomonas aeruginosa* alter their lipid A in response to low Mg^{2+} concentrations resulting in increased virulence (Guo *et al.,* 1997; Hajjar *et al.,* 2002), the findings prompted our investigation into the LPS of *B. pseudomallei.*.

Since lipid A changes in *Salmonella* serovar Typhimurium are well characterized, LPS was extracted from this bacteria cultured in Mg^{2+} rich or poor N-minimal medium and used as a positive control to confirm changes in lipid A fatty acid composition. When cultured in Mg^{2+} poor medium, C14:0 (2-OH) and C16:0 were found in addition to the fatty acids present in LPS of bacteria cultured in Mg^{2+} rich medium. The differences observed in the fatty acid composition were similarly identified by other groups (Guo *et al.,* 1998; Zhou *et al.,* 2001). In this study, no changes were detected in the lipid A fatty acid composition of either *B. pseudomallei* or *B. thailandensis* cultured in different Mg^{2+} concentrations.

Exposure to low magnesium environment not only alters the fatty acid composition of lipid A, but may alter other structural components of lipid A. For example, the phosphate groups

of lipid A from *Salmonella* serovar Typhimurium and *P. aeruginosa* become modified with 4-amino-4-deoxy-arabinose (Ara4N) upon exposure to low concentrations of Mg^{2+} (Ernst *et al.,* 2006; Guo *et al.,* 1997). Modification with Ara4N increases the virulence of bacteria by conferring resistance to host cationic antimicrobial peptides. Based on this knowledge, we further investigated the effect of magnesium on the virulence of *B. pseudomallei* LPS *in-vitro*. However, the LPS from *B. pseudomallei* cultured in either Mg^{2+} rich or Mg^{2+} poor medium were similarly recognized by murine macrophages (RAW 264.7).

Lipid A modifications in *P. aeruginosa* are differentially recognized by murine and human cells *in-vitro* (Hajjar *et al.,* 2002). Since mouse macrophage cells could not distinguish *B. pseudomallei* LPS extracted from bacteria cultured in either Mg^{2+} rich or Mg^{2+} poor medium, we further investigated the ability of human monocytes (THP-1) to differentiate the two LPS. Similar to RAW 264.7, THP-1 was unable to distinguish *B. pseudomallei* LPS extracted from Mg^{2+} rich from Mg^{2+} poor medium.

The results in this study indicates that Mg^{2+} does not modulate the virulence phenotype of *B. pseudomallei* and *B. thailandensis* LPS. The contrary findings in our preliminary study, where under magnesium deficient conditions *B. pseudomallei* exhibited increased virulence in *C. elegans,* could be a consequence of the primitive immune system of *C. elegans* (Wiles *et al.,* 2006). Environmental magnesium may also modify the virulence phenotype of *B. pseudomallei* by inducing modifications other than LPS.

In conclusion, contrary to our hypothesis, Mg^{2+} deficiency does not alter the fatty acid composition of lipid A from *B. pseudomallei and B. thailandensis*. In addition, both murine and human macrophages are unable to differentiate the LPS extracted from bacteria cultured in Mg^{2+} rich or Mg^{2+} poor N-minimal medium in both *B. pseudomallei* and *B. thailandensis in-vitro*. Interestingly, differences were observed in the LPS fatty acid composition between *B. pseudomallei* and *B. thailandensis*. These changes may contribute to the different virulence observed in humans and requires further investigation.

Chapter 4

Structural and biological characterization of lipid A from *B. pseudomallei* and *B. thailandensis*

4.1 INTRODUCTION

A key pathway for the induction of inflammation in response to Gram-negative bacteria is the activation of innate immune system by lipopolysaccharide (LPS). LPS is recognized by the immune system as a marker of bacterial invasion. It is responsible for the development of inflammatory response to combat the infection (Medzhitov, 2001). However, the induction of uncontrolled and excessive levels of innate inflammatory response leads to septic shock. The endotoxic activity of lipid A varies strongly with its primary structure, namely the fatty acids, polar heads and carbohydrate components (Seydel *et al.*, 2000).

The architecture of lipid A is usually the most conserved within a genus, compared to the core polysaccharide and O-antigen (Leone *et al.*, 2007). The fatty acid composition of LPS from *Burkholderia mallei*, *Burkholderia cepacia* and *Burkholderia caryophylli* consist of tetradecanoic acid (C14:0), 3-hydroxy-tetradecanoic acid [C14:0(3-OH)] and 3-hydroxy-hexadecanoic acid [C16:0 (3-OH)] (Brett *et al.*, 2007; Molinaro *et al.*, 2003; Silipo *et al.*, 2005). These fatty acids were also detected in the LPS of *B. pseudomallei* and *B. thailandensis* as described in Chapter 3 of this thesis. Of note is that, although no differences were found in the fatty acid composition of *B. pseudomallei* or *B. thailandensis* when cultured under varying magnesium concentration, the presence of 2-hydroxy-tetradecanoic acid [C14:0(2-OH)] was detected only in the LPS of *B. pseudomallei* (Table 3.1, Chapter 3) and not in *B. thailandensis*. The fatty acid analysis carried out in chapter 3 was able to determine only the fatty acid components of

lipid A, and could not be used to determine the detailed structure of the lipid A moiety of LPS. As the shape of the lipid A component of LPS may influence its biological activity (Netea *et al*., 2002; Seydel *et al*., 2000), this chapter sought to characterize the unique structural features of the lipid A moiety of *B. pseudomallei*, and compare to that of *B. thailandensis*.

Preliminary mass spectrometry data indicated the presence of impurities that made structural interpretation challenging. Hence, the use of a recently published method on LPS purification was explored and its suitability in purifying the extracted LPS was assessed. In addition, a side by side comparison of the biological activities of *B. pseudomallei* LPS and *B. thailandensis* LPS were investigated to determine if the differences detected in the lipid A structure in *B. pseudomallei* and *B. thailandensis* were recognized by immune cells *in-vitro*.

4.2 MATERIALS AND METHODS

4.2.1 Removal of proteins, DNA and RNA from LPS

LPS was dissolved in phosphate buffered saline (PBS) containing 0.001M $CaCl_2$ and 0.001M $MgCl_2$ at a concentration of 10mg/ml. RNase A and DNase I was added to a final concentration of 10µg/ml and the mixture was incubated at 37^{o}C for 4 hours. After incubation, proteinase K was added to a final concentration of 10µg/ml and the sample was incubated for 2 hours at 37^{o}C. The sample was then ultra-centrifuged at 100,000g for 6 hours. The supernatant was removed, and the pellet was washed twice with double distilled water. Finally, the sample was dissolved in water and lyophilized in a pre-weighed micro-centrifuge tube. The removal of proteins and nucleic acids were monitored by taking the absorbance of the LPS at 280nm and 260nm, respectively with a spectrophotometer.

4.2.2 Removal of TLR2 activating contaminants from LPS

After removal of proteins and nucleic acids, LPS was further purified to remove TLR2 activating contaminants (Tirsoaga *et al.*, 2007). 10mg of LPS was suspended in 1M hydrochloric acid (HCl) at a concentration of 20mg/ml, and sonicated in an ultrasonic bath for 2 minutes. Chloroform and methanol were added to the mixture to achieve a final ratio of 3:2:0.25 (chloroform: methanol: 1M HCl, v:v:v). The suspension was sonicated for 2 minutes and then centrifuged at 2000g for 10 minutes. The supernatant was separated from the pellet and the pellet was dried under a stream of nitrogen. The above extraction was repeated twice and followed by two extractions using water instead of

HCl. Finally, the dried pellet was re-suspended in water and centrifuged at 300,000g for 45 minutes at 4^{o}C. The supernatant was discarded, and the pellet corresponding to purified LPS was dissolved in water and lyophilized overnight.

4.2.3 LPS stimulation assay

RAW 264.7 were seeded in 24 well plates at a density of 2.5 x 10^4 cells/well and incubated overnight prior to LPS stimulation assay. The next day, RAW 264.7 cells were stimulated with increasing concentrations of crude or purified LPS from *B. pseudomallei* and *B. thailandensis* for 16 hours. Purified LPS from *Escherichia coli* strain K12 (InvivoGen, San Diego, CA) was used as a control. The supernatant was analyzed for TNF-α, IL-6 and IL-10 release using Quantikine ELISA (R&D Systems, Minneapolis, MN). Cytokine production was analyzed by unpaired Student's *t* test (2-tailed distribution). Values of $p<0.05$ were considered statistically significant.

4.2.4 Isolation of lipid A

Lipid A was isolated from LPS using modified mild acid hydrolysis (Yi *et al.,* 2000). 1mg of LPS was dissolved in 500µl of 1% SDS in 10mM sodium acetate, pH 4.5 and placed in a ultrasound bath to dissolve the sample. The sample was heated at 100 °C for 1 hour, after which the mixture was dried under vacuum in a speed-vac. To remove SDS, the dried pellet was suspended in 100µl of distilled water and sonicated in an ultrasonic bath to disperse the sample. 500µl of acidified ethanol (prepared by adding 100µl of 4M HCl to 20ml of 95% ethanol) was added to the suspension and centrifuged at 2000*g*

for 10 minutes. The pellet was washed twice more with 500µl of non-acidified ethanol and lyophilized to obtain lipid A.

4.2.5 ESI/QTOF mass spectrometry

Electrospray Ionization Mass Spectrometry (ESI/MS) was performed on a Waters Micromass Q-TOF micro mass spectrometer (Waters Corp., Milford, MA). The capillary voltage and sample cone voltage were maintained at 3.0kV and 55V, respectively. The source temperature was 80°C, and the desolvation temperature was set at 250°C. Mass spectra were acquired in the negative ion mode. Isolated lipid A was re-suspended in chloroform:methanol 2:1 (v/v) containing 3% of 300mM piperidine as an ion signal enhancer. Sample was directly infused into mass spectrometer at a flow rate of 10µl/min. Negative ESI/MS/MS analyses were carried out under similar conditions except for collision energies. The major peaks identified in the ESI/QTOF mass spectra were investigated using tandem MS either through ESI/MS/MS or HPLC/ESI/MS/MS with collision energies varying from 35 to 80 volts, and argon was used as the collision gas.

4.2.6 Contribution of authors towards experimental data in this chapter

Removal of proteins, DNA, RNA and TLR2 contaminating proteins from LPS, LPS stimulation assay, fatty acid analysis and significance of structural differences were carried out by Vidhya Novem. Isolation of lipid A was jointly carried out by Vidhya Novem and Dr Shui Guanghou in Associate Professor Markus Wenk's laboratory (Department of Biochemistry, NUS). ESI/QTOF mass spectrometry and ESI/MS/MS analyses of lipid A were carried out by Dr Shui Guanghou.

4.3 RESULTS

4.3.1 Repurification of LPS

Preliminary mass spectra data of lipid A from *B. pseudomallei* indicated the presence of impurities, which made interpretation of data difficult. Figure 4.1 shows the mass spectra of the crude and purified lipid A from *B. pseudomallei*. After purification, a good signal to noise ratio was achieved and this enabled the characterization of lipid A structure. The purity of LPS from *B. pseudomallei* and *B. thailandensis* was also monitored by taking the absorbance of the LPS at 260nm and 280nm with a spectrophotometer (Table 4.1). Crude LPS showed high levels of protein and nucleic acid content. Upon purification, the levels of protein and nucleic acid were significantly decreased.

4.3.2 Comparison of LPS from *B. pseudomallei* and *B. thailandensis in-vitro*

To investigate if LPS from *B. pseudomallei* and *B. thailandensis* are recognized differently by host cells, RAW 264.7 were stimulated with increasing concentrations of LPS from the two organisms. When stimulated with crude LPS from *B. pseudomallei* and *B. thailandensis*, no significant difference ($p>0.05$) was detected in the levels of TNF-α produced (Figure 4.2). However, RAW 264.7 produced significantly higher levels of TNF-α, IL-6 and IL-10 ($p<0.05$) when stimulated with purified LPS from *B. thailandensis* than from *B. pseudomallei* (Figure 4.3). On average, purified LPS from *B. pseudomallei* was approximately two times less potent in inducing cytokines production in RAW 264.7 than purified *B. thailandensis* LPS. LPS from *E.coli*, a potent stimulator of cells, was used as a positive control and was found to induce significantly

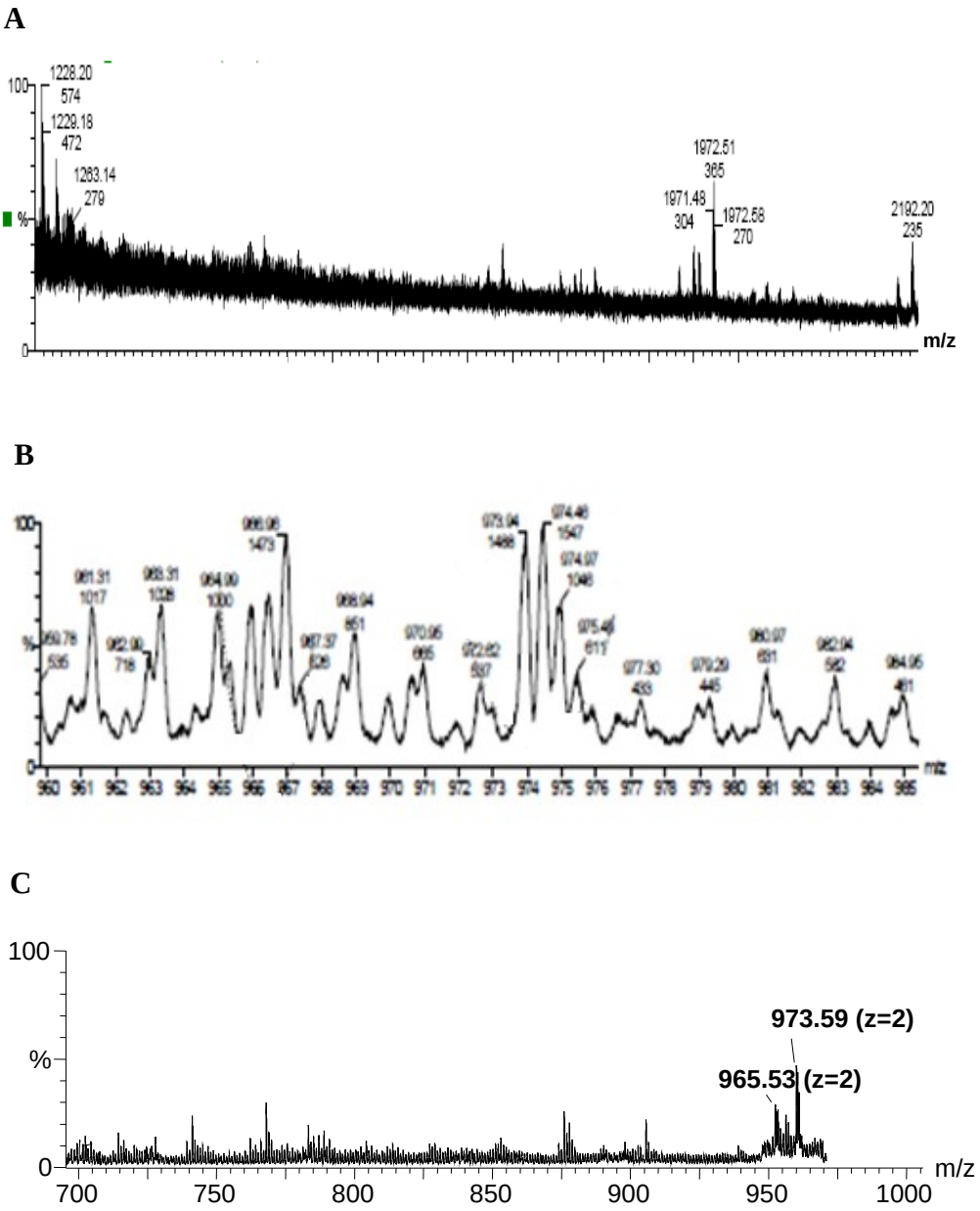


Figure 4.1: Representative ESI/QTOF mass spectra of lipid A extracted from crude and purified LPS of *B. pseudomallei.* Data illustrates singly charged ions (A) and doubly charged ions (B) from crude LPS, and doubly charged ions (C) from purified LPS of *B. pseudomallei.*

Table 4.1: Comparison of nucleic acid (260nm) and protein (280nm) content of crude and purified LPS from *B. pseudomallei* and *B. thailandensis.*

Absorbance wavelength	*B. pseudomallei*		*B. thailandensis*	
	Crude	Purified	Crude	Purified
260nm	2.933	0.034	2.895	0.482
280nm	>3	0.058	>3	0.396

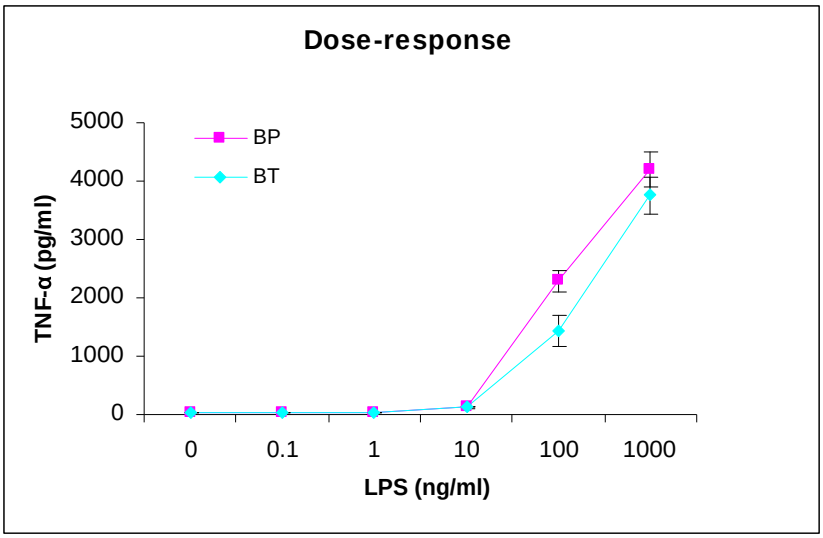
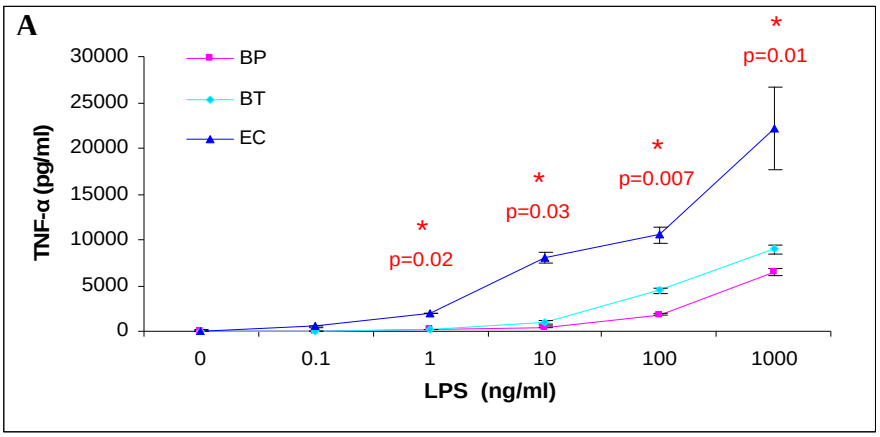
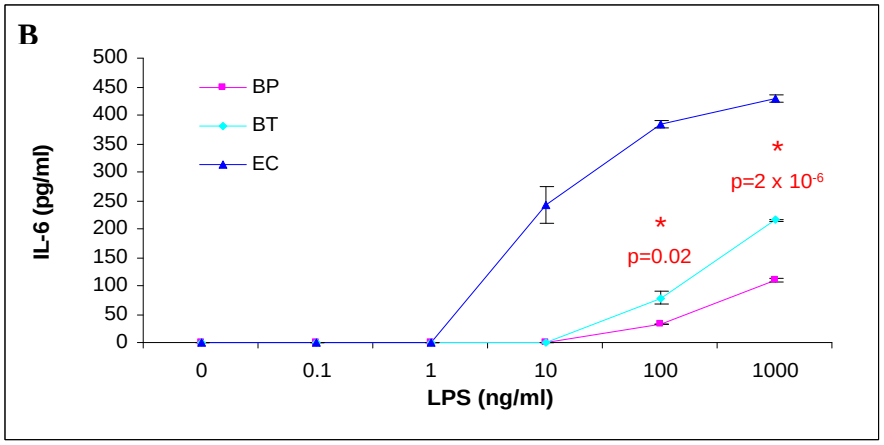


Figure 4.2: TNF-α production in RAW 264.7 stimulated with crude LPS from *B. pseudomallei* (BP) and *B. thailandensis* (BT). Data represent the means ± standard errors of the means from triplicate samples. Results shown are representative of 2 independent experiments.





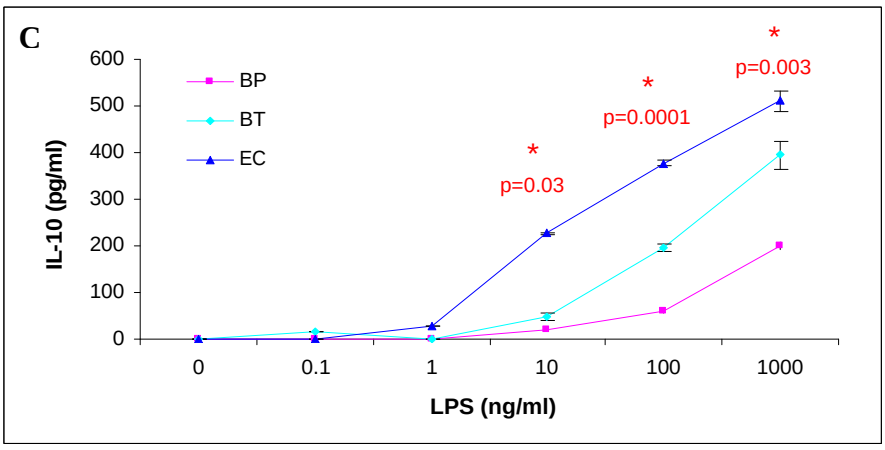


Figure 4.3: TNF-α production in RAW 264.7 stimulated with purified LPS from *B. pseudomallei* (BP) and *B. thailandensis* (BT). The cell culture supernatant was measured for TNF-α (A), IL-6 (B) and IL-10 (C). EC represents LPS from *E. coli*. Data represent the means ± standard errors of the means from triplicate samples. Results shown are representative of 2 independent experiments. * indicates significant differences of $p<0.05$ for comparisons between *B. pseudomallei* and *B. thailandensis.*

higher levels of cytokines than *B. pseudomallei* and *B. thailandensis* LPS.

4.3.3 Structural analysis of lipid A from *B. pseudomallei* and *B. thailandensis*

The lipid A from *B. pseudomallei* and *B. thailandensis* were subjected to a combination of mass-spectrometric analysis to investigate their structures (Figure 4.4). The negative ion ESI-QTOF mass spectra of purified lipid A from *B. pseudomallei* showed a heterogeneous mixture of doubly charged ions at m/z 973.59 and 965.53 (Figure 4.4a). Minor peaks of doubly charged ions at m/z 908.05 and 900.09 (not labeled) were detected as well. The mass differences of 131 units observed between doubly charged ions at m/z 965.53 and 900.09, and between ions at m/z 973.59 and 908.05, indicated a likely difference of 4-amino-4-deoxy-arabinose (Ara4N) residue. The doubly charged ion at m/z 973.59 contained one more hydroxyl (-OH) group than the doubly charged ion at m/z 965.53. Based on the accurate masses of the doubly charged species, the ions at m/z 973.59, 965.53, 908.05, and 900.09, were assigned to lipid A with molecular formulae of $C_{96}H_{182}N_4O_{31}P_2$, $C_{96}H_{182}N_4O_{30}P_2$, $C_{91}H_{173}N_3O_{28}P_2$ and $C_{91}H_{173}N_3O_{27}P_2$, respectively.

The negative ion ESI-TOF mass spectra of purified lipid A from *B. thailandensis* showed major doubly charged ions at m/z 965.53, 900.09 852.59 and 787.01 and a minor doubly charged ion at m/z 834.50 (not labeled) (Figure 4.4b). The doubly charged ions at m/z 973.59 and 908.05 detected in *B. pseudomallei* were not detected in *B. thailandensis*. Similar to the lipid A species in *B. pseudomallei*, the mass differences of 131 units observed between doubly-charged ions at m/z 965.53 and 900.09 indicated a difference of

Ara4N. The mass differences of 226 units between doubly charged ions at m/z 965.53 and 852.59, or between ions at m/z 900.09 and 787.01, was attributed to a difference of one fatty acid, 3-hydroxy-tetradecanoic acid [C14:0(3-OH)]. Based on the accurate masses of the doubly charged species, the ions at m/z 965.53, 900.09, 852.59, 834.50 and 787.01, were assigned to lipid A with molecular formulae of $C_{96}H_{182}N_4O_{30}P_2$, $C_{91}H_{173}N_3O_{27}P_2$, $C_{82}H_{156}N_4O_{28}P_2$, $C_{86}H_{164}N_2O_{24}P_2$ and $C_{77}H_{147}N_3O_{25}P_2$, respectively.

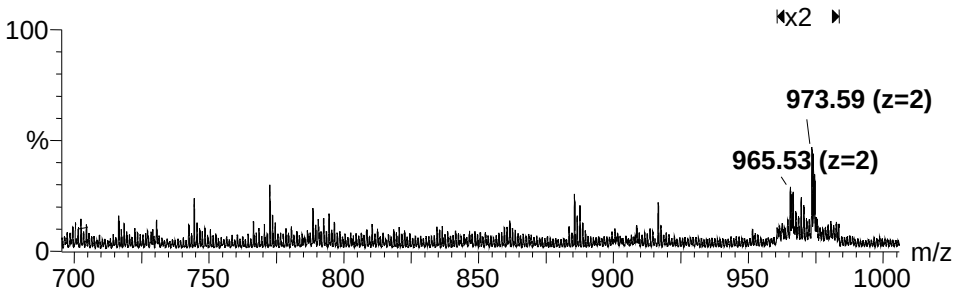
The major lipid A peaks identified in the ESI-QTOF mass spectra (965.53, 900.09, 852.59 and 787.01) were further subjected to tandem mass spectrometry (Figure 4.5). The MS/MS fragmentation spectrum of lipid A species at m/z 965.53 showed doubly charged daughter ion at m/z 900.07 (Δm/z=131) (Figure 4.5a). This indicates a loss of Ara4N from the doubly charged parental ion at m/z 965.53 (fragment II, Figure 4.5a). Similarly, the loss of phosphorylated Ara4N (fragment III, Figure 4.5a-d) was also observed among parental ions at m/z 965.53, 900.09, 852.59, and 787.01 (Figure 4.5a-d). The fragmented ion at m/z 210.21 (Fig. 4.5a) indicates the loss of one water molecule from phosphorylated Ara4N (fragment I, Figure 4.5a). These results indicate that the Ara4N is attached to a phosphate group in the lipid A backbone.

The sequential loss of C14:0(3-OH) (Δm/z=226) from the doubly charged parental ion at m/z 965.53 to doubly charged daughter ions at m/z 852.48 and 739.5 (not labeled) were observed (Figure 4.5a). Furthermore, the doubly charged daughter ions at m/z 787.06 and 673.93 arose from the sequential loss of C14:0(3-OH) from the doubly charged parental

on at m/z 900.09 (Fig. 4.5b). These results imply that C14:0(3-OH) is the major acyl chain attached to the disaccharide backbone.

By combining the structural data from fatty acid compositional and mass spectrometry analysis, we have proposed the structure for the major lipid A fraction present in both *B. pseudomallei* and *B. thailandensis* at m/z 965.53 (Figure 4.6b). This lipid A consists of a bi-phosphorylated disaccharide backbone modified with Ara4N at both phosphate groups and penta-acylated with C14:0(3-OH), tetradecanoic acid (C14:0) and 3-hydroxy-hexadecanoic acid [C16:0(3-OH)]. The lipid A fraction with doubly charged ion at m/z 973.59 present only in *B. pseudomallei* is proposed to have the same structure as the ion at m/z 965.53 but with the hydroxylation of C14:0 to 2-hydroxy-tetradecanoic acid [C14:0(2-OH)] (Figure 4.6a). Similarly, lipid A with m/z at 900.09 was assigned to be a penta-acylated lipid A, but containing only one Ara4N group; lipid A with m/z at 852.59 was assigned to be a tetra-acylated lipid A, containing two Ara4N groups; lipid A with m/z at 787.01 was assigned to be a tetra-acylated lipid A, containing one Ara4N group; and lipid A with m/z at 834.50 was assigned to be a penta-acylated lipid A containing no Ara4N group.

A



B

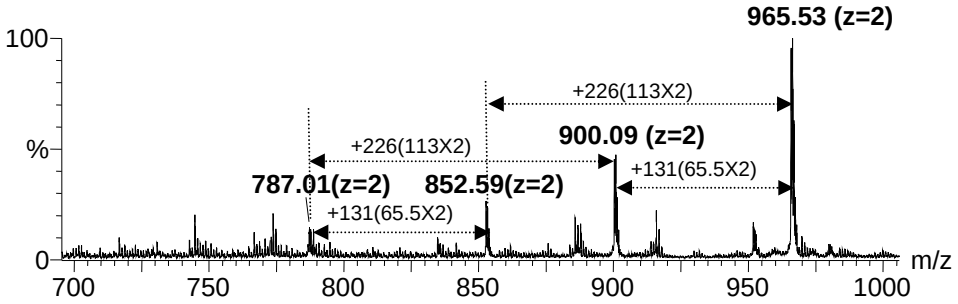


Figure 4.4: ESI-QTOF mass spectra of lipid A from *B. pseudomallei* and *B. thailandensis* obtained in the negative ion mode. (A) Mass spectrum of *B. pseudomallei* shows major doubly charged ions (z=2) of lipid A species at m/z 973.59 and 965.53. (B) Mass spectrum of *B. thailandensis* shows doubly charged ions (z=2) of lipid A species at m/z 965.53, 900.09, 852.59, 834.50 (not labeled) and 787.01.

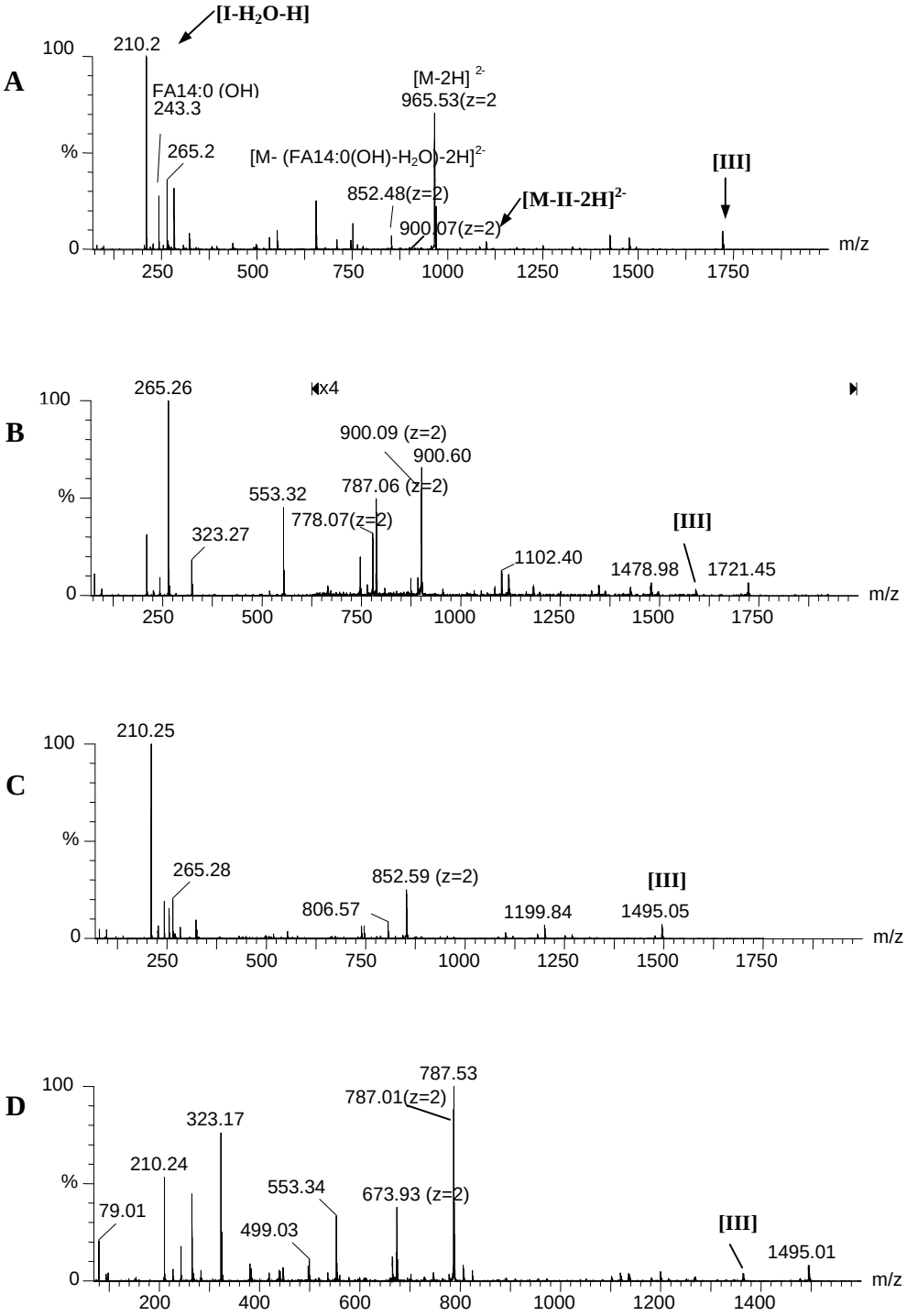


Figure 4.5: ESI/MSMS mass spectra of the major lipid A species. MSMS of doubly charged ions (z=2) at m/z 965.53(A), 900.09 (B), 852.59 (C) and 787.01 (D). [I] indicates the loss of one water molecule from phosphorylated Ara4N, [II] indicates the loss of Ara4N from the doubly charged parental ion and [III] indicates singly charged parental ions with loss of phosphorylated Ara4N.

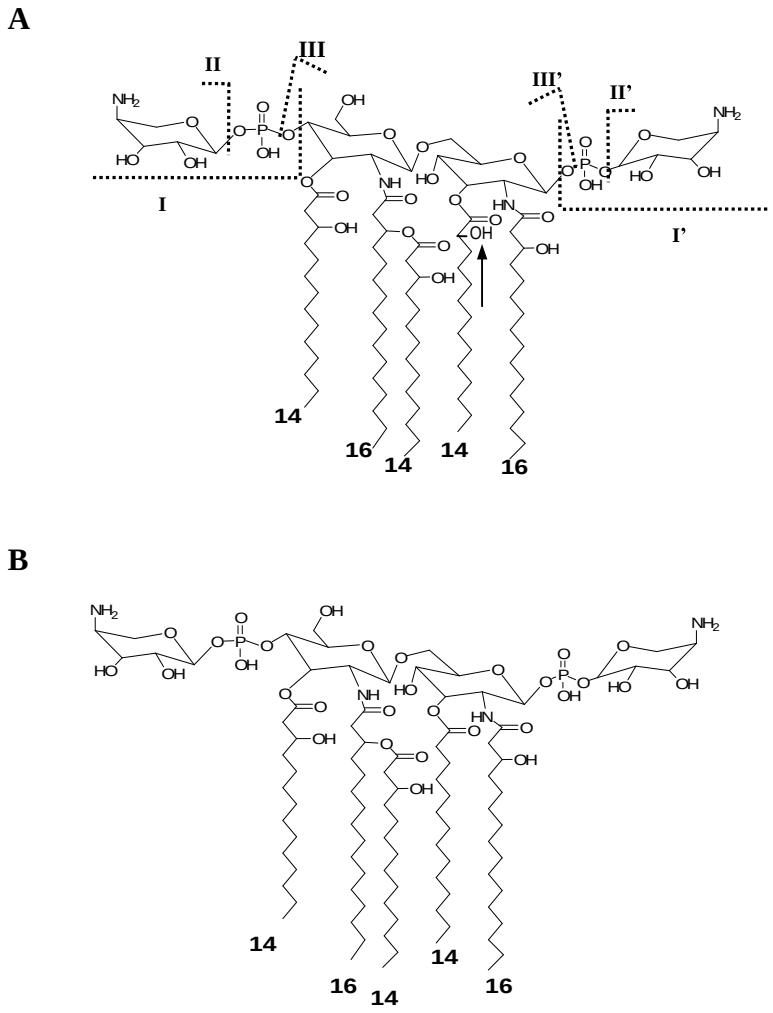


Figure 4.6: Proposed structures of the major lipid A species present in *B. pseudomallei* and *B. thailandensis*. (A) Proposed structure of lipid A species at m/z 973.59 present only in *B. pseudomallei*; (B) Proposed structure of lipid A species at m/z 965.53 present in both *B. pseudomallei* and *B. thailandensis*. Arrow indicates the unique presence of C14:0(2-OH) in the lipid A of *B. pseudomallei* only. Substitution of fatty acids to the lipid A backbone is in no particular order. [I] indicates the loss of one water molecule from phosphorylated Ara4N, [II] indicated the loss of Ara4N from the parental ion and [III] indicates parental ions with loss of phosphorylated Ara4N.

4.4 DISCUSSION

The endotoxic activity of lipid A varies strongly with its primary structure, namely the fatty acids and polar heads that make it up (Seydel *et al.*, 2000). The finding in chapter 3 that the LPS fatty acid composition was different between *B. pseudomallei* and *B. thailandensis* prompted our investigations into the biological and structural differences in these two pathogens. *B. thailandensis* is a close relative of *B. pseudomallei* and co-exists with *B. pseudomallei* in the soil. However, *B. thailandensis* rarely causes disease and is more than 10^5 fold less virulent than *B. pseudomallei* in Syrian Hamsters or mice (Brett *et al.*, 1998). Hence, the difference in LPS fatty acid composition observed between *B. pseudomallei* and *B. thailandensis* may contribute towards the different pathogenesis observed.

Initial stimulation assays showed that LPS from *B. pseudomallei* and *B. thailandensis* activated murine macrophages similarly, despite the difference observed in the fatty acid composition. This suggested that LPS may not play an important role in the virulence of *B. pseudomallei* since no difference was observed in the host recognition of LPS from *B. pseudomallei* and *B. thailandensis*. However, mass-spectrometry analysis revealed that the LPS of *B. pseudomallei* was associated with contaminating sugars and proteins, which made structural interpretation challenging. Hence, the observed similarity in recognition of LPS from the two species may have arisen as an artifact of contaminants present in the LPS. This prompted the search for a suitable method to purify the LPS. Upon purification, it was found that murine macrophages (RAW 264.7) produced two

times more cytokines in response to purified LPS from *B. thailandensis* than to purified LPS from *B. pseudomallei*. Structural analysis further revealed that the major lipid A species in *B. pseudomallei* consisted of a bi-phosphorylated disaccharide backbone modified with Ara4N at both phosphate groups and penta-acylated with C14:0(3-OH), C16:0(3-OH) and either C14:0 or C14:0(2-OH). On the contrary, the major lipid A species identified in *B. thailandensis* was a heterogenous mixture of penta- and tetra-acylated structures varying in Ara4N substitution and acylation of C14:0(3-OH). The substitution of C14:0(2-OH) to the lipid A backbone was unique to *B. pseudomallei* and not found in *B. thailandensis.*

Lipid A is the endotoxic and bioactive centre of LPS (Miller *et al.,* 2005). Some Gram-negative bacteria have evolved to modify the structure of their lipid A in order to evade anti-bacterial mechanisms initiated by the host innate immune system (Montminy *et al.,* 2006). One such modification is the synthesis of LPS with low biological activities. For example, the hexa-acylated lipid A of *E. coli* represents the most biologically active form of the molecule (Caroff *et al.,* 2003). However, when *E. coli* was genetically modified to express penta-acylated lipid A, it showed reduced immunogenicity and acted antagonistically by inhibiting pro-inflammatory signaling induced by wild-type *E. coli* and LPS from other bacteria (Backhed *et al.,* 2003). The LPS of *B. pseudomallei* has been previously shown by other groups to stimulate host cells weakly compared to *E. coli* LPS *in-vitro* (Matsuura *et al.,* 1996; Utaisincharoen *et al.,* 2001). Similarly, we found that although purified, LPS from *B. pseudomallei* induced low levels of cytokines from murine macrophages *in-vitro*. The ability of LPS from *B. pseudomallei* and

B. thailandensis to activate murine macrophages weakly compared to *E.coli* LPS may be attributed to their penta-acylated structures, which are substituted with longer fatty acids [C14:0(3-OH) and C16:0(3-OH)] than *E.coli* LPS. Differences in the relative amounts of individual lipid A species and the existence of lipid A species acylated with C14:0(2-OH) only in *B. pseudomallei* may contribute to the differential recognition of *B. pseudomallei LPS* compared to *B. thailandensis* LPS by murine macrophages *in-vitro*.

The lipid A of *Burkholderia mallei* consist of a mixture of tetra- and penta-acylated lipid A structures substituted with C14:0, C14:0 (3-OH) and C16:0 (3-OH) (Brett *et al.,* 2007). Although its lipid A is similar to that of *B. thailandensis*, the LPS from *B. mallei* stimulates cells in a manner similar to *E.coli* LPS. The authors suggested that by optimizing the order its LPS fatty acids, *B. mallei* may produce LPS molecules with biological properties similar to the potent hexa-acylated species (Brett *et al.,* 2007). As the order of fatty acid acylation was not determined in the study here, it is likely that that the differences in the immunological activities of LPS from *B. pseudomallei* and *B. thailandensis* compared to *B. mallei* may be contributed by differences in the order of fatty acyl chains to the lipid A backbone and the relative amounts of individual lipid A species. In addition, minor lipid A species differentially substituted with longer fatty acyls C16:0 and C18:0 were detected in the MS/MS spectra of *B. pseudomallei* and *B. thailandensis*. These lipid A species may further contribute to the lower immunological activities of these species *in-vitro.*

The phosphate groups of lipid A in some pathogenic bacteria are modified with Ara4N, allowing the bacteria to resist the bactericidal effects of endogenously produced host cationic antimicrobial peptides (CAMPS) (Gunn *et al.*, 2001). The lipid A of *Salmonella* serovar Typhimurium becomes modified with Ara4N residues when grown under magnesium deficient conditions, which mimic the environment of a macrophage phagosome (Guo *et al.,* 1997). This decreases the overall negative charge on the pathogen's cell surface and lowers the affinity for CAMPS (Kawsaki *et al.,* 2005). In our structural analysis, the phosphate groups in the major lipid A species of *B. pseudomallei* were found to be capped with Ara4N residues. Modification with Ara4N may increase the resistance of *B. pseudomallei* to CAMPS and allow the bacterium to survive and replicate within host cells. Similarly, *B. pseudomallei* has been shown to be resistant to the cationic peptides protamine sulfate and purified human defensin HNP-1 *in-vitro* (Jones *et al.*, 1996). On the contrary, in *B. thailandensis*, there were major lipid A species identified with only one phosphate group substituted with Ara4N. Minor lipid A species without Ara4N substitution were also present in *B. thailandensis*. This may contribute to the difference in pathogenesis observed between *B. pseudomallei* and *B. thailandensis*.

In *Salmonella* serovar Typhimurium, hydroxylation of C14:0 to C14:0(2-OH) is observed in response to low magnesium concentrations in the host micro-environments. This has been postulated to confer resistance to cationic anti-microbial peptides (CAMPs), permitting a prolonged survival of the bacteria inside the host cell (Guo *et al.*, 1997; Gibbons *et al.*, 2000). Likewise, the hydroxylation of C14:0 to C14:0(2-OH) in *B. pseudomallei* may contribute to the pathogenesis by allowing the bacterium to subvert

host cellular responses and survive within host cells. Lipid A species acylated with C14:0(2-OH) was not found in *B. thailandensis*. The presence of C14:0(2-OH) in *B. pseudomallei* and not in *B. thailandensis* was similarly demonstrated in a study that compared the cellular fatty acid profiles of *B. pseudomallei* and *B. thailandensis* (Inglis *et al.*, 2003).

In conclusion, the ability of *B. pseudomallei* LPS to evade strong immune cell activation may be due to the unique structural features of its lipid A. The presence of longer acyl chains, C14:0(2-OH) and Ara4N capped phosphate groups may allow the bacterium to evade host recognition through weak activation of innate immune system and resistance to CAMPS. In addition, the differences in lipid A of *B. pseudomallei* and *B. thailandensis* may contribute to the observed differences in the ability to cause disease.

Chapter 5

Determination of the Toll-like receptor (TLR) involved in the recognition of LPS from *B. pseudomallei* and *B. thailandensis*

5.1 INTRODUCTION

The recognition of bacteria as non-self agents by mammalian cells is an important innate response against infection. Innate immune responses to pathogens are mainly carried out by monocyte or macrophages, granulocytes and dendritic cells, which act as the first line of defence against invading micro-organisms (Medzhitov *et al.,* 1997). Pattern recognition receptors on these immune cells are able to recognize micro-organism specific molecules that are essential and conserved. One such group of pattern recognition receptors is the family of Toll-like receptors (TLRs) (Medzhitov, 2001). TLRs can be divided into 2 distinct subpopulations, those that are on cell-surface and those that are found intracelluarly. Cell surface TLRs include TLR4/MD-2 that recognizes lipopolysaccharide (LPS), and TLR2/TLR1 and TLR2/TLR6 heterodimers that recognizes bacterial lipoproteins.

LPS is a major component of the outer membrane of Gram-negative bacteria. Recognition of LPS by TLR4 depends on the structure of lipid A, the bioactive and endotoxic region of LPS (Miller *et al.,* 2005). Some preliminary reports suggest that TLR2 may be able to recognize some LPS structures (Braedel-Ruoff *et al.,* 2005; Hirschfeld *et al.,* 2001); however others have shown that this is an artifact caused by the presence of contaminating lipoproteins in the LPS preparation (Lee *et al.,* 2002).

In this chapter, since we have managed to obtain purified LPS fractions, we aim to identify the TLR that is involved in recognizing the LPS from *B. pseudomallei* and

B. thailandensis. HEK 293, a human embryonic kidney cell-line does not have endogenous TLRs (Hornung *et al.,* 2002), and was thus chosen to study TLR-dependent activation of cells. This was achieved by transfection to over-express either TLR2 or TLR4 in these cells. Binding of specific ligands to specific TLRs initiates a complex signal transduction cascade, ultimately leading to the activation of nuclear factor kappa beta (NF-κβ), which then induces the transcription of a plethora of inflammatory cytokines and chemokines (Miller *et al.,* 2005). Hence NF-κβ was chosen to quantify the activation of HEK 293 cells in the assays here.

Like previous reports (Wiersinga *et al.,* 2007), preliminary experiments in our laboratory indicated that *B. pseudomallei* LPS signals through TLR2. Given the possibility that lipoprotein contamination may complicate such experiments, we investigated if further purification of the LPS to remove contaminating proteins would change the recognition receptor to TLR4.

5.2 MATERIALS AND METHOD

5.2.1 *In-vitro* culture

HEK 293 cell-lines, transfected with either the human TLR2 or the human TLR4-MD2-CD14 complex were purchased from InvivoGen (San Diego, CA). The complete medium used to maintain the cell-lines was DMEM containing 4.5g/l glucose, and 10% fetal calf serum with 10µg/ml of blasticidin and 50µg/ml of hydrogold. Untransfected HEK 293 cells not expressing any TLR was also purchased from InvivoGen and used as a negative control.

5.2.2 Flow cytometry

Cells were harvested and washed twice in FACS (fluorescent-activated cell sorting) staining buffer (sterile PBS containing 1% fetal calf serum). A cell count was performed and cells were reconstituted to a concentration of 5 x 10^6 cells/ml. 200µl was added to FACS tubes and the relevant fluorescent tagged antibodies or their isotypes were added to each tube at the pre-determined concentration. The antibodies used were human TLR4, human TLR2 and human CD14. The cells were incubated with the antibodies on ice for 30 minutes. After incubation, the cells were washed twice to remove unbound antibodies and reconstituted in 200µl of staining buffer. Data was acquired on a Becton Dickinson FACS Calibur using the CellQuest software (Becton Dickinson).

5.2.3 Plasmid preparation

Plasmid (pNifty2) encoding NF-κβ directed luciferase expression was purchased from InvivoGen. pNifty2 plasmid was provided as lyophilized transformed *Escherichia* **coli**. 1ml of LB medium was added to re-suspend the lyophilized *E.coli* transformed cells. After mixing gently by inverting the tube several times, the bacteria were streaked out from the suspension to LB agar plates containing zeocin for the selection of transformed cells. The plate was placed into an incubator overnight at 37^{o}C. The next day, a single colony was picked, and the bacteria were grown in terrific broth (TB) supplemented with zeocin for 14 hours at 37^{o}C. The pNifty2 plasmid DNA was then extracted from the transformed *E. coli* using the QIAprep spin miniprep kit (Qiagen, Singapore) according to manufacturer's instructions.

5.2.4 Site directed mutagenesis

As a plasmid similar to pNifty2 but without NF-κβ binding sites was not available from InvivoGen, site directed mutagenesis was carried out on the pNifty2 plasmid to obtain an empty vector plasmid, which would serve as a control to demonstrate that the induction of luciferase was specific to NF-κβ.

pNifty2 contains a Not1 restriction site at one end of the NF-κβ binding site. Another Not1 restriction site had to be added to the other end of the NF-κβ site to effectively excise this section. Primers encoding a Not1 restriction site and the corresponding flanking sequence of pNifty2 plasmid where the restriction site was to be inserted were synthesized from 1st Base (Singapore). The Not1 restriction site was inserted into pNifty2

using Quikchange mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. After transforming XL10 Gold Ultracomp competent cells with the mutated plasmid, transformed cells were plated onto zeocin coated LB plates, and incubated at 37^{o}C overnight, following which about 10 colonies were picked and inoculated into TB supplemented with zeocin. The cultures were grown overnight and miniprep was performed as described. Glycerol stocks were prepared for the 10 colonies. Plasmid DNA extracted was then subjected to Not1 restriction and an analytical DNA gel was run to check for the presence of inserted gene. If the gene is inserted correctly, an additional 450 base-pair (bp) fragment should be present compared to unrestricted samples. This 450 bp corresponds to the section of the plasmid that encodes for the NF-κβ binding site.

After identifying the plasmid that contains the inserted gene, a preparative DNA gel was run using 800ng of the plasmid restricted with Not1. The high molecular weight band was excised and DNA was extracted using the QIAquick Gel extraction kit (Qiagen). The plasmid DNA was transformed into TOP10 competent cells and the cells were plated onto zeocin coated LB plates overnight. 10 colonies was chosen and inoculated into TB supplemented with zeocin and grown overnight. The next day, glycerol stocks were prepared and the miniprep was performed to extract the plasmid DNA. An analytical gel was run to check the absence of Not1 restriction site on the plasmid. This plasmid DNA corresponds to a vector similar to pNifty but without the NF-κβ binding site, and was used as a control plasmid.

5.2.5 Transfection

pGL4.74 [hRluc/TK] that encodes renilla luciferase expression constitutively and pSV-β-Galactosidase, a reporter vector that expresses β-galactosidase constitutively were purchased from Promega (Singapore). Both vectors were used as control plasmids to normalize the transfection. As β-galactosidase activity could not be detected in our system, renilla luciferase (pRen) was used as the control plasmid for all subsequent experiments.

2×10^4 of HEK 293 cells were seeded per well of a 96 well plate. Cells were allowed to adhere overnight before proceeding with the transfection. Fugene 6 (Roche, Singapore) was brought to room temperature and mixed by inversion gently. For one sample to be transfected, 0.15µl of Fugene 6 reagent was added to 4.6µl of serum free medium (DMEM) in a sterile tube without touching the sides of the tube. The mixture was vortexed for 1 second and allowed to incubate at room temperature for 10 minutes. 0.2µl of pNifty2 or the mutated pNifty2, and 0.05µl of pRen were added to the diluted Fugene 6 (ratio of Fugene 6 to DNA = 6:1). The transfection complex was allowed to incubate at room temperature for 30 minutes and 5µl of the transfection complex was added to the cells in a drop wise manner. The plates were swirled to ensure equal distribution of complex to the entire surface of cells. The cells were returned to the incubator for 24 hours before proceeding with the cell stimulation.

5.2.6 Cell stimulation assay

Phorbol 12-myristate 13-acetate (PMA), purified *E.coli* LPS that activates only TLR4 and not TLR2, and purified lipoteichoic acid (LTA) that activates TLR2 were purchased from InvivoGen. LPS extracted using the hot phenol-water method from *B. pseudomallei* wildtype and capsular mutant strain K96243 was obtained as a gift from Dr. Joann Prior from the Defence Science and Technology Laboratory (United Kingdom).

24 hours after transfection, cells were stimulated with PMA, LTA or LPS from *E.coli*, *Salmonella enetrica* serovar Typhimurium (Sigma), *B. thailandensis* strain ATCC 700388, and *B. pseudomallei* strains BP22 and K96243. The final concentration used was 100ng/ml for PMA and 1000ng/ml for LTA and LPS. Control cells were stimulated with medium only. Cells were allowed to incubate with the agonists for 7 hours for the NF-κβ activation assay and 18 hours for the IL-8 assay.

5.2.7 NF-κβ luciferase assay

Dual-lucifease reporter assay system (Promega) was used to quantify NF-κβ activation. Cell culture supernatant was removed, and the cells were washed twice with sterile PBS. 30µl of 1x passive lysis buffer was then added to the cells, and the cells were allowed to lyse for 15 minutes on a shaking platform. After lysis, 20µl of the cell lysate was transferred to white luminescence plates and placed into a dual auto-injector luminometer platform (GloMax, Promega). The injector was programmed to add the reagents in sequential order and take the reading before moving to the next well. Relative light units

were obtained by normalizing the firefly luciferase value (pNifty or mutated pNifty) to the Renilla Luciferase value (pRen).

5.2.8 IL-8 analysis

After 18 hours of incubation with the various ligands, the cell culture supernatant was analyzed for the expression of IL-8 by ELISA (Cytometric Bead Assay, BD Pharmingen).

5.2.9 Statistical analysis

All results were analysed by unpaired Student's *t* test using 2-tailed distribution. Differences with $p<0.05$ was considered significant.

5.3 RESULTS

5.3.1 Distribution of receptors on cells

To confirm the cellular responsiveness of HEK 293 to the different TLR ligands, we examined the cell surface expression of TLR2, TLR4 and CD14 in untransfected HEK 293 cells, TLR2 transfected HEK 293 cells and TLR4-CD14-MD2 transfected HEK 293 cells (Figure 5.1). Untransfected HEK 293 cells did not express TLR2, TLR4 or CD14 (Figure 5.1a). TLR2 transfected HEK 293 cells expressed only TLR2 (Figure 5.1b). Expression of CD14 and TLR4 was not detected in these cells.

In HEK 293 cells that were transfected with the TLR4-CD14-MD2 complex, both TLR4 and CD14 expression was detected (Figure 5.1c). TLR2 was not detected in these cells. A small proportion of cells not expressing any of the markers was also detected in the TLR4 transfected cells.

5.3.2 Specificity of TLR for its ligands

To check the specificity of TLR2 and TLR4 in recognizing its particular ligand, TLR transfected HEK 293 cells were stimulated with various ligands (PMA, LTA or *E.coli* LPS). For a read-out of NF-κβ activity, cells were additionally transfected with a NF-κβ inducible reporter (luciferase) plasmid (pNifty). Renilla luciferase was used as a control to normalize the transfection. Figure 5.2 shows the results of the control experiment. PMA is direct stimulator of protein kinase C and hence is able to induce NF-κβ activation regardless of the presence of TLRs. In line with this, PMA was able to

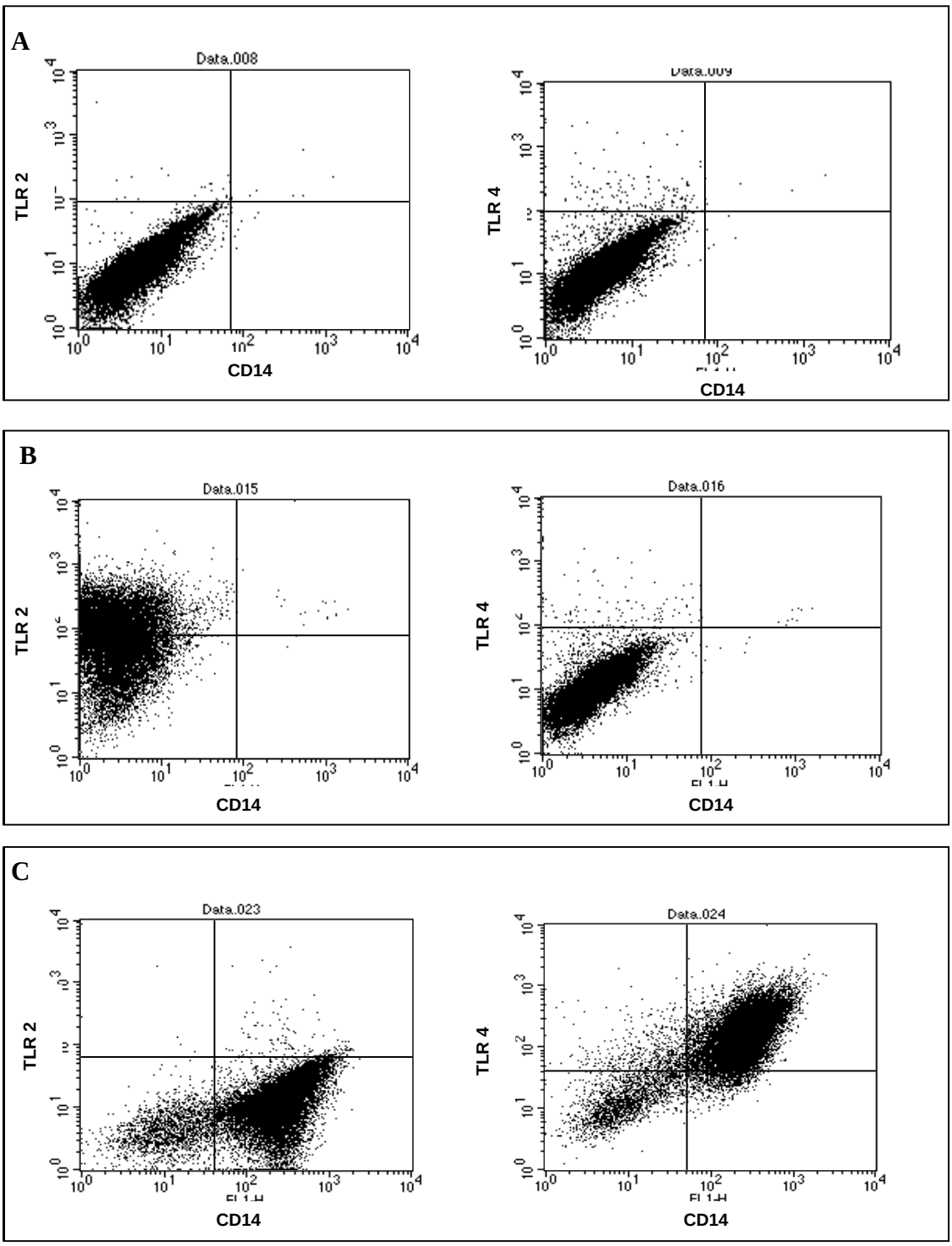


Figure 5.1: Distribution of TLR2, TLR4 and CD14 in transfected and untransfected HEK 293. The cell surface expressions of TLR2, TLR4 and CD14 in untransfected (A) and TLR2 (B) or TLR4-MD2-CD14 (C) transfected HEK 293 cells were determined by flow cytometry.

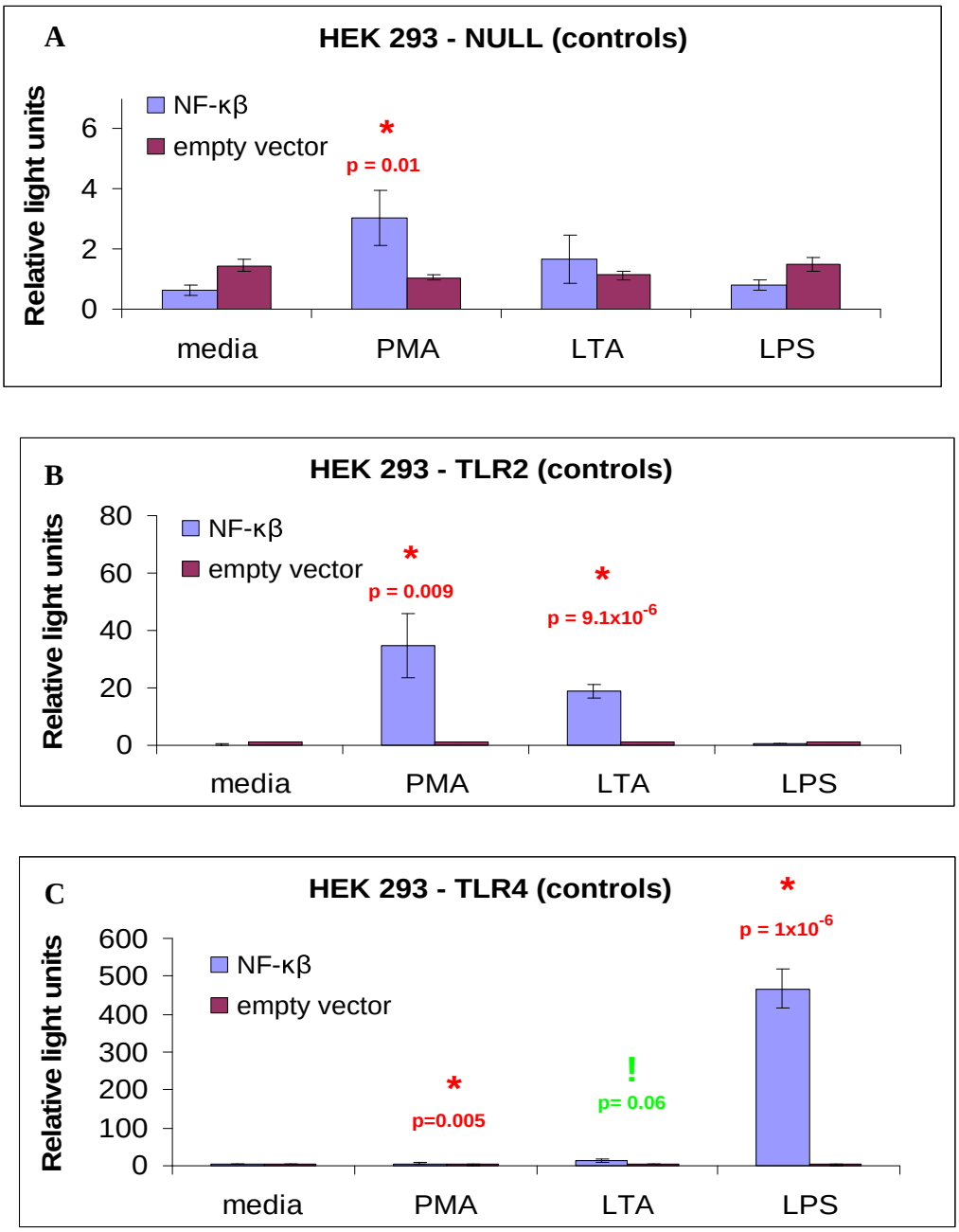


Figure 5.2: Stimulation of HEK 293 cells with various ligands. Untransfected (A), and TLR2 (B) or TLR4-CD14-MD2 complex (C) transfected HEK 293 cells were stimulated with phorbol 12-myristate 13-acetate (PMA), purified lipoteichoic acid (LTA), or purified *E.coli* LPS (LPS). Media indicates mock stimulated cells. Experiments were repeated twice and results represents the mean with error bars indicating the standard error (n=7). ***** indicate significant difference ($p<0.05$) between the ligand and mock stimulated cells. ! indicates no significant difference ($p>0.05$) in comparison to mock stimulated cells.

induce NF-κβ activation in untransfected HEK 293 and in TLR2 and TLR4 transfected cell-lines. This stimulation was significant ($p<0.05$) compared to mock stimulated cells. LTA was used as a positive control for TLR2 transfected cells and negative control for TLR4 transfected cells. Likewise, LTA was able to induce NF-κβ activation only in TLR2 transfected cells ($p=9.1x10^{-6}$ compared to mock stimulated cells). Although it appears that LTA was able to induce NF-κβ activity in TLR4 transfected cells, the activation was not significant compared to mock stimulated cells ($p=0.06$). Purified LPS from E.coli was used as a positive control for TLR4 transfected cells. LPS from E.coli was able to induce NF-κβ activity only in TLR4 transfected cells ($p=1x10^{-6}$). Empty vectors (reporter plasmids not containing the NF-κβ binding sites) were used as a negative control and they did not respond to any of the ligands used. Although the basal level of the empty vector was slightly higher than the NF-κβ plasmid, it remained constant throughout the assay.

5.3.3 Effect of purity on the recognition of *B. pseudomallei* and *B. thailandensis* LPS by TLR2 and TLR4

To determine whether LPS from *B. pseudomallei* and *B. thailandensis* is recognized by TLR2 or TLR4, HEK 293 cells were used. Figure 5.3 shows the results of the stimulation assay using LPS from *B. pseudomallei* strain BP22. BP22 LPS was unable to induce NF-κβ activation in untransfected cells. The crude LPS obtained directly from the kit was recognized by both TLR2 ($p=0.01$, compared to mock stimulated) and TLR4 ($p=2.4x10^{-7}$). On the other hand, purified BP22 LPS was recognized only by TLR4 ($p=9.9x10^{-11}$, compared to mock stimulated). No significant difference was observed

between the recognition of crude and purified BP22 LPS in the TLR4 transfected cell-lines.

LPS from *B. thailandensis* could induce NF-κβ activation only in TLR4 transfected cell-lines regardless of the purity status (Figure 5.4). TLR4-dependent activation of NF-κβ was significant compared to mock stimulated cells when stimulated with crude LPS ($p=2x10^{-6}$) and purified LPS ($p=9.2x10^{-9}$).

5.3.4 IL-8 production in response to various ligands

As HEK 293 cells produce only IL-8, this precluded the examination of other cytokines and chemokines that are NF-κβ regulated (Fan *et al.*, 2007). Hence, the stimulation of IL-8 by HEK 293 was investigated in response to the various ligands and LPS (Figure 5.5). In untransfected cells, only PMA induced IL-8 production. In TLR2 transfected cells, only PMA and LTA induced IL-8 production. LPS from BP22 and *B. thailandensis* did not stimulate TLR2 regardless of purity status. All the LPS tested were able to induce IL-8 production by TLR4 transfected cells. Only LTA, the negative control could not stimulate IL-8 production in these cells.

5.3.5 Effect of LPS extraction method on recognition by TLR2 and TLR4

To investigate if the method of extraction affects the recognition of LPS by TLR2 or TLR4, LPS from *B. pseudomallei* strain K96243 was extracted using the hot phenol-water method and compared to the kit extraction method (Figure 5.6). K96243 LPS was unable to induce NF-κβ activation in non-transfected cells. In TLR2 transfected cells,

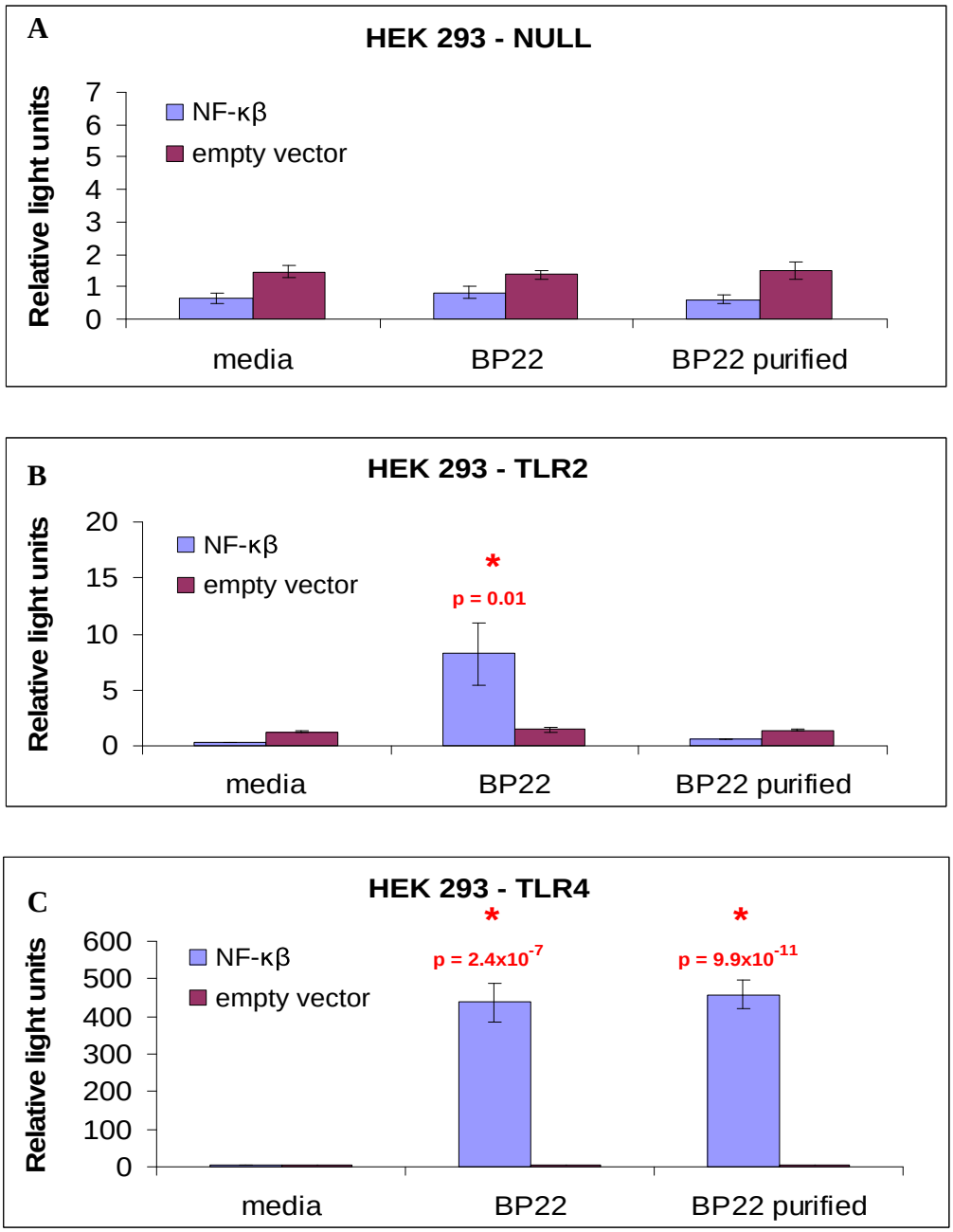


Figure 5.3: Stimulation of HEK 293 cells with LPS from *B. pseudomallei* strain BP22. Untransfected (A), and TLR2 (B) or TLR4-CD14-MD2 complex (C) transfected HEK 293 cells were stimulated with either crude LPS (BP22) or purified LPS (BP22 purified) from *B. pseudomallei* strain BP22. Media indicates mock stimulated cells. Experiments were repeated twice and results represents the mean with error bars indicating the standard error (n=7). ***** indicate significant difference ($p<0.05$) between the ligand and mock stimulated cells.

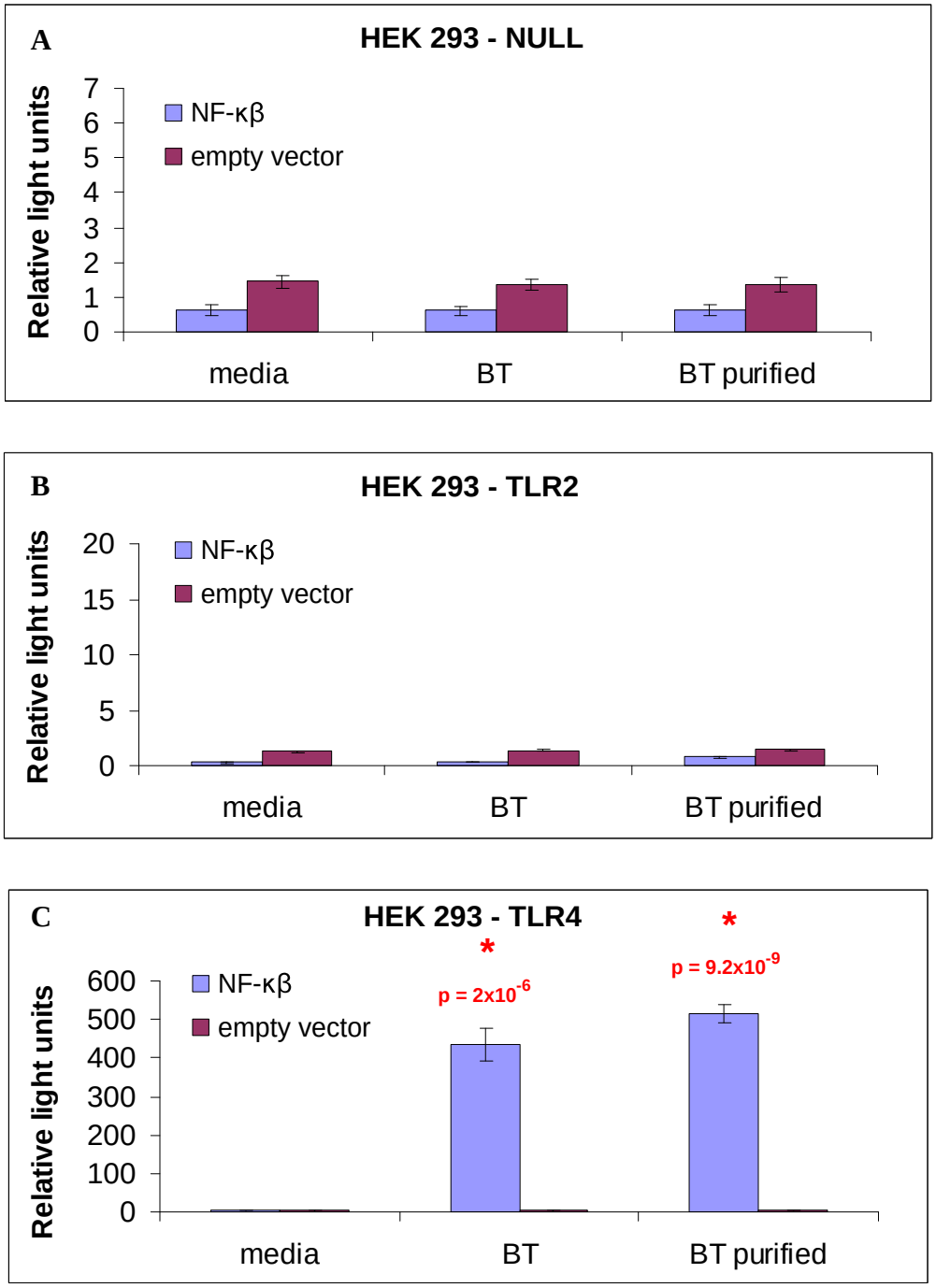


Figure 5.4: Stimulation of HEK 293 cells with *B. thailandensis* LPS. Untransfected (A), and TLR2 (B) or TLR4-CD14-MD2 complex (C) transfected HEK 293 cells were stimulated with either crude LPS (BT) or purified LPS (BT purified) from *B. thailandensis*. Media indicates mock stimulated cells. Experiments were repeated twice and results represents the mean with error bars indicating the standard error (n=7). ***** indicate significant difference ($p<0.05$) between the ligand and mock stimulated cells.

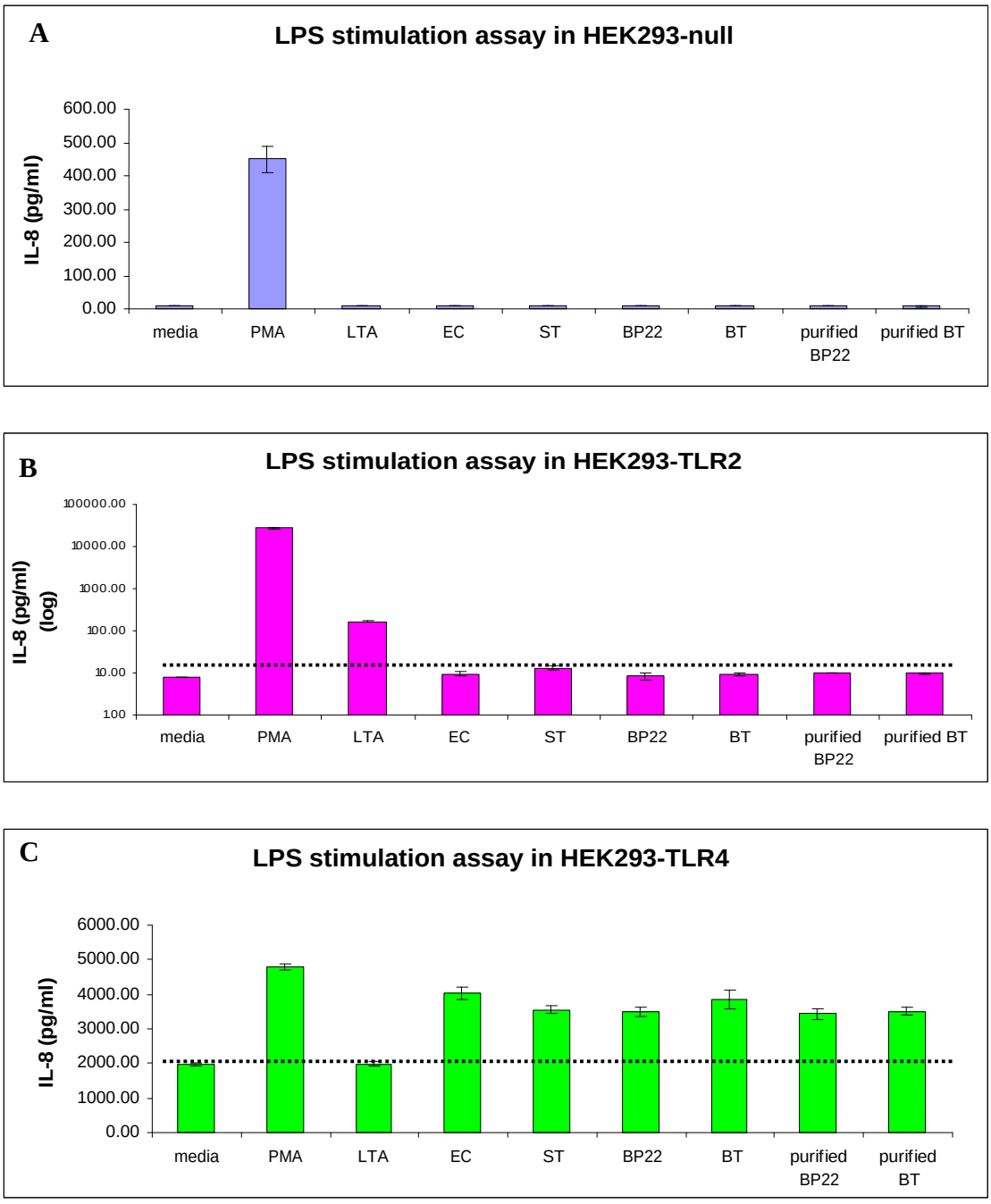


Figure 5.5: IL-8 production by HEK 293 cells stimulated with various ligands. Untransfected (A), and TLR2 (B) or TLR4-CD14-MD2 complex (C) transfected HEK 293 cells were stimulated with phorbol 12-myristate 13-acetate (PMA), purified lipoteichoic acid (LTA), or LPS from *E.coli* (EC), *Salmonella* serovar Typhimurium (ST), *B. pseudomallei* (BP22) or *B. thailandensis* (BT). Media indicates mock stimulated cells. Experiments were repeated twice and results represents the mean with error bars indicating the standard error (n=3). (⋯⋯) indicate the basal level expression.

LPS extracted by the hot aqueous phenol method (p=0.002, compared to mock stimulated) and kit (p=0.01) were able to induce NF-κβ activity. However, the activation by the kit extracted LPS was 20 fold less than the LPS extracted by hot aqueous phenol method (p=0.002). Similar to the LPS from BP22, upon further purification, LPS extracted with the kit could not induce NF-κβ activation in the TLR2 transfected cells. In the TLR4 transfected cells, NF-κβ activation was induced by both the LPS and this activation was significant compared to activation in mock stimulated cells ($p < 0.05$). No significance difference was observed in the LPS stimulation between the hot aqueous phenol extraction method and the kit extraction method in the TLR4 transfected cells.

5.3.6. Effect of capsular polysaccharide on recognition by TLR2 and TLR4

Finally, to investigate if the presence of capsular polysaccharide would contribute towards NF-κβ activation, LPS extracted from a capsular mutant strain of K96243 was compared to LPS from the wild-type strain (Figure 5.7). LPS from the capsular mutant strain also did not induce NF-κβ activation in the untransfected cells. Similar to the wild-type strain, LPS from the capsular mutant strain was able to induce NF-κβ activation in both TLR2 ($p=1.5 \times 10^{-5}$, compared to mock stimulated) and TLR4 ($p=3.1 \times 10^{-5}$) transfected cells. There was no significant difference in NF-κβ activation induced by LPS extracted from capsular mutant strain and wild-type strain ($p>0.05$) in TLR2 and TLR4 transfected cells.

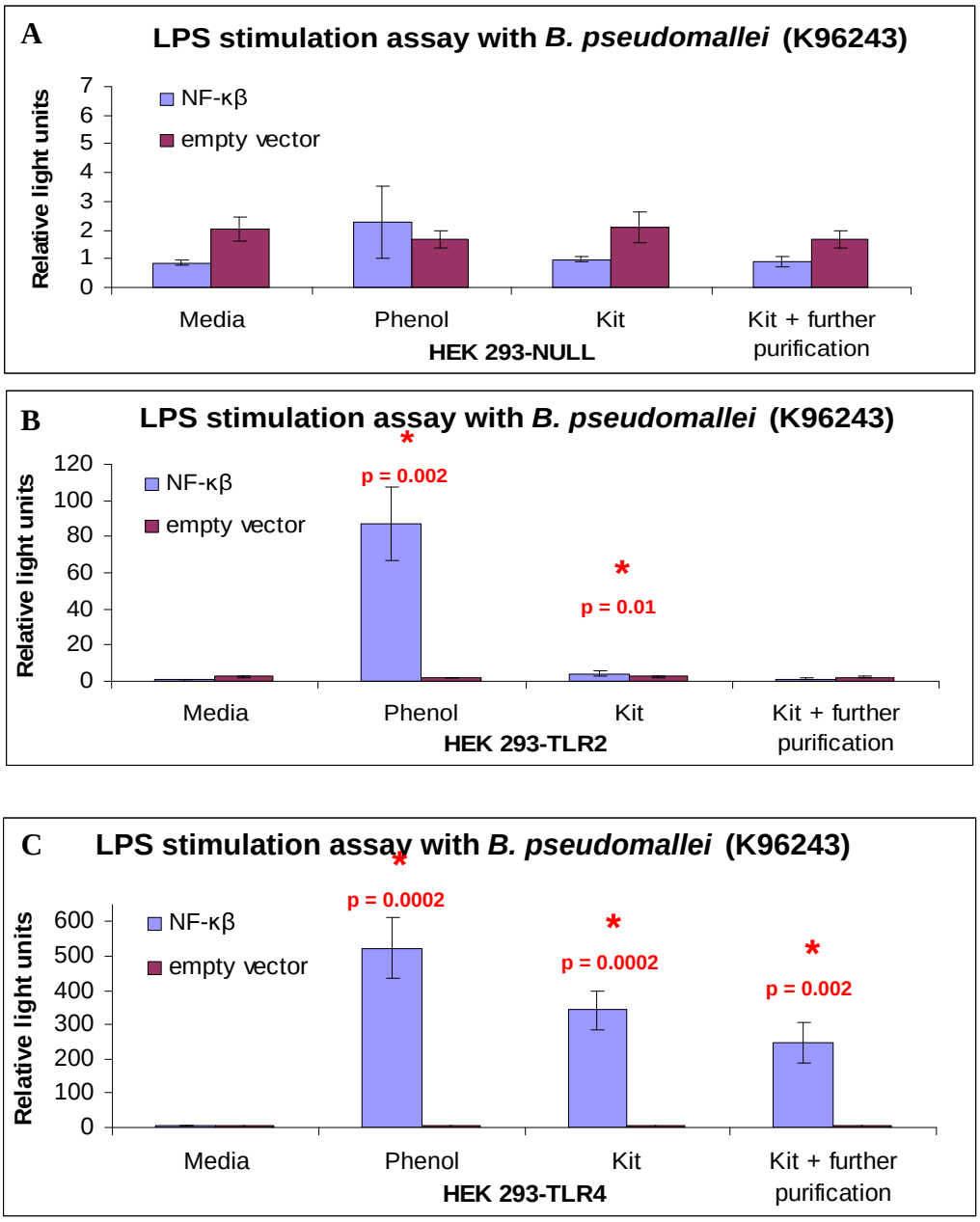


Figure 5.6: Comparison of LPS extraction method used on the stimulation of HEK 293 cells. Untransfected (A), and TLR2 (B) or TLR4-CD14-MD2 complex (C) transfected HEK 293 cells were stimulated with LPS from *B. pseudomallei* strain K96243, extracted either by the hot phenol-water method (phenol) or the LPS extraction kit (kit). Kit extracted LPS that was purified further was also used as a ligand (kit + further purification). Media indicates mock stimulated cells. Experiments were repeated twice and results represents mean with error bars indicating the standard error (n=6). ***** indicate significant difference between the ligand and mock stimulated cells.

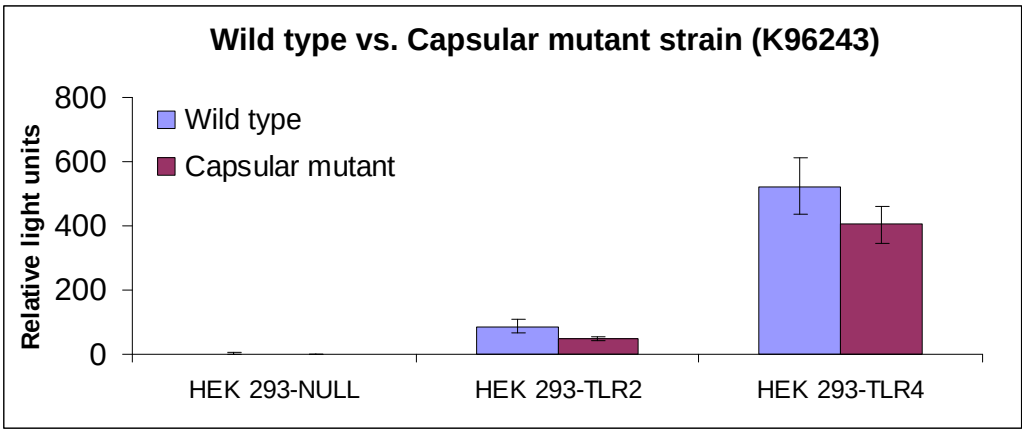


Figure 5.7: Effect of the presence of capsule on the stimulation of HEK 293 cells. Untransfected (HEK 293-null), and TLR2 (HEK 293-TLR2) or TLR4-CD14-MD2 complex (HEK 293 – TLR4) transfected HEK 293 cells were stimulated with LPS from *B. pseudomallei* strain K96243 wildtype or capsular mutant. Experiments were repeated twice and results represents mean with error bars indicating the standard error (n=6).

5.4 DISCUSSION

Understanding the contribution of receptors involved in the innate recognition of LPS is essential in the context of potential therapeutic intervention. The LPS extracted from *B. pseudomallei* was able to stimulate cells via both TLR2 and TLR4. However, upon further purification to remove contaminants, signaling via TLR2 was abrogated, and the purified LPS was recognized only by TLR4. Our data indicates that TLR4 is the true receptor for the recognition of *B. pseudomallei* LPS.

Two groups demonstrated the ability of TLR2 to signal in the response to LPS (Kirschning *et al.,* 1998; Yang *et al.,* 1998). Kirschning and group (1998) demonstrated that only overexpression of TLR2, but not TLR1, TLR4, or CD14 conferred LPS stimulation of NF-κβ activation in mammalian HEK 293 cells, supporting the notion that TLR2 was the specific receptor for LPS (Kirschning *et al.,* 1998). However, a genetic lesion identified in C3H/HeJ mice, which corresponded to a mutation in tlr4 gene (Poltorak *et al.,* 1998), made the mice highly susceptible to *Salmonella* infection and resistant to its LPS, compared to C3H/HeN mice with intact response to LPS. This finding prompted the search for the true receptor of LPS and it was initially found that both TLR2 and TLR4 could respond to LPS (Chow *et al.,* 1999). The failure of Kirschning and group (1998) to observe LPS inducible NF-κβ activation of HEK 293 cells transfected with TLR4 was attributed to inherent differences in stocks of HEK 293 cells. Some stocks of HEK 293 cells could respond to LPS without any transfection

(Chow *et al.,* 1999), emphasizing the importance in ascertaining the basal stimulation of untransfected cells by the ligands used.

Hirschfeld and group (2000) demonstrated that the stimulation of TLR2 by LPS was due to lipoprotein contamination in LPS preparations (Hirschfeld *et al.,* 2000). Similarly, the ability of *Porphyromonas gingivalis* LPS and its natural lipid A fraction to induce NF-κβ activation in Ba/F3 cells expressing mouse TLR2 was attributed to minor contaminants present. The purified contaminants induced NF-κβ activation through TLR2 (Ogawa *et al.,* 2007)

A recent publication reported that TLR2 was the receptor involved in the recognition of LPS from *B. pseudomallei* (Wiersinga *et al.,* 2007). On the contrary, Hii *et al.,* (2008) showed that *B. pseudomallei* activates NF-κβ through TLR4. Although their data was based on whole bacteria rather than purified LPS, activation of NF-κβ through TLR4 indirectly indicated recognition of *B. pseudomallei* LPS by TLR4. Supporting their data was a study by West and colleagues, who confirmed that *B. pseudomallei* LPS was a TLR4 agonist (West *et al.,* 2008). Hence, the observations by Wiersinga *et al.,* (2007) could be due to either the use of different strain of bacteria or the presence of contaminants in the extracted LPS that are strongly recognized by TLR2. In fact, the method of LPS extraction used may have contributed significantly to their observation. The LPS used in their studies were extracted by the modified hot phenol-water method, the established procedure for the extraction of LPS from *B. pseudomallei* (Perry *et al.,*

1995). As LPS from *B. pseudomalle*i partitions into both the phenol and water phase, this LPS was subsequently recovered from both phases (Perry *et al.*, 1995).

To investigate whether the method of extraction used may influence the TLR recognition of LPS, we compared NF-κβ activation in TLR2 or TLR4 transfected cells using LPS (*B. pseudomallei* strain K96243) that was extracted by the hot phenol-water method to the kit extraction method. LPS extracted by the classical hot phenol-water method was recognized by both TLR2 and TLR4 and the recognition by TLR2 was 20x more stimulatory than LPS extracted by the kit. However, when LPS extracted by the kit was purified to remove contaminants, signaling through TLR2 was abrogated. As LPS extracted using the hot phenol-water method was obtained as a gift, the amount was too little to undergo further purification. Consequently, we were unable to investigate if the removal of contaminants from this LPS preparation would abrogate signaling through TLR2.

Several groups have shown that lipoproteins enter the phenol phase and purifying LPS from this phase results in LPS preparations that may be contaminated with lipoproteins (Tirsoaga et al., 2007). Over-expression of TLR in human or murine cell-lines makes them very sensitive to minute amounts of endotoxic proteins, thus pure preparations of LPS are prudent in such studies. It is likely the observation made that TLR2 was the recognition receptor for *B. pseudomallei* LPS was due to lipoproteins present in the preparation (Wiersinga *et al.*, 2007). In support, both the strains of LPS tested here

(K96243 and BP22) were recognized by only TLR4 after the contaminants had been removed.

For *B. thailandensis*, TLR4 was found to be the receptor that recognizes its LPS. This is the first time a study has shown the receptor involved in the recognition of *B. thailandensis* LPS. Surprisingly, for this LPS, even the crude preparation before purification did not stimulate TLR2. While the reason for this has not been investigated in this study, we postulate that unlike in some bacteria where LPS is associated strongly with outer-membrane proteins, LPS is not so associated in *B. thailandensis*.

When LPS of *B. pseudomallei* was compared to LPS of *B. thailandensis*, we found no significant difference in the recognition of these LPS by TLR4. However, these LPS were differently recognized by RAW 264.7 (Chapter 4). We also found that the potent LPS from *Salmonella* serovar Typhimurium and *E. coli* activated TLR4 transfected HEK 293 cells similarly to LPS from *B. pseudomallei* and *B. thailandensis.* It is likely that the amount of LPS used in these studies was at saturation point, and hence no difference in stimulation was observed among the different LPS. Further investigations will be needed to confirm this suggestion.

LPS purified from *B. pseudomallei* is known to co-precipitate with capsular polysaccharide (Isshiki *et al.*, 2001). To investigate if the presence of capsule, if any, would affect the recognition by TLR4, the activation of NF-κβ was compared between LPS extracted from the wild type strain and a capsular mutant strain of K96243. No

difference was observed in the stimulation of TLR4 transfected HEK 293 cells by the two LPS. This suggests that capsule polysaccharide, if present, does not contribute to the observed activation of NF-κβ.

In-vivo data by Wiersinga and co-workers demonstrated that TLR4 knock-out mice had similar survival rates, bacterial loads and lung inflammation to the wild-type animals in experimental melioidosis (Wiersinga *et al*., 2007). Although they conclude that TLR4, and indirectly *B. pseudomallei* LPS play no role in the immune response *in-vivo*, their data further emphasizes our point that the reduced stimulatory potential of *B. pseudomallei* LPS plays an important role in the pathogenicity of *B. pseudomallei* by allowing it to evade the innate immune system.

Identification of the TLR involved in the recognition of LPS may be important for therapeutic intervention. The data here indicates that the pathogenicity of *B. pseudomallei* is partly linked to its ability to evade host defenses as its LPS is poorly recognized by TLR4. Hence, the design of exogenous TLR4 ligands or agonists that boost the innate immune response to infection with *B. pseudomallei* and compensate for the reduced TLR4 stimulation may be beneficial in clearing the disease.

Chapter 6

Overall Conclusions

6.1 CONCLUSIONS

PhoP-PhoQ is a two-component environmental regulatory system that governs virulence and mediates the adaptation to magnesium (Mg^{2+}) limiting environments in several Gram-negative species (Groisman, 2001). The finding that a similar system may exist in *B. pseudomallei* prompted our investigations in the effect of magnesium on the virulence of *B. pseudomallei.* Mg^{2+} has been described to regulate lipid A modifications in *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*. As preliminary experiments in our laboratory demonstrated that *B. pseudomallei* was more virulent when cultured in magnesium limiting culture medium, we hypothesized that the increase in virulence observed in response to low magnesium concentrations was a result of surface remodeling of lipid A in *B. pseudomallei*. However, when the fatty acid composition of lipopolysaccharide (LPS) extracted from *B. pseudomallei* cultured in magnesium supplemented medium was compared to that of bacteria cultured in low magnesium concentration medium, no qualitative differences were found. We further compared the ability of LPS extracted from the two different media to induce cytokine production in both murine and human macrophage cell-lines *in-vitro*. No difference was observed in the ability of *B. pseudomallei* LPS extracted from either medium to induce cytokine production in these cell-lines. Based on these findings, we concluded that magnesium does not play a role in the surface remodeling of *B. pseudomallei* LPS.

Burkholderia thailandensis is a close relative of *B. pseudomallei*. Although it shares the same ecological niche, it rarely causes disease (Glass *et al.,* 2006; Smith *et al.,* 1997).

Several groups have used *B. thailandensis* as a model system to identify virulence mechanisms in *B. pseudomallei* (Haraga *et al.*, 2008). Similarly, we compared the LPS of *B. pseudomallei* to the LPS of *B. thailandensis* to understand the role of LPS in the pathogenesis of *B. pseudomallei* . Our experiments showed that the purity of LPS was very important when comparing biological activities. Although initial experiments showed no difference in the ability of LPS from *B. pseudomallei* and *B. thailandensis* to induce cytokine production in murine macrophage cells, upon further purification to remove contaminating proteins, *B. thailandensis* LPS induced higher production of cytokines than *B. pseudomallei* LPS *in-vitro*. This difference correlated with the structure variability of lipid A between the two organisms.

The major lipid A species identified in *B. pseudomallei* consisted of a bi-phosphorylated disaccharide backbone modified with 4-amino-4-deoxy-arabinose (Ara4N) at both phosphate groups and penta-acylated with C14:0(3-OH), C16:0(3-OH) and either C14:0 or C14:0(2-OH). On the contrary, the major lipid A species identified in *B. thailandensis* was a heterogenous mixture of penta- and tetra-acylated structures varying in Ara4N substitution and acylation of C14:0(3-OH). In summary, two specific differences were found in the lipid A of the two pathogens. Firstly, the presence of C14:0(2-OH) was unique to *B. pseudomallei* and not found in *B. thailandensis*. Secondly, all the major species identified in *B. pseudomallei* had both phosphate groups modified with Ara4N. On the contrary, in *B. thailandensis,* there were major lipid A species identified with either no Ara4N modification or only one phosphate group modified with Ara4N. Based on these findings, and supporting evidence on the role of these structural features in other

Gram-negative bacteria, we postulate that the differences in structure may contribute to the variation in the virulence of *B. pseudomallei* and *B. thailandensis.* In *B. pseudomallei*, synthesis of lipid A with long fatty acyl chains and C14:0(2-OH) may contribute to the low immunological activities observed *in-vitro.* The presence of Ara4N modified phosphate groups and C14:0(2-OH) in its lipid A may further confer resistance to the effects of CAMPS. Collectively, these modifications may allow *B. pseudomallei* to evade host recognition through weak activation of innate immune system and resistance to CAMPS, allowing the pathogen to survive intracellularly. On the contrary, the more potent LPS synthesized by *B. thailandensis* may activate the innate immune system more strongly. Consequently, *B. thailandensis* becomes more susceptible to the bactericidal effects of host innate immune responses, resulting in efficient clearance of the pathogen.

Toll like receptor (TLR) 4 is the pattern recognition receptor of LPS (Miller *et al.,* 2005). However, a recently published data indicated that TLR2, and not TLR4 was involved in the signal transduction *of B. pseudomallei* LPS (Wiersinga *et al*., 2007). As our initial experiments indicated the presence of contaminating proteins in *B. pseudomallei* LPS, we hypothesized that the presence of contaminating proteins were responsible for the signaling through TLR2. In accordance with this, we found that LPS extracted using the hot phenol-water method showed significant signaling through TLR2 and TLR4. However, when LPS was purified to remove contaminating proteins, signaling through TLR2 was eliminated and signaling was observed through TLR4 only. Similarly, purified *B. thailandensis* LPS stimulated host cells through TLR4. This study further emphasizes the need of pure preparations of LPS in biological assays.

6.2 FUTURE WORK

In this study, the structures of lipid A from *B. pseudomallei* and *B. thailandensis* were proposed. To confirm the structures proposed here and to determine the order of fatty acids to the lipid A backbone, further structural analysis such as nuclear magnetic resonance (NMR) spectroscopy is required. Such analysis would give a clearer picture on the structure-function relationship of LPS in pathogenesis.

The differences in the ability of *B. pseudomallei* LPS and *B. thailandensis* LPS to activate murine macrophages were observed *in-vitro*. *In-vivo* experiments need to be carried out to determine if the differences seen in cell culture will also be observed *in-vivo*. In addition, to confirm the postulations on the impact of structural differences on the virulence and intracellular survival of *B. pseudomallei*, it will be necessary to purify or synthesize individual lipid A species and observe their effects *in-vitro* and *in-vivo*.

The studies here indicated that the pathogenicity of *B. pseudomallei* is linked to its ability to evade or suppress the activation of mammalian defenses as it gains access to and replicates in its intracellular niche. This is facilitated by a LPS structure that is poorly recognized by TLR4. Hence, the design of exogenous TLR4 ligands or agonists that boost the innate immune response to infection with *B. pseudomallei* and compensate for the reduced TLR4 stimulation may be beneficial in clearing the disease. The administration of such synthetic TLR4 agonists have been described to provide protection against experimental pneumonic tularemia in mice upon infection with *Francisella*

tularensis novicida, a Gram-negative bacterium with an unusual LPS structure that is also poorly recognized by TLR4 (Lembo *et al.*, 2008).

Bibliography

Bibliography

1. Aballay, A., Drenkard, E., Hilbun, L. R., and Ausubel, F. M. 2003. *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway. Curr Biol **13:** 47.
2. Abbink, F. C., Orendi, J. M., and de Beaufort, A. J. 2001. Mother-to-child transmission of *Burkholderia pseudomallei*. N Engl J Med **344:** 1171.
3. Anuntagool, N., Aramsri, P., Panichakul, T., Wuthiekanun, V. R., Kinoshita, R., White, N. J., and Sirisinha, S. 2000. Antigenic heterogeneity of lipopolysaccharide among *Burkholderia pseudomallei* clinical isolates. Southeast Asian J Trop Med Public Health **31 Suppl 1:** 146.
4. Anuntagool, N., Intachote, P., Wuthiekanun, V., White, N. J., and Sirisinha, S. 1998. Lipopolysaccharide from nonvirulent Ara+ *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent Ara- clinical isolates. Clin Diagn Lab Immunol **5:** 225.
5. Anuntagool, N., Wuthiekanun, V., White, N. J., Currie, B. J., Sermswan, R. W., Wongratanacheewin, S., Taweechaisupapong, S., Chaiyaroj, S. C., and Sirisinha, S. 2006. Lipopolysaccharide heterogeneity among *Burkholderia pseudomallei* from different geographic and clinical origins. Am J Trop Med Hyg **74:** 348.
6. Arjcharoen, S., Wikraiphat, C., Pudla, M., Limposuwan, K., Woods, D. E., Sirisinha, S., and Utaisincharoen, P. 2007. Fate of a *Burkholderia pseudomallei* lipopolysaccharide mutant in the mouse macrophage cell line RAW 264.7:

possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. Infect Immun **75:** 4298.

7. Aussel, L., Therisod, H., Karibian, D., Perry, M. B., Bruneteau, M., and Caroff, M. 2000. Novel variation of lipid A structures in strains of different *Yersinia* species. FEBS Lett **465:** 87.

8. Backhed, F., Normark, S., Schweda, E. K., Oscarson, S., and Richter-Dahlfors, A. 2003. Structural requirements for TLR4-mediated LPS signalling: a biological role for LPS modifications. Microbes Infect **5:** 1057.

9. Baldauf, N. A., Rodriguez-Romo, L. A., Mannig, A., Yousef, A. E., and Rodriguez-Saona, L. E. 2007. Effect of selective growth media on the differentiation of *Salmonella enterica* serovars by Fourier-Transform Mid-Infrared Spectroscopy. J Microbiol Methods **68:** 106.

10. Bosshart, H., and Heinzelmann, M. 2007. Targeting bacterial endotoxin: two sides of a coin. Ann N Y Acad Sci **1096:** 1.

11. Braedel-Ruoff, S., Faigle, M., Hilf, N., Neumeister, B., and Schild, H. 2005. *Legionella pneumophila* mediated activation of dendritic cells involves CD14 and TLR2. J Endotoxin Res **11:** 89.

12. Brett, P. J., Burtnick, M. N., Snyder, D. S., Shannon, J. G., Azadi, P., and Gherardini, F. C. 2007. *Burkholderia mallei* expresses a unique lipopolysaccharide mixture that is a potent activator of human Toll-like receptor 4 complexes. Mol Microbiol **63:** 379.

13. Brett, P. J., DeShazer, D., and Woods, D. E. 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int J Syst Bacteriol **48 Pt 1:** 317.

14. Brett, P. J., and Woods, D. E. 2000. Pathogenesis of and immunity to melioidosis. Acta Trop **74:** 201.

15. Bryn, K., and Jantzen, E. 1982. Analysis of lipopolysaccharides by methanolysis, trifluoroacetylation and gas chromatography on a fused-silica capillary column. J. Chromatogr. **240:** 405.

16. Bucki, R., Byfield, F. J., and Janmey, P. A. 2007. Release of the antimicrobial peptide LL-37 from DNA/F-actin bundles in cystic fibrosis sputum. Eur Respir J **29:** 624.

17. Caroff, M., and Karibian, D. 2003. Structure of bacterial lipopolysaccharides. Carbohydr Res **338:** 2431.

18. Caroff, M., Karibian, D., Cavaillon, J. M., and Haeffner-Cavaillon, N. 2002. Structural and functional analyses of bacterial lipopolysaccharides. Microbes Infect **4:** 915.

19. Chaowagul, W., White, N. J., Dance, D. A., Wattanagoon, Y., Naigowit, P., Davis, T. M., Looareesuwan, S., and Pitakwatchara, N. 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J Infect Dis **159:** 890.

20. Charuchaimontri, C., Suputtamongkol, Y., Nilakul, C., Chaowagul, W., Chetchotisakd, P., Lertpatanasuwun, N., Intaranongpai, S., Brett, P. J., and Woods, D. E. 1999. Antilipopolysaccharide II: an antibody protective against fatal melioidosis. Clin Infect Dis **29:** 813.

21. Cheng, A. C., and Currie, B. J. 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev **18:** 383.

22. Chierakul, W., Winothai, W., Wattanawaitunechai, C., Wuthiekanun, V., Rugtaengan, T., Rattanalertnavee, J., Jitpratoom, P., Chaowagul, W., Singhasivanon, P., White, N. J., Day, N. P., and Peacock, S. J. 2005. Melioidosis in 6 tsunami survivors in southern Thailand. Clin Infect Dis **41:** 982.

23. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem **274:** 10689.

24. Currie, B. J. 2008. Advances and remaining uncertainties in the epidemiology of *Burkholderia pseudomallei* and melioidosis. Trans R Soc Trop Med Hyg **102:** 225.

25. Currie, B. J., and Jacups, S. P. 2003. Intensity of rainfall and severity of melioidosis, Australia. Emerg Infect Dis **9:** 1538.

26. Currie, B. J., Mayo, M., Anstey, N. M., Donohoe, P., Haase, A., and Kemp, D. J. 2001. A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. Am J Trop Med Hyg **65:** 177.

27. Dance, D. A. 2000. Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia spp*. and human-animal hosts. Acta Trop **74:** 159.

28. Dance, D. A. 1991. Melioidosis: the tip of the iceberg? Clin Microbiol Rev **4:** 52.

29. Darveau, R. P., and Hancock, R. E. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J Bacteriol **155:** 831.

30. Dharakul, T., and Songsivilai, S. 1999. The many facets of melioidosis. Trends Microbiol **7:** 138.

31. Dixon, D. R., and Darveau, R. P. 2005. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. J Dent Res **84:** 584.

32. Egan, A. M., and Gordon, D. L. 1996. *Burkholderia pseudomallei* activates complement and is ingested but not killed by polymorphonuclear leukocytes. Infect Immun **64:** 4952.

33. El Hamidi, A., Tirsoaga, A., Novikov, A., Hussein, A., and Caroff, M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res **46:** 1773.

34. Ernst, R. K., Adams, K. N., Moskowitz, S. M., Kraig, G. M., Kawasaki, K., Stead, C. M., Trent, M. S., and Miller, S. I. 2006. The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. J Bacteriol **188:** 191.

35. Ernst, R. K., Hajjar, A. M., Tsai, J. H., Moskowitz, S. M., Wilson, C. B., and Miller, S. I. 2003. *Pseudomonas aeruginosa* lipid A diversity and its recognition by Toll-like receptor 4. J Endotoxin Res **9:** 395.

36. Ernst, R. K., Moskowitz, S. M., Emerson, J. C., Kraig, G. M., Adams, K. N., Harvey, M. D., Ramsey, B., Speert, D. P., Burns, J. L., and Miller, S. I. 2007. Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. J Infect Dis **196:** 1088.

37. Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M., and Miller, S. I. 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa.* Science **286:** 1561.

38. Fan, H., Luttrell, L. M., Tempel, G. E., Senn, J. J., Halushka, P. V., and Cook, J. A. 2007. Beta-arrestins 1 and 2 differentially regulate LPS-induced signaling and pro-inflammatory gene expression. Mol Immunol **44:** 3092.

39. Fields, P. I., Groisman, E. A., and Heffron, F. 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:** 1059.

40. Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A **83:** 5189.

41. Friedland, J. S., Suputtamongkol, Y., Remick, D. G., Chaowagul, W., Strieter, R. M., Kunkel, S. L., White, N. J., and Griffin, G. E. 1992. Prolonged elevation of interleukin-8 and interleukin-6 concentrations in plasma and of leukocyte interleukin-8 mRNA levels during septicemic and localized *Pseudomonas pseudomallei* infection. Infect Immun **60:** 2402.

42. Galanos, C., Luderitz, O., and Westphal, O. 1969. A new method for the extraction of R lipopolysaccharides. Eur J Biochem **9:** 245.

43. Garcia Vescovi, E., Soncini, F. C., and Groisman, E. A. 1996. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. Cell **84:** 165.

44. Gibbons, H. S., Kalb, S. R., Cotter, R. J., and Raetz, C. R. 2005. Role of Mg2+ and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. Mol Microbiol **55:** 425.

45. Gibbons, H. S., Lin, S., Cotter, R. J., and Raetz, C. R. 2000. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, A new Fe2+/alpha-ketoglutarate-dependent dioxygenase homologue. J Biol Chem **275:** 32940.

46. Glass, M. B., Gee, J. E., Steigerwalt, A. G., Cavuoti, D., Barton, T., Hardy, R. D., Godoy, D., Spratt, B. G., Clark, T. A., and Wilkins, P. P. 2006. Pneumonia and septicemia caused by *Burkholderia thailandensis* in the United States. J Clin Microbiol **44:** 4601.

47. Groisman, E. A. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol **183:** 1835.

48. Gunn, J. S. 2001. Bacterial modification of LPS and resistance to antimicrobial peptides. J Endotoxin Res **7:** 57.

49. Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. 1997. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. Science **276:** 250.

50. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell **95:** 189.

51. Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol **3:** 354.

52. Haraga, A., West, T. E., Brittnacher, M. J., Skerrett, S. J., and Miller, S. I. 2008. *Burkholderia thailandensis* as a model system for the study of the virulence-associated type III secretion system of *Burkholderia pseudomallei*. Infect Immun **76:** 5402.

53. Hii, C. S., Sun, G. W., Goh, J. W., Lu, J., Stevens, M. P., and Gan, Y. H. 2008. Interleukin-8 induction by *Burkholderia pseudomallei* can occur without Toll-like receptor signaling but requires a functional type III secretion system. J Infect Dis **197:** 1537.

54. Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol **165:** 618.

55. Hirschfeld, M., Weis, J. J., Toshchakov, V., Salkowski, C. A., Cody, M. J., Ward, D. C., Qureshi, N., Michalek, S. M., and Vogel, S. N. 2001. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun **69:** 1477.

56. Ho, M., Schollaardt, T., Smith, M. D., Perry, M. B., Brett, P. J., Chaowagul, W., and Bryan, L. E. 1997. Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. Infect Immun **65:** 3648.

57. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. 2003.

Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature **424:** 743.

58. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., and Hartmann, G. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol **168:** 4531.

59. Howe, C., Sampath, A., and Spotnitz, M. 1971. The *pseudomallei* group: a review. J Infect Dis **124:** 598.

60. Inglis, T. J., Aravena-Roman, M., Ching, S., Croft, K., Wuthiekanun, V., and Mee, B. J. 2003. Cellular fatty acid profile distinguishes *Burkholderia pseudomallei* from avirulent *Burkholderia thailandensis*. J Clin Microbiol **41:** 4812.

61. Inglis, T. J., Rolim, D. B., and Sousa Ade, Q. 2006. Melioidosis in the Americas. Am J Trop Med Hyg **75:** 947.

62. Inglis, T. J., and Sagripanti, J. L. 2006. Environmental factors that affect the survival and persistence of *Burkholderia pseudomallei*. Appl Environ Microbiol **72:** 6865.

63. Isshiki, Y., Matsuura, M., Dejsirilert, S., Ezaki, T., and Kawahara, K. 2001. Separation of 6-deoxy-heptan [correction of 6-deoxy-heptane] from a smooth-type lipopolysaccharide preparation of *Burkholderia pseudomallei*. FEMS Microbiol Lett **199:** 21.

64. Jerala, R. 2007. Structural biology of the LPS recognition. Int J Med Microbiol **297:** 353.

65. Jones, A. L., Beveridge, T. J., and Woods, D. E. 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect Immun **64:** 782.

66. Kanaphun, P., Thirawattanasuk, N., Suputtamongkol, Y., Naigowit, P., Dance, D. A., Smith, M. D., and White, N. J. 1993. Serology and carriage of *Pseudomonas pseudomallei*: a prospective study in 1000 hospitalized children in northeast Thailand. J Infect Dis **167:** 230.

67. Kawahara, K., Tsukano, H., Watanabe, H., Lindner, B., and Matsuura, M. 2002. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. Infect Immun **70:** 4092.

68. Kawasaki, K., Ernst, R. K., and Miller, S. I. 2005. Inhibition of *Salmonella enterica* serovar Typhimurium lipopolysaccharide deacylation by aminoarabinose membrane modification. J Bacteriol **187:** 2448.

69. Kespichayawattana, W., Rattanachetkul, S., Wanun, T., Utaisincharoen, P., and Sirisinha, S. 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun **68:** 5377.

70. Kirschning, C. J., Wesche, H., Merrill Ayres, T., and Rothe, M. 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med **188:** 2091.

71. Knirel, Y. A., Lindner, B., Vinogradov, E., Shaikhutdinova, R. Z., Senchenkova, S. N., Kocharova, N. A., Holst, O., Pier, G. B., and Anisimov, A. P. 2005. Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of *Yersinia pestis*. Carbohydr Res **340:** 1625.

72. Le Hello, S., Currie, B. J., Godoy, D., Spratt, B. G., Mikulski, M., Lacassin, F., and Garin, B. 2005. Melioidosis in New Caledonia. Emerg Infect Dis **11:** 1607.

73. Lee, H. K., Lee, J., and Tobias, P. S. 2002. Two lipoproteins extracted from *Escherichia coli* K-12 LCD25 lipopolysaccharide are the major components responsible for Toll-like receptor 2-mediated signaling. J Immunol **168:** 4012.

74. Leelarasamee, A. 2004. Recent development in melioidosis. Curr Opin Infect Dis **17:** 131.

75. Leelarasamee, A., and Bovornkitti, S. 1989. Melioidosis: review and update. Rev Infect Dis **11:** 413.

76. Lembo, A., Pelletier, M., Iyer, R., Timko, M., Dudda, J. C., West, T. E., Wilson, C. B., Hajjar, A. M., and Skerrett, S. J. 2008. Administration of a synthetic TLR4 agonist protects mice from pneumonic tularemia. J Immunol **180:** 7574.

77. Leone, S., Sturiale, L., Pessione, E., Mazzoli, R., Giunta, C., Lanzetta, R., Garozzo, D., Molinaro, A., and Parrilli, M. 2007. Detailed characterization of the lipid A fraction from the nonpathogen *Acinetobacter radioresistens* strain S13. J Lipid Res **48:** 1045.

78. Li, J., Thibault, P., Martin, A., Richards, J. C., Wakarchuk, W. W., and van der Wilp, W. 1998. Development of an on-line preconcentration method for the analysis of pathogenic lipopolysaccharides using capillary electrophoresis-electrospray mass spectrometry. Application to small colony isolates. J Chromatogr A **817:** 325.

79. Liu, Y., Loh, J. P., Aw, L. T., Yap, E. P., Lee, M. A., and Ooi, E. E. 2006. Rapid molecular typing of *Burkholderia pseudomallei*, isolated in an outbreak of

melioidosis in Singapore in 2004, based on variable-number tandem repeats. Trans R Soc Trop Med Hyg **100:** 687.

80. Matsuura, M., Kawahara, K., Ezaki, T., and Nakano, M. 1996. Biological activities of lipopolysaccharide of *Burkholderia (Pseudomonas) pseudomallei*. FEMS Microbiol Lett **137:** 79.

81. Mays, E. E., and Ricketts, E. A. 1975. Melioidosis: recrudescence associated with bronchogenic carcinoma twenty-six years following initial geographic exposure. Chest **68:** 261.

82. Medzhitov, R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol **1:** 135.

83. Medzhitov, R., and Janeway, C. A., Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell **91:** 295.

84. Miller, S. I., Ernst, R. K., and Bader, M. W. 2005. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol **3:** 36.

85. Miller, W. R., Pannell, L., Cravitz, L., Tanner, W. A., and Ingalls, M. S. 1948. Studies on Certain Biological Characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*: I. Morphology, Cultivation, Viability, and Isolation from Contaminated Specimens. J Bacteriol **55:** 115.

86. Ministry of Health. 2006. Communicable Diseases Surveillance in Singapore 2006, 80pp. Ministry of Health, Singapore.

87. Ministry of Health. 2004a. Communicable Diseases Surveillance in Singapore 2004, xivpp. Ministry of Health, Singapore.

88. Ministry of Health. 2004b. Epidemiological and clinical features of melioidosis in Singapore (Jan 2002 – Jul 2004). Epidemiol. News Bull **30:** 19.

89. Ministry of Health. 2004c. Epidemiological investigations into an epidemic of melioidosis, January–June 2004. Epidemiol. News Bull. **30:** 24.

90. Miyake, K. 2006. Roles for accessory molecules in microbial recognition by Toll-like receptors. J Endotoxin Res **12:** 195.

91. Mohamed, R., Nathan, S., Embi, N., Razak, N., and Ismail, G. 1989. Inhibition of macromolecular synthesis in cultured macrophages by Pseudomonas *pseudomallei* exotoxin. Microbiol Immunol **33:** 811.

92. Molinaro, A., Lindner, B., De Castro, C., Nolting, B., Silipo, A., Lanzetta, R., Parrilli, M., and Holst, O. 2003. The structure of lipid A of the lipopolysaccharide from *Burkholderia caryophylli* with a 4-amino-4-deoxy-L-arabinopyranose 1-phosphate residue exclusively in glycosidic linkage. Chemistry **9:** 1542.

93. Montminy, S. W., Khan, N., McGrath, S., Walkowicz, M. J., Sharp, F., Conlon, J. E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K., Akira, S., Cotter, R. J., Goguen, J. D., and Lien, E. 2006. Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. Nat Immunol **7:** 1066.

94. Moore, R. A., Tuanyok, A., and Woods, D. E. 2008. Survival of *Burkholderia pseudomallei* in Water. BMC Res Notes **1:** 11.

95. Nelson, M., Prior, J. L., Lever, M. S., Jones, H. E., Atkins, T. P., and Titball, R. W. 2004. Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. J Med Microbiol **53:** 1177.

96. Netea, M. G., van Deuren, M., Kullberg, B. J., Cavaillon, J. M., and Van der Meer, J. W. 2002. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? Trends Immunol **23:** 135.

97. Ngauy, V., Lemeshev, Y., Sadkowski, L., and Crawford, G. 2005. Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. J Clin Microbiol **43:** 970.

98. Nieminen, T., and Vaara, M. 2005. *Burkholderia pseudomallei* infections in Finnish tourists injured by the December 2004 tsunami in Thailand. Euro Surveill **10:** E050303 4.

99. Ogawa, T., Asai, Y., Makimura, Y., and Tamai, R. 2007. Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. Front Biosci **12:** 3795.

100. Peacock, S. J. 2006. Melioidosis. Curr Opin Infect Dis **19:** 421.

101. Perry, M. B., MacLean, L. L., Schollaardt, T., Bryan, L. E., and Ho, M. 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. Infect Immun **63:** 3348.

102. Petkanjanapong, V., Naigowit, P., Kondo, E., and Kanai, K. 1992. Use of endotoxin antigens in enzyme-linked immunosorbent assay for the diagnosis of *P. pseudomallei* infections (melioidosis). Asian Pac J Allergy Immunol **10:** 145.

103. Pitt, T. L., Aucken, H., and Dance, D. A. 1992. Homogeneity of lipopolysaccharide antigens in *Pseudomonas pseudomallei*. J Infect **25:** 139.

104. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P.,

Layton, B., and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282:** 2085.

105. Pourtaghva, M., Dodin, A., Portovi, M., Teherani, M., and Galimand, M. 1977. [1st case of human pulmonary melioidosis in Iran]. Bull Soc Pathol Exot Filiales **70:** 107.

106. Raetz, C. R., and Whitfield, C. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem **71:** 635.

107. Reckseidler-Zenteno, S. L., DeVinney, R., and Woods, D. E. 2005. The capsular polysaccharide of *Burkholderia pseudomallei* contributes to survival in serum by reducing complement factor C3b deposition. Infect Immun **73:** 1106.

108. Reinhart, R. A. 1988. Magnesium metabolism. A review with special reference to the relationship between intracellular content and serum levels. Arch Intern Med **148:** 2415.

109. Rolim, D. B., Vilar, D. C., Sousa, A. Q., Miralles, I. S., de Oliveira, D. C., Harnett, G., O'Reilly, L., Howard, K., Sampson, I., and Inglis, T. J. 2005. Melioidosis, northeastern Brazil. Emerg Infect Dis **11:** 1458.

110. Rosner, M. R., Tang, J., Barzilay, I., and Khorana, H. G. 1979. Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. J Biol Chem **254:** 5906.

111. Sarkar-Tyson, M., Thwaite, J. E., Harding, S. V., Smither, S. J., Oyston, P. C., Atkins, T. P., and Titball, R. W. 2007. Polysaccharides and virulence of *Burkholderia pseudomallei*. J Med Microbiol **56:** 1005.

112. Seydel, U., Schromm, A. B., Blunck, R., and Brandenburg, K. 2000. Chemical structure, molecular conformation, and bioactivity of endotoxins. Chem Immunol **74:** 5.

113. Silipo, A., Molinaro, A., Cescutti, P., Bedini, E., Rizzo, R., Parrilli, M., and Lanzetta, R. 2005. Complete structural characterization of the lipid A fraction of a clinical strain of *B. cepacia* genomovar I lipopolysaccharide. Glycobiology **15:** 561.

114. Smith, M. D., Angus, B. J., Wuthiekanun, V., and White, N. J. 1997. Arabinose assimilation defines a nonvirulent biotype of *Burkholderia pseudomallei*. Infect Immun **65:** 4319.

115. Stevens, M. P., and Galyov, E. E. 2004. Exploitation of host cells by *Burkholderia pseudomallei*. Int J Med Microbiol **293:** 549.

116. Stevens, M. P., Wood, M. W., Taylor, L. A., Monaghan, P., Hawes, P., Jones, P. W., Wallis, T. S., and Galyov, E. E. 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Mol Microbiol **46:** 649.

117. Suputtamongkol, Y., Hall, A. J., Dance, D. A., Chaowagul, W., Rajchanuvong, A., Smith, M. D., and White, N. J. 1994. The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. Int J Epidemiol **23:** 1082.

118. Suputtamongkol, Y., Kwiatkowski, D., Dance, D. A., Chaowagul, W., and White, N. J. 1992. Tumor necrosis factor in septicemic melioidosis. J Infect Dis **165:** 561.

119. Tanamoto, K., and Azumi, S. 2000. *Salmonella*-type heptaacylated lipid A is inactive and acts as an antagonist of lipopolysaccharide action on human line cells. J Immunol **164:** 3149.

120. Tirsoaga, A., Novikov, A., Adib-Conquy, M., Werts, C., Fitting, C., Cavaillon, J. M., and Caroff, M. 2007. Simple method for repurification of endotoxins for biological use. Appl Environ Microbiol **73:** 1803.

121. Trent, M. S. 2004. Biosynthesis, transport, and modification of lipid A. Biochem Cell Biol **82:** 71.

122. Utaisincharoen, P., Anuntagool, N., Arjcharoen, S., Limposuwan, K., Chaisuriya, P., and Sirisinha, S. 2004. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-beta is distinct from IFN-gamma in *Burkholderia pseudomallei*-infected mouse macrophages. Clin Exp Immunol **136:** 277.

123. Utaisincharoen, P., Tangthawornchaikul, N., Kespichayawattana, W., Anuntagool, N., Chaisuriya, P., and Sirisinha, S. 2000. Kinetic studies of the production of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-alpha) in macrophages stimulated with *Burkholderia pseudomallei* endotoxin. Clin Exp Immunol **122:** 324.

124. Utaisincharoen, P., Tangthawornchaikul, N., Kespichayawattana, W., Chaisuriya, P., and Sirisinha, S. 2001. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. Microbiol Immunol **45:** 307.

125. Vietri, N. J., and Deshazer, D. 2007. Melioidosis, 147 pp. *In* Dembek Z. F. (ed.), Medical Aspects of Biological Warfare, rev ed. Falls Church, VA.

126. Warawa, J., and Woods, D. E. 2005. Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. FEMS Microbiol Lett **242:** 101.

127. Weinbaum, G., Kadis, S., and Ajl, S. J. 1971. Microbial toxins. Bacterial toxins **4**.

128. West, T. E., Ernst, R. K., Jansson-Hutson, M. J., and Skerrett, S. J. 2008. Activation of Toll-like receptors by *Burkholderia pseudomallei*. BMC Immunol **9:** 46.

129. Westphal, O., and Jann, K. 1965. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of the procedure. Methods in Carbohydrate Chemistry **5:** 83.

130. White, N. J. 2003. Melioidosis. Lancet **361:** 1715.

131. Wiersinga, W. J., van der Poll, T., White, N. J., Day, N. P., and Peacock, S. J. 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nat Rev Microbiol **4:** 272.

132. Wiersinga, W. J., Wieland, C. W., Dessing, M. C., Chantratita, N., Cheng, A. C., Limmathurotsakul, D., Chierakul, W., Leendertse, M., Florquin, S., de Vos, A. F., White, N., Dondorp, A. M., Day, N. P., Peacock, S. J., and van der Poll, T. 2007. Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by *Burkholderia pseudomallei* (Melioidosis). PLoS Med **4:** e248.

133. Wiles, S., Hanage, W. P., Frankel, G., and Robertson, B. 2006. Modelling infectious disease - time to think outside the box? Nat Rev Microbiol **4:** 307.

134. Wong, K. T., Puthucheary, S. D., and Vadivelu, J. 1995. The histopathology of human melioidosis. Histopathology **26:** 51.

135. Woods, D. E., DeShazer, D., Moore, R. A., Brett, P. J., Burtnick, M. N., Reckseidler, S. L., and Senkiw, M. D. 1999. Current studies on the pathogenesis of melioidosis. Microbes Infect **1:** 157.

136. Wuthiekanun, V., and Peacock, S. J. 2006. Management of melioidosis. Expert Rev Anti Infect Ther **4:** 445.

137. Wuthiekanun, V., Smith, M. D., and White, N. J. 1995. Survival of *Burkholderia pseudomallei* in the absence of nutrients. Trans R Soc Trop Med Hyg **89:** 491.

138. Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol **36:** 1251.

139. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395:** 284.

140. Yi, E. C., and Hackett, M. 2000. Rapid isolation method for lipopolysaccharide and lipid A from Gram-negative bacteria. Analyst **125:** 651.

141. Zhou, Z., Ribeiro, A. A., Lin, S., Cotter, R. J., Miller, S. I., and Raetz, C. R. 2001. Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. J Biol Chem **276:** 43111.

142. Zughaier, S. M., Zimmer, S. M., Datta, A., Carlson, R. W., and Stephens, D. S. 2005. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. Infect Immun **73:** 2940.

Appendix

Appendix A

Preparation of stocks and solutions

1. N-minimal broth and agar
1.1 Reagents used to prepare N-minimal media (prepared in laboratory)
To make 1M KCl (Sigma), 100ml: Dissolve 7.46g in 100ml of distilled water
To make 0.1M K_2SO_4 (Sigma), 100ml: Dissolve 1.74g in 100ml of distilled water
To make 1M KH_2PO_4 (Merck), 100ml: Dissolve 13.61g in 100ml of distilled water
To make 1M glucose (Sigma), 100ml: Dissolve 18.02g in 100ml of distilled water
To make 1M $MgSO_4$ (Sigma), 100ml: Dissolve 12.04g in 100ml of distilled water
To make 0.1M $MgSO_4$ (Sigma), 100ml: Dissolve 1.20g in 100ml of distilled water

To make 1.8mM $FeSO_4.7H_20$ (Sigma), 50ml:

Dissolve 25.0mg in 50ml of distilled water (prepare fresh)

To make 2mg/ml thiamine-HCl, 50ml:

Dissolve 100mg in 50ml of distilled water (prepare fresh)

1.2 To prepare N-minimal broth or agar, 1000ml:

1g of Bacto-casamino acid

17g Bactor agar (only used when making agar, not for broth medium)

Dissolve in 865ml of distilled water and autoclave at 121^oC for 20 minutes. Cool and maintain at ~50^oC in a water bath. Add filter sterilized volumes of the following:

5ml of 1M KCl

5ml of 0.1M K_2SO_4

1ml of 1M KH_2PO_4

1ml of 1.8mM $FeSO_4$

1ml of 2mg/ml thiamine HCl

100ml of 1M tris-HCl, pH 7.4 (1st Base, Singapore)

22ml of 1M glucose

For magnesium supplemented medium: 10ml of 1M $MgSO_4$

For low concentration magnesium medium: 80µl of 0.1M $MgSO_4$

2. Solubilisation buffer (whole cell profile)
2.1 To prepare base buffer, 32ml:
2ml of 1M tris-HCl, pH 6.8 (1st Base, Singapore) 3.2ml of glycerol (Sigma) 9.6ml of 10% SDS (prepared fresh) 15.6ml of double distilled water Trace amount of bromophenol blue (Sigma) Add 50µl of β-mercaptoethanol (55mM, Gibco) to 950µl of above base buffer, just before use.
2.2 To prepare solubilisation buffer containing proteinase K, 32ml:
Add 1mg of Proteinase K to 300µl of solubilisation buffer just before use.

3. Cell culture medium

3.1 To prepare fetal calf serum (FCS):

Heat-inactivate at 56^{o}C for 30 minutes and aliquot into 50ml falcon-tubes. Store at -20^{o}C.

3.2 To prepare RAW 264.7 culture medium, 500ml:

10ml of L-glutamine (200mM, Gibco)

5ml of sodium pyruvate (100mM, Gibco)

50ml of fetal calf serum

Make up volume to 500ml with DMEM (Gibco) and filter sterilise.

3.3 To prepare THP-1 culture medium, 500ml:

5ml of L-glutamine (200mM, Gibco)

5ml of sodium pyruvate (100mM, Gibco)

5ml of HEPES (1M, Gibco)

454µl of β-mercaptoethanol (55mM, Gibco)

50ml of fetal calf serum

Make up volume to 500ml with RPMI (Gibco) and filter sterilise.

3.4 To prepare HEK 293 culture medium, 500ml:

10ml of L-glutamine (200mM, Gibco)

5ml of sodium pyruvate (100mM, Gibco)

50ml of fetal calf serum

Make up volume to 500ml with DMEM (Gibco) and filter sterilise.

3.5 To prepare TLR2 or TLR4 transfected HEK 293 culture medium, 500ml:

10ml of L-glutamine (200mM, Gibco)

5ml of sodium pyruvate (100mM, Gibco)

50ml of fetal calf serum

500µl of blasticidine (10mg/ml, Invivogen)

250µl of hydrogold (100mg/ml, Invivogen)

Make up volume to 500ml with DMEM (Gibco) and filter sterilise.

4. LPS re-purification buffer
To make repurification buffer, 500ml: 0.055g of $CaCl_2$ (Sigma) 0.102g of $MgCl_2.6H_20$ (Sigma) Dissolve in 400ml of PBS and make up volume to 500ml with PBS.

5. Lipid A isolation buffer
5.1 To make 1% SDS in 10mM sodium acetate, pH 4.5, 100ml:
0.082g of sodium acetate (Sigma) 1g of SDS (Sigma) Dissolve in 100ml of double distilled water. Adjust pH to 4.5 using 1M HCl.
5.2 To make acidified ethanol, 20.1ml:
Add 100µl of 4M HCl to 20ml of 95% ethanol.